WOMEN IN NEUROSCIENCE

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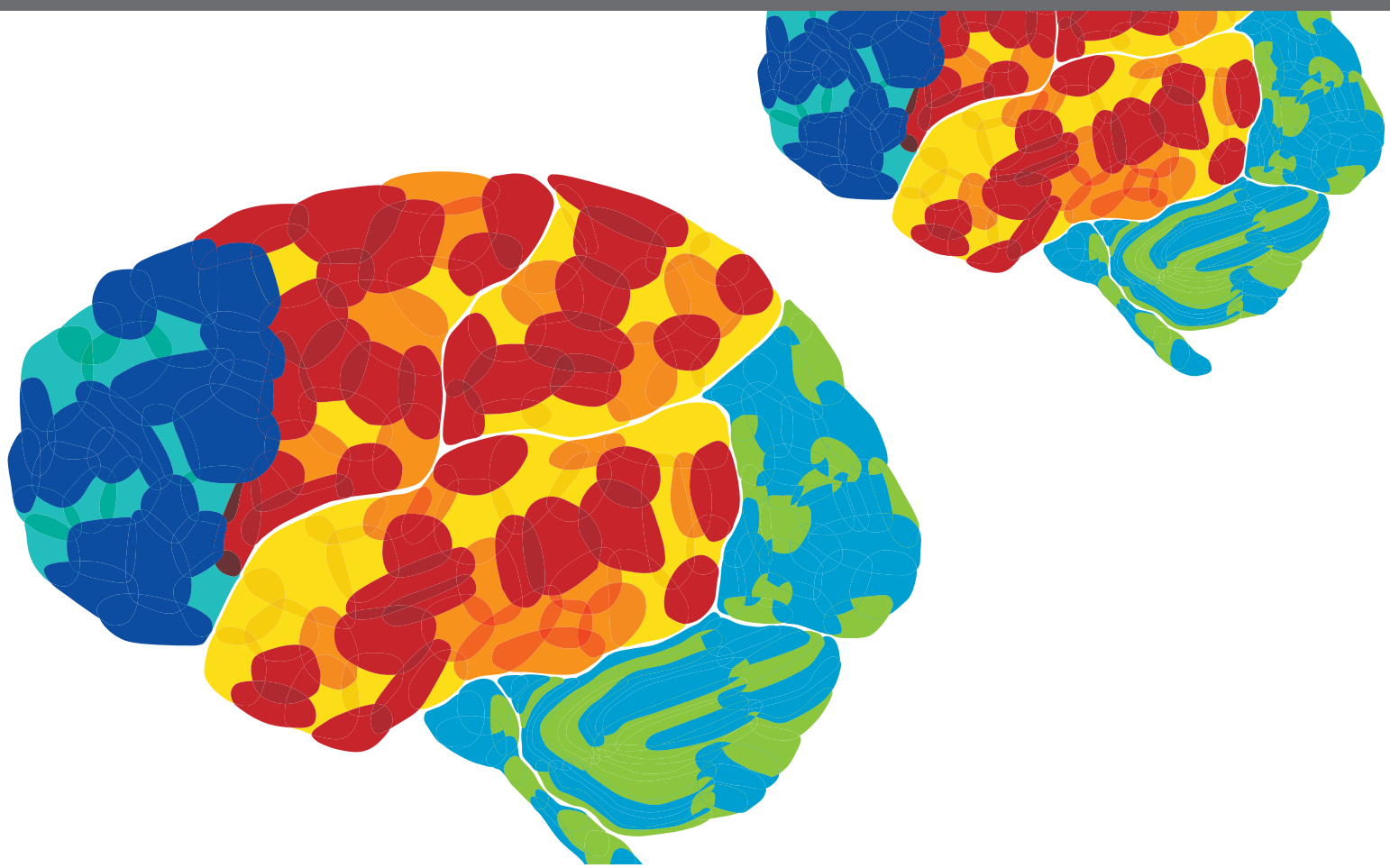

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WOMEN IN NEUROSCIENCE

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Editorial: Women in neuroscience

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Editorial on the Research Topic
Women in neuroscience

The "Matilda effect" is an expression coined in 1993 by Margaret Rossiter, a prominent science historian, to describe the faint recognition of the contribution of women to the scientific enterprise. The expression derived from the realization that just like the work of Matilda Gage, a suffragist who also wrote about women in science, the discoveries and inventions of many women scientists had been forgotten over the course of history. Indeed, women's contributions to science have been often misappropriated, forgotten or, in some cases, even actively removed from the records. This resulted in a misplaced historic assumption that women lack the intellectual ability and interest for scientific disciplines, and left younger generations of women with very few role models to look up to. Over the past few years, the awareness of this lack of recognition has increased and, despite encountering some resistance, active efforts have been made to make science a more inclusive enterprise. Neuroscience is a multidisciplinary field that encompasses all scientific disciplines including biology, psychology, cognitive sciences, physics, engineering, and mathematics. While women to this day represent a minority of neuroscience faculty, they contribute to all aspects of the field. The goal of the *Women in neuroscience* Research Topic is to oppose the "Matilda effect" by bringing together excellent research by women, or in collaboration with women. The Research Topic brings together 33 articles in which the first or last author are women. The formats include mini-reviews and reviews of the exceptional work done by past and present women neuroscientists, an opinion article, perspectives and specific Research Topic reviews highlighting scholarship and innovative frameworks, and original research articles that push the field forward.

Malerba describes the inspiration of working with Dr. Levi-Montalcini by focusing on the last years of research of one of the few women Nobel Laureates. Heiden et al., Yevoo and Maffei, Gonzalez Osorio et al., and Quattrocolo et al. highlight some of the excellent and impactful work by female researchers in neuroscience and medicine with the goal of emphasizing their scientific discoveries and providing role models for future scientists.

On the technological side, the work of women scientists demonstrates creativity and long-term vision. Le Bars et al. propose an innovative Brain Computer Interface (BCI) paradigm inspired by the ideomotor principle, while Shim et al. present a Machine Learning (ML) method to classify electroencephalographic oscillations, which outperforms current neurophysiological-based approaches to diagnose Post Traumatic Stress Disorder (PTSD). By exploiting fNIRS (functional near-infrared spectroscopy), Zhang M. et al. compared hemodynamic responses to low vs. high Informational Masking speech. Focusing on the human-machine teaming, Hopko and Mehta discuss the neural correlates of trust in automation and Douibi et al. envisage the (not so far) large-scale deployment of BCIs in the Industry 4.0, widening their possible applications in education, entertainment and aviation.

Numerous groups also investigated sex differences and the role of sex hormones in brain functions and behavior. Zhao et al. used a post-menopausal mouse model to verify the hypothesis that mitochondrial damage may contribute to cognitive impairment associated with estrogen deficiency, while Zhang S. et al. conducted a cross-sectional study in early menopausal women to evaluate estradiol-related structural changes in the brain. As suggested by De Filippi et al., Lima et al., Zanolie et al., Qin et al., and Palamarchuk and Vaillancourt, ovarian hormones and menstrual cycle modulate whole-brain turbulent dynamics, contribute to the sex differences in the reserpine-induced progressive animal model of PD, and modulate neural activity and pathways related to stressor detection and coping. Dai et al. reports clinical manifestations of variants of the DDX3X gene, which is associated with intellectual disability mostly in females (and only rarely in males).

Several papers contribute to advancing our understanding of schizophrenia. Rootes-Murdy, Zendehrough et al. and Jensen et al. discuss MRI data analysis methods to describe the structural and cognitive differences of schizophrenia patients and healthy controls. Rootes-Murdy, Goldsmith et al. provide an overview of neural changes and clinical presentations associated with delusions, a hallmark of certain psychotic disorders and neurodegenerative diseases. Liu et al. explore if metabolites of phospolipids may be used as biomarkers for therapeutic response in schizophrenia patients, while Bermperidis et al. use the human transcriptome to more generally differentiate between neuropsychiatric and neurological disorders as human embryonic stem cells become neurons. Cognitive functions in conditions of nutrient deprivation, high stress (associated to maternal post-partum and childhood bullying victimization), neurodegenerative disorders or Small Vessel Disease were addressed by Zhang Q. et al., Palamarchuk and Vaillancourt, Vandenbroucke et al., Gronewold and Engels, Liao et al., and Qin et al. Moreover, Shi et al. demonstrated that berberine treatment significantly restored cognitive impairment in sepsis mice, while Metaxas focused on the molecular interactions characterizing the pathogenesis of Alzheimer's Disease in a *C. elegans* model.

Finally, Saveko et al. and Nosikova et al. report on how motor function may be affected by extreme conditions, such as dry immersion and altered gravity situations in long term space missions, while Putman et al. investigated the effects of Galvanic vestibular stimulation in functional mobility tasks, to be potentially exploited by as astronauts, firefighters, high performance athletes, and soldiers.

The breadth and quality of the papers in this Research Topic provides important ground for future research and will hopefully serve as an inspiration for young neuroscientists. The Research Topic recognizes the breadth of scientific ideas and findings within the field, and given the cross-disciplinary nature of neuroscience, puts on record women's contribution to the scientific effort at large.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Gestational Folic Acid Administration Alleviated Maternal Postpartum Emotional and Cognitive Dysfunction in Mice

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Gestational folic acid (FA) supplementation has been widely recognized for its benefits in preventing offspring defects, but its effect on postpartum females has not yet been adequately assessed. The occurrence of emotional and cognitive dysfunction is common in postpartum women, and its treatment remains limited. Considering the promising results of FA in various psychiatric disorders both in human and redents, we tested the effect of gestational FA administration on postpartum psychiatric behavioral phenotypes and the implicated brain-related mechanisms in a murine model. FA was administered orally in both the hormone-stimulated-pregnancy (HSP) model and pregnant mice at doses of 1 and 5 mg/kg. Postpartum behavioral results showed that the disorders of cognitive performance, depressive, and anxiety-related behaviors were all alleviated in the 5 mg/kg FA group. However, the general development of their offspring remained unaffected. Immunofluorescence and immunoblot results revealed that FA pretreatment significantly activated the maternal hippocampal BDNF-related pathway. Morphological studies have confirmed that FA promotes hippocampal neurogenesis. Moreover, synaptic plasticity and synaptic transmission are enhanced. All of these hippocampal changes play critical roles in rescuing neuronal function and behaviors. Thus, our data suggest that gestational FA administration has a therapeutic effect that improves cognition and reduces depression and anxiety in a murine postpartum model. This may be developed as a preventive and adjuvant therapeutic option for pregnant women.

Keywords: folic acid, postpartum, behavior, hippocampus, brain-derived neurotrophic factor

INTRODUCTION

Women who undergo pregnancy and childbirth experience dramatic hormonal fluctuations, which contribute to the occurrence of mental symptoms, including cognitive and emotional dysfunction, as are widely observed in postpartum women. Postpartum mental symptoms include postpartum cognitive dysfunction (PCD), postpartum depression (PPD), and postpartum anxiety (PPA), which have turned into a serious epidemiological concern in the field of maternity care. Pregnant women have significantly poorer cognitive abilities (Henry and Sherwin, 2012). Simultaneously, women are

twice as likely to develop depression, with comorbid generalized anxiety disorder; most episodes begin postpartum (Wisner et al., 2013). The incidence and prevalence of unique symptoms occurring after birth range from 8 to 26%, and multiple symptoms are often found concurrently (Fairbrother et al., 2016; Meena et al., 2016; Shorey et al., 2018. However, these symptoms in postpartum women are often overlooked, leading to lower diagnosis rates and even lower treatment rates. These symptoms not only affect the quality of life of postpartum women, but also mother-infant interactions and spousal relationships, which in turn affect child-rearing behaviors and healthy development of infants. The conventional treatment for these symptoms often abides by conventional drug treatment protocols. Even if clinically effective, these still might be rejected by pregnant women considering potential risks to the baby (Noble, 2005). Therefore, therapies that can serve as complementary or alternative treatments must be developed.

The hippocampus plays a central role in the processing of emotional and cognitive behaviors (Korotkova et al., 2018). Unlike general major depression disorder and cognitive impairment, with pathogenic mechanisms associated with psychosocial stress or neurological injury, postpartum symptoms are often speculated to be caused by hormonal fluctuations (Becker et al., 2016; Brown and Schaffir, 2019). A growing number of studies have shown that hippocampus-related mechanisms are implicated in the pathogenesis of postpartum emotional and cognitive dysfunction (Pawluski and Galea, 2007; Baka et al., 2017). Anatomical hippocampal neurogenesis and synaptic plasticity functional dysregulation have been shown to mediate the presentation of certain types of cognitive or affective dysfunction behaviors (Brummelte and Galea, 2016; Workman et al., 2012). Thus, discovery of novel therapies targeting these potential candidates targets is urgent and beneficial for these treatment.

The transition to motherhood not only involves hormones, but also the metabolic adaptations of various nutrients that modify behavioral states. Folic acid (FA), an essential B vitamin, is involved in regulating fetal development during pregnancy, and is required at an even higher dose during pregnancy (Chitayat et al., 2016). A consensus regarding the prevention of neural tube defects and other folic acid-sensitive congenital malformations has been internationally suggested. Moreover, existing clinical studies have shown that FA supplementation during pregnancy is correlated with a lower risk of postpartum mental symptoms (Yan et al., 2017). In fact, FA has shown favorable therapeutic effects in multiple experimental and clinical mental symptom models (Fava and Mischoulon, 2009; Shrestha and Singh, 2013; Budni et al., 2013; Lv et al., 2019). Based on this wide range of therapeutic effects of FA, considering that postpartum mental symptoms almost share the same symptoms as the previously mentioned mental illnesses, characterized by like lower cognitive performance, depression, hopelessness, and anxiety, FA offers promise in terms of its clinical benefits, and may represent a cure of PCD, PPD, and PAD. In this study, we sought to assess this potential.

The etiopathogenesis of emotional and cognitive dysfunction within the postpartum period and has been well established in mice (Zhang et al., 2016). In the first part of this study, a hormone-stimulated pregnancy (HSP) "pseudo-gestational" model was established to mimic the effects of hormones on pregnancy in mice, and the effect of FA administration on related behavioral phenotypes was monitored. In addition, these observations were conducted in naturally pregnant female mice. The effect of the gestational administration of FA on offspring generation was also monitored. This observation may shed light on potential postpartum-related maternal mental symptom prevention and treatment strategies.

MATERIALS AND METHODS

Animals

Ten to twelve-week-old male/female C57BL/6 mice (purchased from Beijing Vitong Lihua Co., Ltd.) were raised in an SPF environment at a temperature of 22–24°C and relative humidity of 55–65%. All animals had free access to food and drinking water and were reared according to a 12/12 light-dark cycle. All experimental animal procedures strictly followed the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and were authorized by the Ethics Committee of Guangdong Women and Children Hospital. All efforts were made to minimize animal suffering. After the animals were accommodated to environmental condition, then the experimental procedures started on 15–16-weeks-old animals. In behaviral tests, the animal behavior tests were monitored using Ethovision 7.0 software (Noldus, Wageningen, Netherlands).

Modeling and Folic Acid Administration

In this study, we utilized two postpartum models: the hormone-induced pseudo-pregnancy (HSP) model and the natural delivery postpartum model. The HSP model referred to the modeling method adopted in mice (Zhang et al., 2016; Zhu and Tang, 2020), and animals with bilateral oophorectomy (OVX) were treated with hormones. Briefly, oophorectomy was performed under isoflurane anesthesia in female mice, whereas the control group (Con) underwent a sham operation, except for the oophorectomy. Estrogen (E2) and progesterone (P4) were dissolved in corn oil and subcutaneously injected into OVX mice 9 days after recovery. E2 was administered for 22 consecutive days (20 μg/kg for the first 15 days and 400 μg/kg for the subsequent 7 days), whereas a P4 (32 mg/kg) injection was administered in the first 15 days. Mice in the Con group were injected with a corn oil solvent. Relevant tests were performed within 7 days of E2 withdrawal. In the model of natural pregnancy, mice were caged at a male:female ratio of 1:2, and the appearance of vaginal suppositories was considered the first day of gestation (G0). Pregnant mice and their offspring from postnatal day (PD) were carefully handled and recorded. The body weights of the pregnant mice in each group were measured at G7, G15, and G18. The gestational length, litter size and sex ratio of the offspring, and litter weights during development were recorded until P21. Folic acid was purchased from Sigma Chemical Co. (St. Louis, MO, United States) and dissolved in

distilled water. In this study, two doses of folic acid (1 and 5 mg/kg, gavage) were selected as supplements in addition to normal food sources. In the HSP model, folic acid supplementation was administered daily, along with E2, until E2 was discontinued. In the model of natural pregnancy, folic acid supplementation began with the onset of vaginal suppositories until birth.

Morris Water Maze Test

The MWM was adopted from a previous study (Sun et al., 2020), and the diameter of the water maze device was 1.5 m. It was filled with an opaque water solution dyed with white dye, and the water temperature was maintained at 24°C. The camera device was set above the recording device. The circular area in the device was evenly divided into four quadrants, and the sign markers were set at the periphery of each quadrant. A movable platform was placed 1 cm underwater at a specific position in each quadrant, and each animal tested corresponded to a specific target quadrant. The experiment lasted for 6 days. In the first 5 days, mice were placed in water facing the wall of the pool in one of the four quadrants and the time required to climb up the platform within 90 s was assessed. On day 6, the platform was removed and the total time of the mice passing through the target quadrant within 90 s was recorded. The time ratio was thereafter calculated.

Spontaneous Alternation Y-Maze Test

The Y-maze is composed of three arms of equal length, randomly defined as the starting arm, new arm, and other arms. The mice were placed in a random arm, named the starting arm, and moved freely to explore the other arms for 5 min. A correct alternation was recorded when the mice entered the three different arms. The spontaneous alternation rate (%) was calculated as the total number of correct alternations/(total crossing times of 3 arms − 2) × 100%.

Novel Object Recognition Test

The NOR was adopted as previously described (Muha et al., 2019). Briefly, animals were placed in an organic plastic experimental facility (40 cm × 40 cm × 40 cm) for adaptation every day, and then returned to the cage after 10 min of free exploration for three days. On the fourth day, two identical objects (block-shaped toys, Lego Company) were placed in a relative position on one side of the field. The mice were gently placed in the positions of the two objects with their backs to them (familiarization phase). After 10 min of exploration, the animals were removed and returned to the cage. Exploration was defined as directing the nose to the object (distance of <2 cm) and/or touching the object with the nose. The animals showed a preference for objects during familiarization were excluded from the analysis. After 1.5 h, one of the two identical objects was replaced with a different object, named the old object and the new object, respectively. The time the mice spent exploring the two objects was recorded. The NOR index (%) was calculated as (time to explore novel object/total time to explore two objects: time to explore old object/total time to explore two objects) × 100%.

Open-Field Test

The box for the open field experiment was a square box with the following dimensions: length × width × height = 50 cm × 50 cm × 40 cm. Each mouse was placed in the center of the box, and the activity of the mice was video recorded within 5 min. The residence time in the central region of the square was recorded.

Forced Swim Test

The forced swim test is a desperate behavior test used to assess depression-like behaviors. The experiment was performed as previously described (Guo et al., 2016). Mice were placed in a transparent plexiglass cylinder (40 cm in height and 20 cm in diameter) filled with water at a depth of 25 cm for 6 min, and the time of immobilization (just for the movement of the head on the water surface) for the last 4 min was recorded.

Elevated Plus Maze Test

As previously described (Šutulović et al., 2021), the elevated plus maze consists of two open arms and two closed arms. The mice were placed in the test room for 2 h in advance to adapt to allow for their adaptation to the experimental environment. The mice were gently placed in the central position facing the open arm, and the time and number of mice entering the open arm and the total activity trajectory were recorded within a period of 5 min.

Immunofluorescence

BrdU staining was performed by continuous intraperitoneal injection of BrdU (50 mg/kg) twice daily for 5 days before behavioral tests in advance. After the animals were deeply anesthetized and perfused with ice-cold 0.1% MPBS and 4% paraformaldehyde, the brain tissue was collected and dehydrated in a sucrose solution. Continuous coronal slices were cut into 35 μm slices using a frozen slicer. Hippocampal sections were incubated with 10% BSA (containing 0.3% Triton X-100) at room temperature for 1 h. The corresponding primary antibodies were added and incubated with the sections overnight at 4°C: mouse anti-BDNF (1: 500, Abcam; ab203573), rat anti-BrdU (1:100, AbD Serotec; OBT0030), and mouse anti-Nestin (1:1000, Millipore, MAB5326). After washing with PBS, fluorescent secondary antibody (Invitrogen) was added and incubated at room temperature for 2 h without light. After washing with PBS, a DAPI staining solution was added, and the plates were sealed and observed under a confocal microscope (LSM510, Carl Zeiss, Goettingen, Germany). A total of 7–8 sections were randomly selected from each mouse for image acquisition and statistical analysis.

Immunoblotting Analysis

The animals were sacrificed with CO2 and operated on ice to rapidly isolate the hippocampus. A BCA protein assay kit was used to determine the total protein concentration in the tissue samples. Loading protein was separated by 12% sodium dodecyl sulfate (SDS) -polyacrylamide gel (PAGE) and then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, Massachusetts, United States). After blocking with 5% skimmed milk for 2 h at room temperature, the membranes were incubated overnight at 4°C with primary antibody

(anti-BDNF: 1:500, Santa Cruz Biotechnology, United States; anti-TrkB: 1:400, Santa Cruz Biotechnology, United States; anti-p-PIK3: 1:500, Cell Signaling, United States; anti-PIK3: 1: 400, Cell Signaling, United States; anti-p-AKT: 1:200, Cell Signaling, United States; anti-AKT: 1:200, Cell Signaling, United States; anti-p-mTOR: 1:400, Cell Signaling, United States; anti-mTOR: 1:400, Cell Signaling, United States; anti-synaptophysin: 1:600, Abcam, United States; anti-Synapsin-1: 1:800, Abcam, United States; anti-PSD95: 1:800, Cell Signaling, United States; anti-GAPDH: 1:600, Santa Cruz Biotechnology, United States). After washing with PBST solution, the membranes were incubated with HRP-conjugated secondary antibody (1:10,000) for 2 h at 25°C. After treatment with ECL, bands were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and analyzed with ImageJ software according to the gray levels of the target protein and reference protein (GAPDH).

Long-Term Potentiation Electrophysiological Recordings

Mice were anesthetized with sevoflurane and immediately decapitated (Bieschke et al., 2011). Their brain tissue was placed in ACSF (206 glucose, 2.4 KCl, 2.0 MgSO4, 1.0 NaH2PO4, 1.0 CaCl2,25.0 NaHCO3, 1.0 MgCl2, 25 NaHCO3, 10 mM D-glucose, in mM) containing 95% 02 and 5% CO2 at 4°C for 1 min. Subsequently, coronal hippocampal slices of 400 μm thickness were transferred to the above ACSF solution and incubated for 1 h at 37°C. When recording, the temperature in the irrigation groove was kept at 25°C, and the continuous rate of ACSF (pH = 7.4) perfusion flow was set to 1.0–1.5 ml/min. The stimulation electrode uses a bipolar tungsten stimulation electrode, placed on the Schaffer collaterals of CA3 region, to provide a constant current pulse through the stimulator (Sen. 3301, Nihon Kohden, Japan). The recording pipettes were pulled with a standard borosilicate glass tube filled with 2M NaCl, and the impedance was 3-9MQ and were placed on in the stratum radiatum of the CA1 area. The 50% stimulation intensity of the maximum responses of field excitatory postsynaptic potentials (fEPSP) was selected as the final stimulus intensity, and fEPSP over 20 min was recorded as a stable baseline. Data were normalized with respect to the mean values of fEPSP slope recorded during this period. Two consecutive trains (1 s) of stimuli at 100 Hz separated by 20 s were applied to the slices to induce LTP. The recorded electrical signals were amplified with an amplifier (MEZ-8201), and the fEPSP signals were digitized and saved using a pCLAMP system (Axon Instrument Inc.).

Statistical Analysis

The collected data are shown as the mean ± standard error of the mean (SEM) and analyzed with GraphPad Prism software (version 6.0; GraphPad Inc., La Jolla, CA, United States). Statistical differences between group means were evaluated using a Student's t-test, one-way analysis of variance (ANOVA) followed by post hoc test, and two-way RM ANOVA according to the test. Statistical significance was set to $p < 0.05$.

RESULTS

Hormone-Simulated Pregnancy Postpartum Model Show Impaired Emotional and Cognitive Dysfunction

An OVX combined with hormone-simulated pregnancy mouse model was utilized to assess potential postpartum cognitive and emotional outcomes, and the behavioral test was begun 7 days after the withdrawal of progesterone (P4) (**Figure 1A**). The MWM test showed that HSP mice exhibited longer latency to reach the hidden platform during the acquisition phase, and a lower percentage of time to explore the target quadrant during the probe test phase when the platform was removed (**Figures 1B,C**). Consistently, impaired cognition was also observed in the Y-maze test and novel object recognition memory in HSP female mice compared with the Sham-treated OVX control, while the total number of entries and object recognition during familiarization phase had little changes. (**Figures 1D,E**). These results indicate that the HSP model induces impaired spatial working memory and short-term non-spatial memory. To assess the effects of HSP on emotion-related behaviors induced by HSP treatment, we performed a FST, OFT, and EPM. The HSP mice showed increased immobility time in the FST (**Figure 1F**). The open field test and elevated plus maze test were performed to assess patterns of anxiety-like behavior. The analysis of OFT demonstrated that the HSP mice spent significantly less time in the central area within the arena (**Figure 1G**). In the EPM test, the trajectory diagram showed that the time in the open arms and the number of entries were markedly reduced in the HSP group compared to the control group (**Figures 1H,I**). No differences were observed between the two groups in the total arm entries in the EPM test (**Figure 1J**). These results indicate that the HSP model exhibited impaired emotional and cognitive dysfunction.

Behavioral Analysis for Hormone-Stimulated Pregnancy Model After Folic Acid Treatment

We then investigated whether cognitive and emotional behavioral impairment in HSP mice could be improved by gestational FA administration. In addition to normal food sources for control HSP mice, considering the dose of normal daily requirement, two supplementary concentrations of FA were chosen to determine whether a therapeutic dose response existed. The HSP mice were orally administered once per day with either FA (1 mg/kg [HSP-L] or 5 mg/kg [HSP-H]) or vehicle (HSP) (**Figure 2A**) during HSP modeling. Behavioral results showed that animals from the FA-administered groups learned better than the HSP controls, as evidenced by significantly faster escape

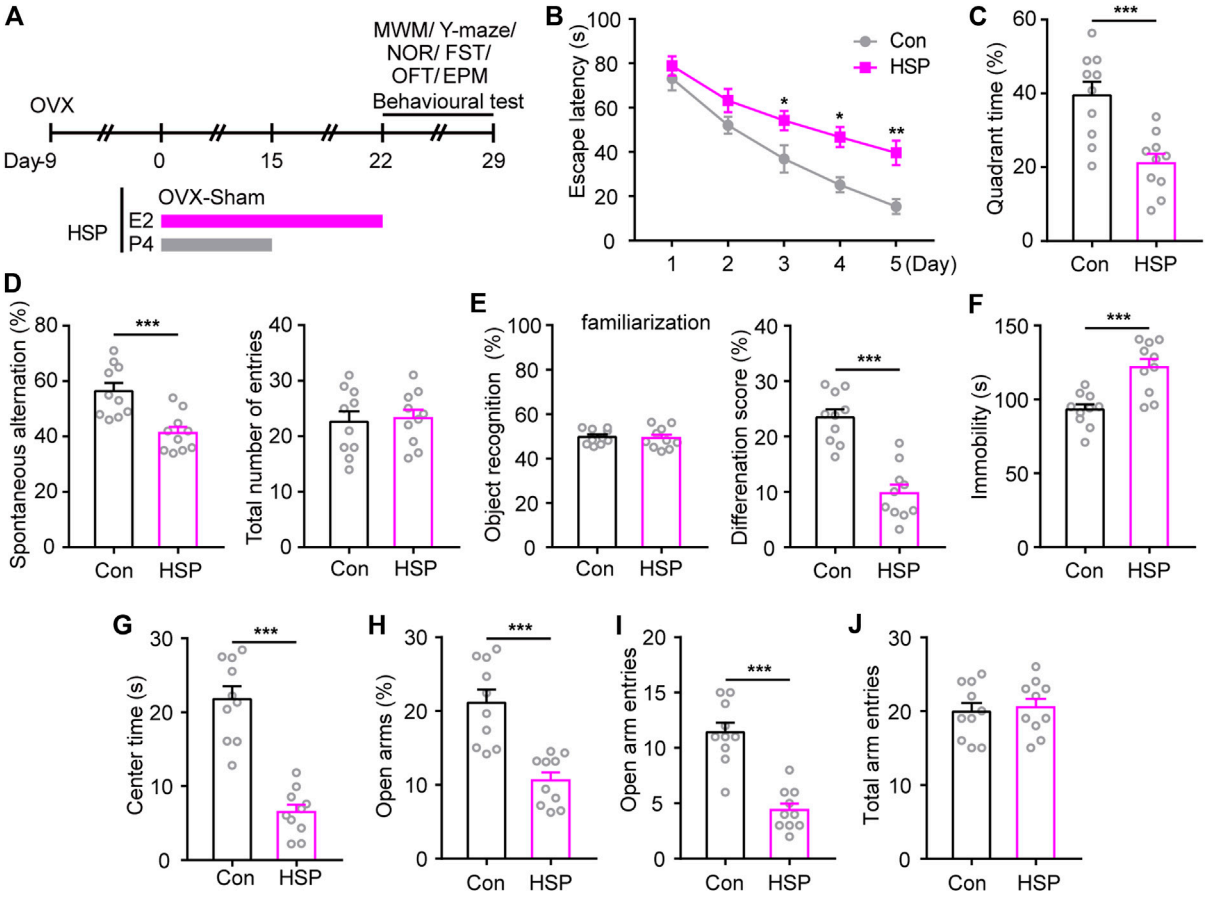

FIGURE 1 | Behavioral changes induced by HSP modeling. **(A)** Schematic timeline representation of HSP modeling and experimental design. **(B)** The latency to reach the hidden platform during the acquisition phase from training days 1–5. The latency during the navigation training was analyzed using two-way repeated measures ANOVA: F (4, 36) = 35.86, $p < 0.0001$. **(C)** The time spent exploring the target quadrant of each group on the 6th day of probe tests with the removed platform. Student's *t*-test: t = 4.096, $p = 0.0007$. **(D)** The performance of spontaneous alterations and total number of entries in the Y-maze test. Student's *t*-test: t = 4.118, $p = 0.0006$; Student's *t*-test: t = 0.2468, $p = 0.8079$. **(E)** The preference for objects during familiarization and performance to distinguish familiar/novel objects in the NOR test. Student's *t*-test: t = 0.2549, $p = 0.8017$; Student's *t*-test: t = 6.449, $p < 0.0001$. **(F)** Immobility time in FST. Student's *t*-test: t = 4.288, $p = 0.0004$. **(G)** Time spent in the central area of the open-field test. Student's *t*-test: t = 7.649, $p < 0.0001$. Percentage of time spent in the open arm **(H)**, entries into open arms **(I)**, and total arm entries **(J)** in the elevated plus maze (EPM) test. Student's *t*-test: t = 7.649, $p < 0.0001$; Student's *t*-test: t = 6.614, $p < 0.0001$; Student's *t*-test: t = 0.3619, $p = 0.7217$. Data are presented as scatter points plus the mean ± SEM. $n = 10$ for each group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared with Con.

latencies during training sessions and a longer time spent exploring the platform in the target quadrant during the probe trial of the MWM task (**Figures 2B,C**). In addition, mice treated with FA were more likely to explore alternate arms in the Y-maze test and spent more time exploring the novel object than the familiar object, compared with the HSP control, while the total number of entries and object recognition during familiarization phase had little changes among three groups (**Figures 2D,E**). FA-treated HSP mice also showed decreased immobility in the FST (**Figure 2F**) and displayed increased time and number of entries into the open arms of the EPM as compared to controls (**Figures 2G,H**), and no differences were observed between the three groups in the total arm entries in the EPM test (**Figure 2I**). The effect of FA observed in the above results showed that FA had a dose-dependent relationship with a certain behavioral phenotype. A higher dose of FA resulted in a better effect than the low-dose group.

Effects of Gestational Folic Acid Administration on Postpartum Behavioral Analysis

Next, we investigated whether gestational FA administration could protect against impaired cognitive and emotional performance in natural postpartum female mice and its effect on maternal and offspring characteristics (**Figure 3A**). Interestingly, the MWM results showed that the escape latency improved more obviously in the FA-H group during both the trial training (**Figure 3B**) and probe test (**Figure 3C**). Similarly, FA administration also improved cognitive performance in both the Y-maze and NOR tasks, while the FA administration had little effect on the total number of entries and

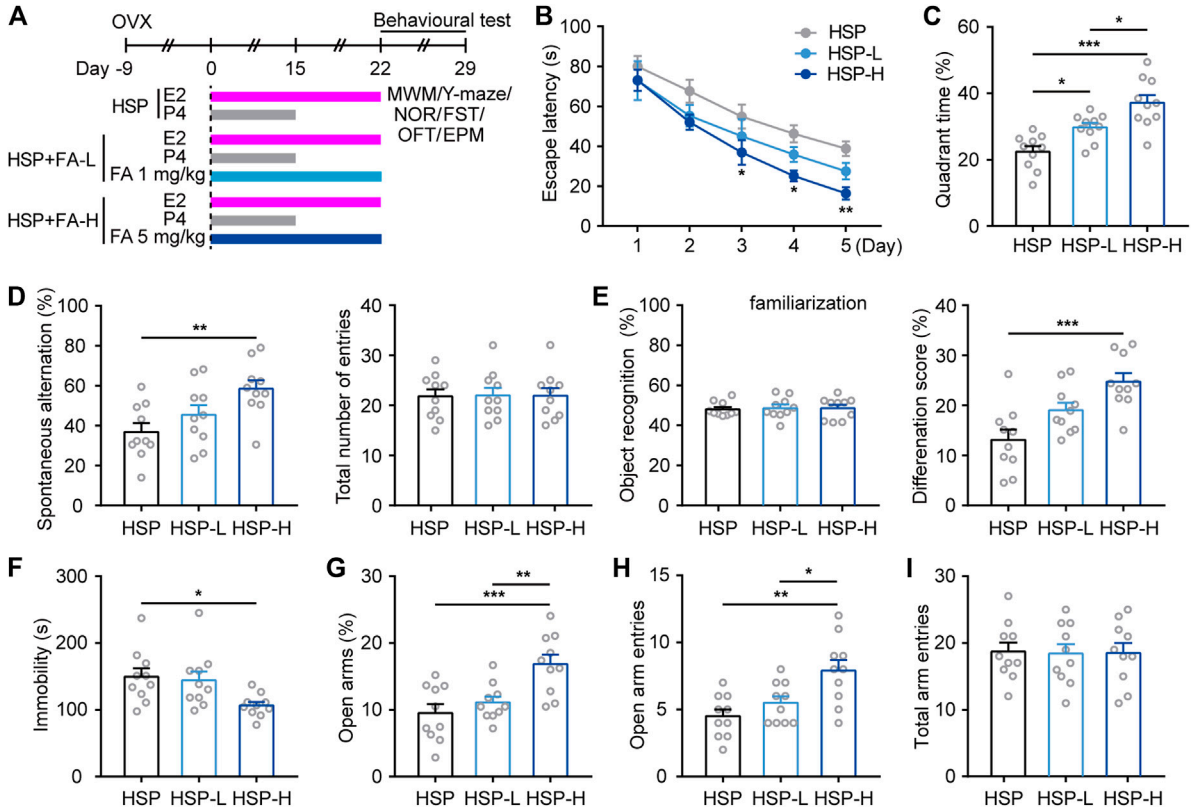

FIGURE 2 | Effects of FA on cognitive- and emotional-related behaviors in female HSP mice. **(A)** Schematic representation of the experimental procedure for assessing the effects of FA administration in HSP mice. **(B)** Escape latency to the hidden platform in the training phase test of the MWM. Two-way repeated measures ANOVA was used to analyze the latency during the navigation training: $F(4, 36) = 45.19$, $p < 0.0001$. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ HSP-H compared with HSP. **(C)** The percentage of time spent exploring the target quadrant. One-way ANOVA: $F(2, 27) = 16.31$, $p < 0.0001$. **(D)** The spontaneous alteration rate and total number of entries were analyzed in the Y-maze task. One-way ANOVA: $F(2, 27) = 5.796$, $p = 0.0081$. One-way ANOVA: $F(2, 27) = 0.004526$, $p = 0.9955$. **(E)** FA significantly improved the ability to discriminate a novel object with a familiar object in HSP mice while had little effect on the preference for objects during familiarization. One-way ANOVA: $F(2, 27) = 0.06243$, $p = 0.9396$. One-way ANOVA: $F(2, 27) = 10.89$, $p = 0.0003$. **(F)** FA-treated mice also exert less immobility in the FST. One-way ANOVA: $F(2, 27) = 4.464$, $p = 0.0211$. **(G)** FA administration increased open arm exploring time in HSP mice. One-way ANOVA: $F(2, 27) = 9.777$, $p = 0.0006$. FA administration increased open arm entries number in HSP mice **(H)**, with little changes on total arm entries **(I)**. One-way ANOVA: $F(2, 27) = F(2, 27) = 15.25$, $p < 0.0001$. One-way ANOVA: $F(2, 27) = 0.1126$, $p = 0.8939$. Data were presented as scatter points plus mean ± SEM. $n = 10$ for each group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

object recognition during familiarization phase among three groups (**Figures 3D,E**). Moreover, in the despair-like behavior measured in the FST, the immobility time was significantly shorter in the FA-H group than in the control group (**Figure 3F**). In the EPM test, the FA-H group mice exhibited more time and entries into the open arms than the control group (**Figures 3G,H**). No differences were observed between the three groups in the total arm entries in the EPM test (**Figure 3I**). The OFT study (**Figure 3J**) further confirmed this trend, suggesting that the FA-H group had a significant improvement in learning, memory, and emotional performance.

Gestational Folic Acid Administration on Pregnancy Outcomes and Offspring Development

Previous FA results on postpartum outcomes were inspiring. Thereafter, we examined whether gestational FA administration had a negative effect on pregnancy or offspring outcomes. FA administration had little effect on maternal body weight gain (**Figure 4A**), gestational length (**Figure 4B**), or abortion during pregnancy. No significant differences were observed in the litter size and sex ratio of the offspring (**Figures 4C,D**). Average pup weights per litter were recorded until P21, and there was no statistical significance in any group (**Figure 4E**). Gestational FA supplementation had little negative effect on pregnancy and offspring general development.

Gestational Folic Acid Administration Promotes Hippocampal Neurogenesis in Postpartum Female Mice

The hippocampus is a key brain region implicated in cognitive and mood disorders. Since neurogenesis plays an important role in the above process and thus contributes to the pathological process of the disease, we assessed neurogenesis-related molecular changes in FA-treated postpartum female mice. Immunofluorescence staining showed increased BDNF expression in the FA-H group

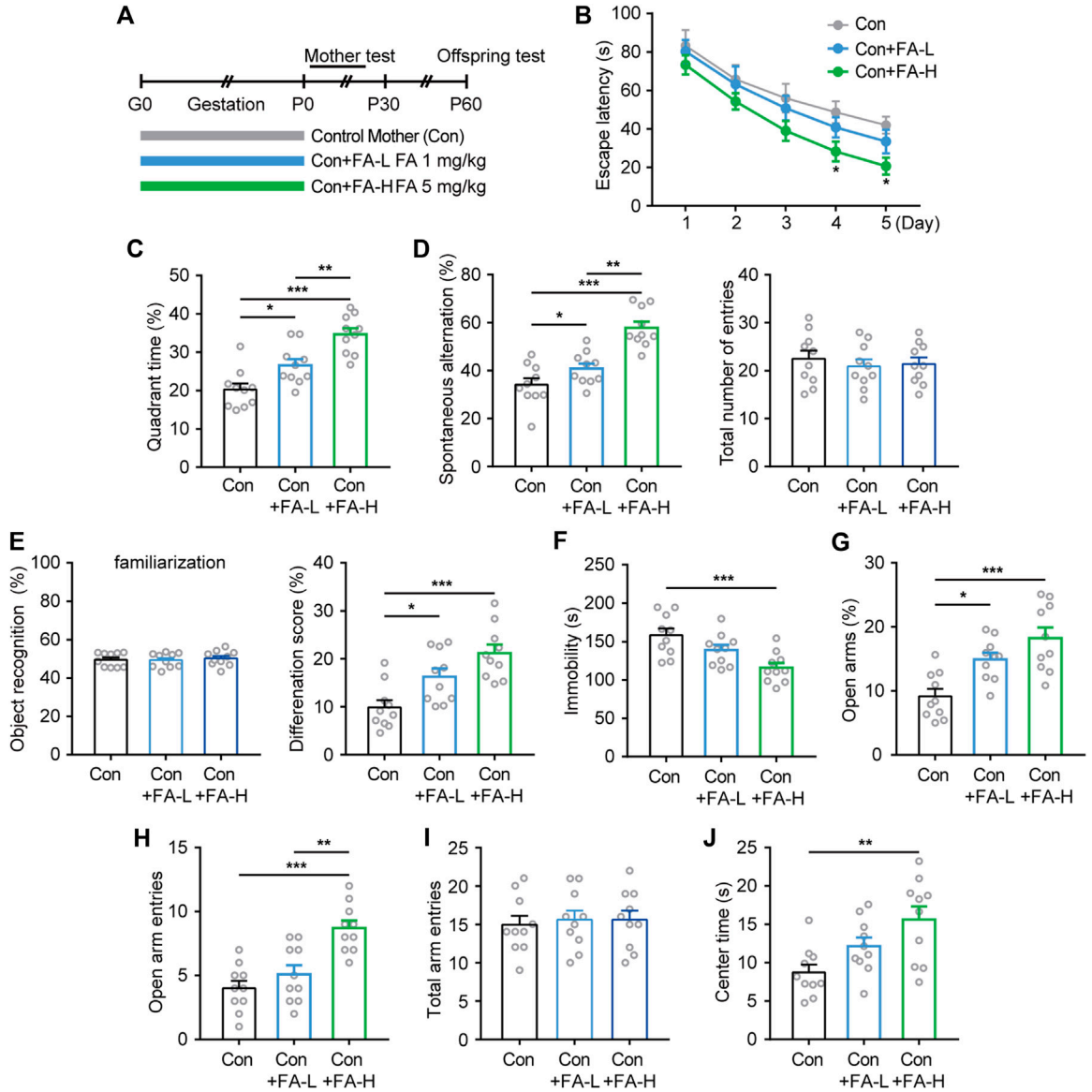

FIGURE 3 | Effects of FA on cognitive- and emotional-related behaviors on postpartum female mice. **(A)** Schematic diagram of the experimental workflow on maternal and offspring. **(B)** Latency to reach the hidden platform in the training phase of the MWM. Two-way repeated measures ANOVA was used to analyze the latency during the navigation training: $F(4, 36) = 31.08$, $p < 0.0001$. $^{*}p < 0.05$ Con + FA-H compared with Con. **(C)** FA administration increase the time spent exploring the target quadrant. One-way ANOVA: $F(2, 27) = 20.45$, $p < 0.0001$. **(D)** The spontaneous alteration rate and total number of entries were analyzed in the Y-maze task. One-way ANOVA: $F(2, 27) = 24.46$, $p < 0.0001$. One-way ANOVA: $F(2, 27) = 0.2525$, $p = 0.7787$. **(E)** Novel object discrimination were significantly improved after FA administration in postpartum female mice while had little effect on the preference for objects during familiarization. One-way ANOVA: $F(2, 27) = 0.2078$, $p = 0.8137$. One-way ANOVA: $F(2, 27) = 11.84$, $p = 0.0002$. **(F)** FA administration decreased the immobility time in the FST in postpartum female mice. One-way ANOVA: $F(2, 27) = 8.665$, $p = 0.0012$. The EPM results of time spent in open arms **(G)**, the frequency of entries into open arms **(H)**, and total arm entries **(I)** revealed treatment effects on anxiety. One-way ANOVA: $F(2, 27) = 12.36$, $p = 0.0002$. One-way ANOVA: $F(2, 27) = 15.25$, $p < 0.0001$. One-way ANOVA: $F(2, 27) = 0.1126$, $p = 0.8939$. **(J)** FA administration increased OFT center area exploring in postpartum female mice. One-way ANOVA: $F(2, 27) = 6.74$, $p = 0.0042$. Data were presented as scatter points plus mean ± SEM. $n = 10$ for each group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

mice (**Figure 5A**). Subsequently, WB assays were conducted to confirm this result. The expression of BDNF in hippocampal tissue was significantly increased in the FA-H group compared to that in the control group (**Figure 5B**). Further verification was provided by Western blotting analysis of neurogenesis-related pathway proteins. Compared with the control group, in the hippocampus of FA-H group mice, the protein expression of TrkB, p-PIK3, p-AKT, and p-mTOR increased. However, this increase was not associated with an increase in total PIK3, AKT, and mTOR expression (**Figure 5B**). Hippocampal neurogenesis was evaluated using neuroepithelial stem cells (marked by Nestin) and mitotic process neural stem cells

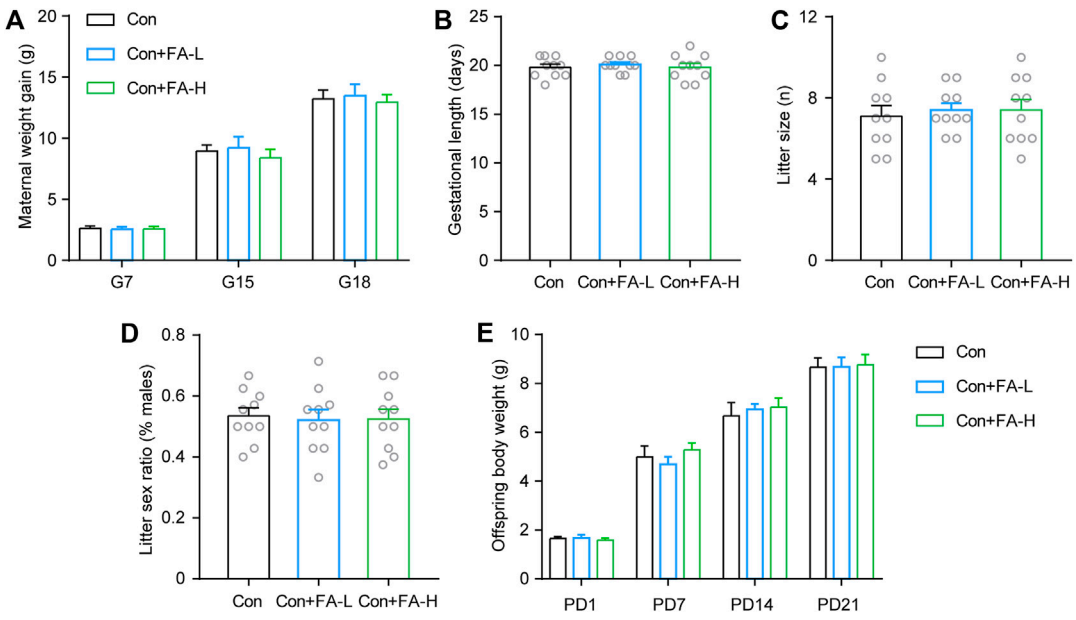

FIGURE 4 | Effects of gestational FA on pregnancy outcomes and offspring characteristics. FA administration during pregnancy had no significant effect on maternal body weight **(A)**, length of gestation **(B)**, dam litter size **(C)**, fraction of pup sex **(D)**, and offspring body weight during development **(E)**. Two-way repeated measures ANOVA was used to analyze the maternal body weight: F (2, 18) = 0.2421, p = 0.7875. Length of gestation via One-way ANOVA: F (2, 27) = 0.2691, p = 0.7661. Dam litter size via One-way ANOVA: F (2, 27) = 0.1357, p = 0.8737. Fraction of pup sex via One-way ANOVA: F (2, 27) = 0.04937, p = 0.9519. Two-way repeated measures ANOVA was used to analyze the offspring body weight: F (2, 18) = 0.3204, p = 0.7299. Data are presented as scatter points plus the mean ± SEM. n = 10 for each group.

(BrdU) in the dentate gyrus (DG). Higher dose FA pretreatment markedly increased the number of Nestin- and BrdU-positive cells in the DG of postpartum female mice when compared with control mice (**Figures 5C,D**). No significant alterations in neurogenesis were observed in mice treated with lower doses of FA.

Gestational Folic Acid Administration Rescued the Synaptic Mechanisms in the Hippocampus of Postpartum Female Mice

To further assess the brain mechanism implied in the therapeutic effect of FA on postpartum female mice, we examined the effects of FA on the expression of synaptic plasticity-related proteins in the hippocampus. The relative expression of synaptophysin, Synapsin-1, and PSD95 proteins was higher in the FA-H group than in the FA-L or Con mice without FA (**Figure 6A**). No differences were found between the FA-L and control groups without FA. We then examined basal synaptic transmission at the CA3-CA1 synapses through fEPSP recordings. In line with the previous results, the FA-H group mice showed clear synaptic enhancement. While both groups exhibited effective enhancement of synaptic transmission, LTP in FA-H slices increased to a greater extent after stimulation, compared to the control group, while no significant difference in LTP was detected between the control + FA-L and Con group mice (**Figures 6B–D**). These results suggest that FA administration enhances synaptic transmission in postpartum female mice.

DISCUSSION

The occurrence of psychiatric disorders after childbirth is well known in postpartum women. However, the treatment options for these symptoms during this special period remain limited, considering the far-reaching toxicity consequences for offspring via *in utero* or breast milk residue. A dietary FA supplements have been well recognized to be helpful, incurring beneficial effects on the nutritional requirements of lactating mothers. In the present study, we further confirmed that gestational FA administration also significantly contributed to the alleviation of emotional and cognitive dysfunction in postpartum female mice.

FA is involved in a variety of metabolic processes and represents an essential micronutrient for fetal development. Currently, there is a consensus that folate supplements during pregnancy help prevent developmental defects caused by FA deficiency in offspring. The birth of a child garners the attention of the entire family, including the parents, while the developmental defects of the offspring are easy to observe and qualitatively assess. However, the postpartum status of mothers garners much less attention and is easily ignored. Although previous studies have found that there may not be a direct correlation between low folate levels and postpartum depression (Lewis et al., 2012), epidemiological evidence has shown that supplementation of folic acid during pregnancy was inversely associated with the manifestation of pregnancy-associated depression (Xu et al., 2014; Yan et al., 2017). Moreover, associations of functional gestational FA supplements with post-

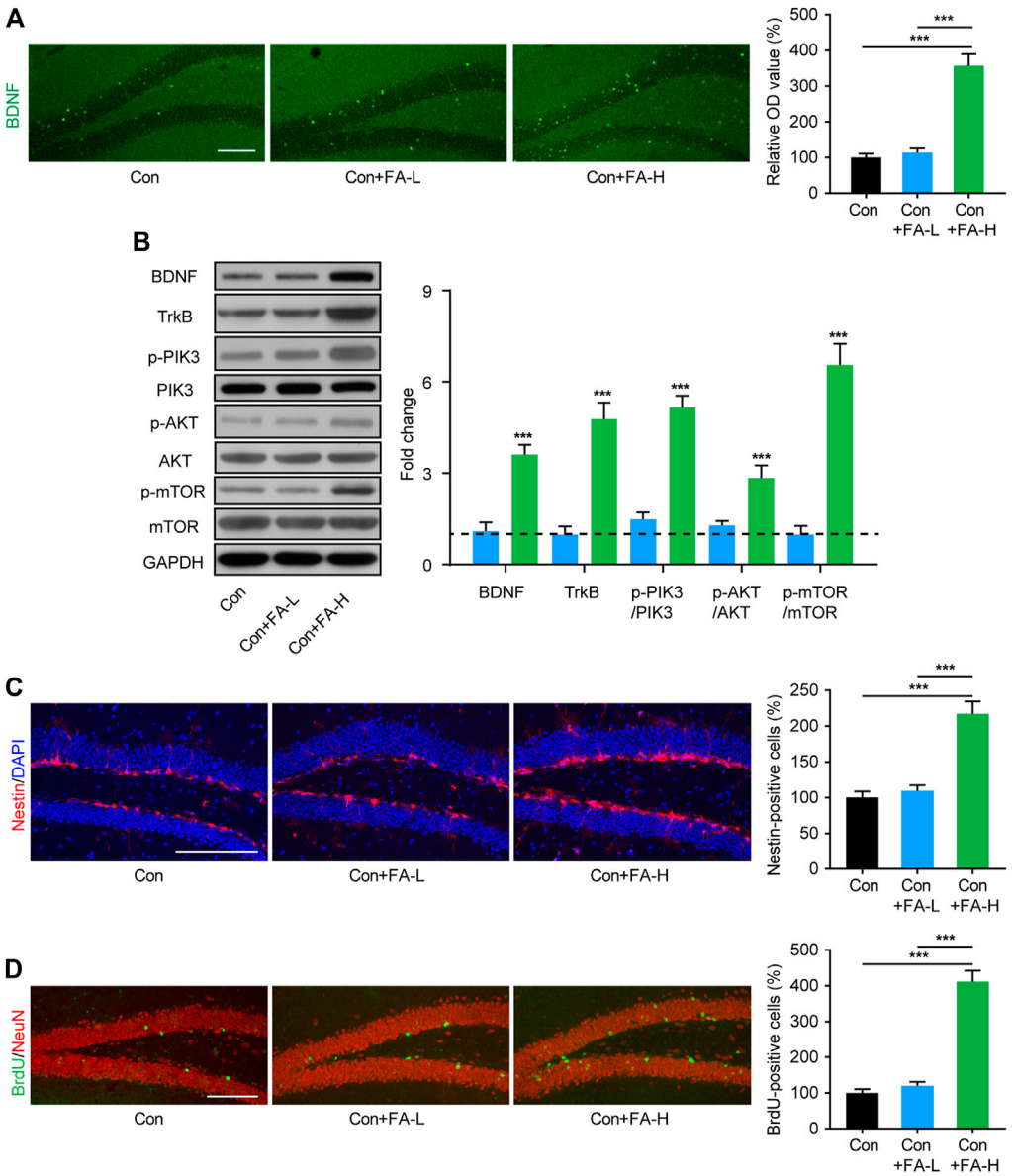

FIGURE 5 | Gestational FA administration promotion of neurogenesis and related molecular expression in postpartum female mice. **(A)** Representative immunohistochemical images for BDNF (Green) in the hippocampus. Histogram showing average fluorescence intensity in O.D. value. One-way ANOVA: $F(2, 15) = 47.97$, $p < 0.0001$. Scale bar = 100 μm. **(B)** Representative immunoblots and quantifications showing BDNF, TrkB, p-PIK3/PIK3, p-AKT/AKT, and p-mTOR/mTOR expression in the hippocampus. BDNF One-way ANOVA: $F(2, 15) = 36.87$, $p < 0.0001$. TrkB One-way ANOVA: $F(2, 15) = 61.06$, $p < 0.0001$. p-PIK3/PIK3 One-way ANOVA: $F(2, 15) = 103.2$, $p < 0.0001$. p-AKT/AKT One-way ANOVA: $F(2, 15) = 21.66$, $p < 0.0001$. p-mTOR/mTOR One-way ANOVA: $F(2, 15) = 70.2$, $p < 0.0001$. The Con group was normalized as a dotted line, and the statistical results were compared with the Con group. **(C)** The immunohistochemistry staining and quantification of Nestin + cells in hippocampus of each group. Nestin (red) and DAPI (blue). One-way ANOVA: $F(2, 15) = 28.99$, $p < 0.0001$. Scale bar = 100 μm. **(D)** The immunohistochemistry staining and quantification of BrdU + cells in hippocampus of each group. BrdU (green) and NeuN (red). One-way ANOVA: $F(2, 15) = 76.08$, $p < 0.0001$. Scale bar = 100 μm. Data were presented as mean ± SEM. Scale bar = 100 μm n = 6 for each group. $^{**}p < 0.01$; $^{***}p < 0.001$.

partum cognition improvement have also been clinically validated (Prado et al., 2018). These results suggest that FA administration may be a suitable treatment strategy in such postpartum patients, and we tested this hypothesis for the first time in a mouse experimental model. To the best of our knowledge, this is the first experimental study to focus on the effects FA supplements on postpartum behavioral outcomes.

As a global mental health problem, depression has a higher incidence rate in women than in men. Moreover, women in the perinatal period (prepartum and postpartum) are at a higher risk of depression. Traditional antidepressants, such as serotonin reuptake inhibitors, have been found to cause negative effects on offspring when administered during pregnancy, which limits their usage and presents clinical challenges. This depressive

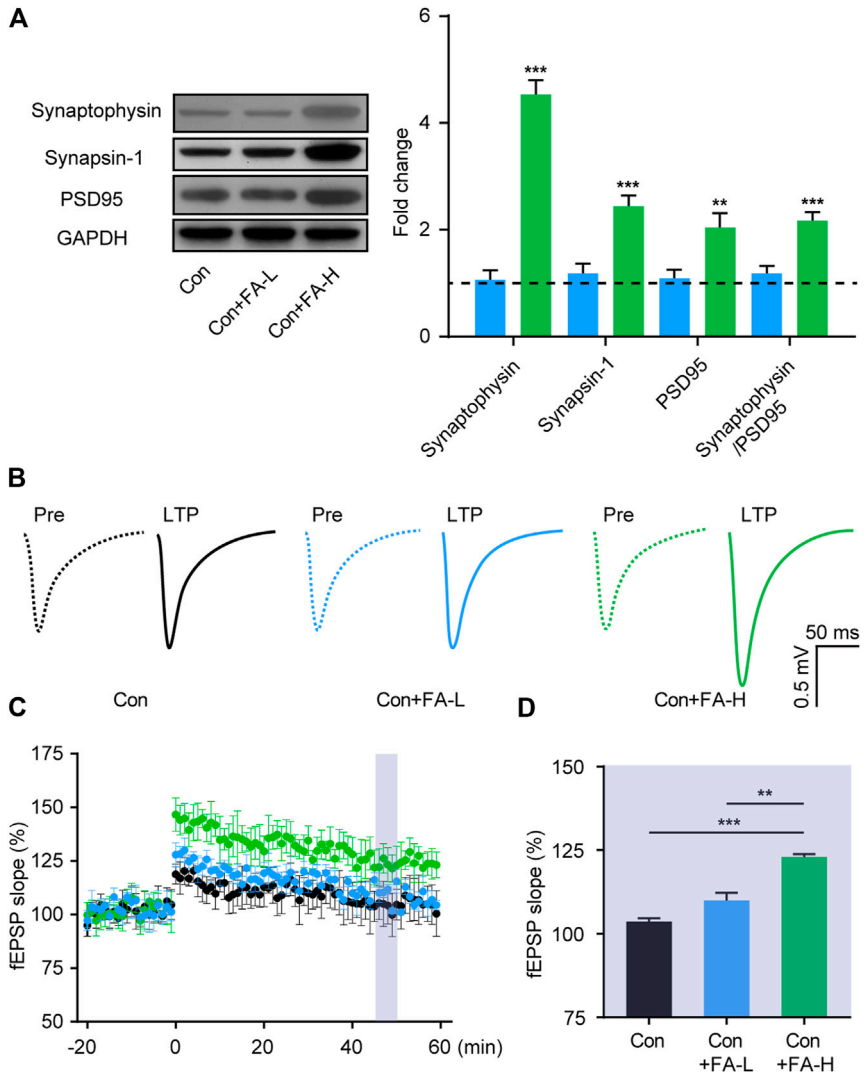

FIGURE 6 | Gestational FA administration promotes synaptic plasticity in postpartum female mice. **(A)** Representative immunoblots and quantifications showing Synaptophysin, Synapsin-1 and PSD95 expression in the hippocampus. Synaptophysin One-way ANOVA: $F(2, 15) = 79.19$, $p < 0.0001$. Synapsin-1 One-way ANOVA: $F(2, 15) = 33.79$, $p < 0.0001$. PSD95 One-way ANOVA: $F(2, 15) = 11.23$, $p = 0.0010$. Synaptophysin/PSD95 One-way ANOVA: $F(2, 15) = 17.57$, $p = 0.0001$. The Con group was normalized as a dotted line, and the statistical results were compared with the Con group. **(B)** Representative traces of fEPSP responses during LTP induction. Scale, 0.5 mV, 50 ms. **(C)** Time-course responses of the evoked LTP potentials following high frequency stimulation (HFS) at CA3-CA1 synapses. The points in the time scale axis were normalized to the average baseline response to show the fEPSP slope. **(D)** Area in the shadow showed the averaged %fEPSP slope during 46–50 min after stimulation in each group. One-way ANOVA was used to analyze the LTP during 46–50 min: $F(2, 15) = 13.22$, $p = 0.0005$. Data were presented as mean ± SEM. $n = 6$ for each group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

phenotype has been well-documented in the HSP hormone withdrawal postpartum model and in postpartum animals (Li et al., 2018; Shoji and Miyakawa, 2019). Our results provide solid evidence to validate the hypothesis pointing to therapeutic effects of FA on postpartum depression (Behzadi et al., 2008). To better mimic this condition in natural situations, this FA supplementary paradigm model was applied to natural pregnancy animals during the gestational period. The effective dose in the HSP model also demonstrated an encouraging rescue effect. Although there are currently no reports in the literature regarding the effect of FA on PAD, it is striking that up to 68.9% PDD also reported anxiety symptoms (Nakić Radoš et al., 2018). Previous studies have reported that HSP mice exhibit anxiety-related behaviors (Zhang et al., 2016; Yang et al., 2017). In the present study, we showed that FA treatment greatly increased open arm exploration in the EPM and center area time in the OFT in HSP and natural postpartum mice, which indicated that FA administration also exerts anxiolytic effects. Despite the emotional symptoms, cognitive deficits have also been observed in the postpartum period (Henry and Rendell, 2007; Pio de Almeida et al., 2012) thereby demonstrating a need for new therapeutic drugs with fewer side effects. Our findings indicate that treatment with FA during pregnancy improves the hippocampus-dependent cognition score, which is consistent

with previous results showing that FA could improve visuospatial ability (Prado et al., 2018). A range of emotional and cognitive symptoms were effectively resolved with regard to the treatment outcomes, considering that the hippocampus is primarily responsible for exerting emotional and cognitive functions, which prompted us to look for hippocampus-related mechanisms that could explain the efficacy of FA.

Elevated nutrition metabolism plays a crucial role in satisfying fetal metabolic demands during pregnancy, contributing to lower maternal levels of BDNF both before and after childbirth (Lommatzsch et al., 2006). BDNF is closely linked to cognition and mood, and plays an important role in neurogenesis and synaptic plasticity. To reveal the possible molecular mechanisms by which FA exerts emotional and cognitive rescue effects, we focused on the changes in BDNF-related pathway expression. In accordance with the previous finding that FA could increase hippocampal BDNF levels in stressed rats (Gao et al., 2017) and neonatal hypoxia-ischemia offspring (Deniz et al., 2018), our results showed that gestational FA caused significant changes in the expression of BDNF-related signaling in the hippocampus of postpartum female mice, thus shedding light on a potential working mechanism. Low BDNF levels correlate with low serotonin (5-HT) levels during peripartum periods (Lommatzsch et al., 2006), and BDNF could interact with the brain 5-HT-system, thus affecting 5-HT expression (Popova et al., 2017). However, the 5-HT related pathway was not assessed in this study, although this association may shed light on a deeper functional interpretation, considering that 5-HT is a key central player in the maintenance of normal brain function. Further research is warranted in order to fully understand the biological mechanisms underlying these associations.

BDNF has multiple important roles in brain development, including supporting the survival and differentiation of selected neuronal populations and modulating synaptic transmission and plasticity. Since neurogenesis plays a central role in cognition and emotion, we monitored neurogenesis after FA administration in postpartum mice. Hippocampal neurogenesis perturbations in rodents during pregnancy are well-known (Kim et al., 2010), such as in the form of PDD, PAD, and PCD, which are linked to emotional and cognitive performance. Here, we examined the immunohistochemical staining results of two stage-specific markers: nestin and BrdU. FA administration enhanced neurogenesis in the hippocampus, as indicated by an increase in Nestin- and BrdU-positive cells. The increased number of generated neurons integrate into the neural ensemble to alter behavioral phenotypes. Neuroelectrophysiological results of LTP and the expression of protein markers related to synaptic transmission consistently proved that FA has a positive impact on synaptic connectivity and subsequent cognition or emotional behavior. These hippocampal mechanisms may act symbiotically to exert their functional effects.

The regular FA dose contained in mice food chow is approximately 1–2 mg/day. Accordingly, we chose two doses of FA in this study. Based on the observed efficacy, the 5 mg/kg dose exerted a much better and more stable effect on the symptoms we observed. The potential gestional toxicity of these two FA doses was preliminary evaluated in this study. Data shown that these two doses had little effect on offspring birth and their body weight development. A dose of 5 mg/kg of FA appears to be safe in mice, both in mothers and offspring. However, additional pharmacological and toxicological studies are needed to prove safety. Though there remain limited potential health hazards data available on humans, these results offers evidence on clinical trial safety data and provide direct evidence of the preclinical potential of FA in postpartum women.

In summary, we systematically observed the effect of FA on common emotional and cognitive complications of pregnancy in experimental HSP and natural pregnant mice. The data obtained shed light on possibilities for clinical investigations of the effects of FA on brain functional parameters in postpartum women. These behavioral improvements were associated with increased BDNF-related pathway expression, neurogenesis, and synaptic transmission in the hippocampus. The validation of FA may help to unveil the biological basis of mental-related behaviors in pregnant women. Although data from further clinical trials are needed for more definitive conclusions regarding the best dietary intervention doses to improve outcomes, these results remain encouraging for pregnant women since FA may not only prevent neural tube defects, but also gestational mineral syndromes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of Guangdong Women and Children Hospital.

AUTHOR CONTRIBUTIONS

All author take part in designed the research, performed experiments, analyzed the data, and wrote the paper. QZ performed the illustrations of the data. All authors have read and approved the final manuscript.

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ORIGINAL RESEARCH
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Estrogen Deficiency Induces Mitochondrial Damage Prior to Emergence of Cognitive Deficits in a Postmenopausal Mouse Model

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Background: Estrogen deficiency contributes to the development of Alzheimer's disease (AD) in menopausal women. In the current study, we examined the impact of estrogen deficiency on mitochondrial function and cognition using a postmenopausal mouse model.

Methods: Bilateral ovariectomy was conducted in adult females C57BL/6J. Cognitive function was examined using the Morris water maze (MWM) test at 2 weeks, 1, 2, and 3 months after ovariectomy. Neurodegeneration was assessed using an immunofluorescence assay of microtubule-associated protein 2 (MAP2) in the hippocampus and immunoblotting against postsynaptic density-95 (PSD95). Mitochondrial function in the hippocampus was assessed using immunoblotting for NDUFB8, SDHB, UQCRC2, MTCO1, and ATP5A1. Mitochondrial biogenesis was examined using immunoblotting for PGC-1α, NRF1, and mtTFA. Mitochondrion fission was assessed with immunoblotting for Drp1, whereas mitochondrion fusion was analyzed with immunoblotting for OPA1 and Mfn2. Mitophagy was examined with immunoblotting for PINK1 and LC3B. Mice receiving sham surgery were used as controls.

Results: Ovariectomy resulted in significant learning and memory deficits in the MWM test at 3 months, but not at any earlier time points. At 2 weeks after ovariectomy, levels of Drp1 phosphorylated at Ser637 decreased in the hippocampus. At 1 month after ovariectomy, hippocampal levels of NDUFB8, SDHB, PGC-1α, mtTFA, OPA1, and Mfn2 were significantly reduced. At 2 months after ovariectomy, hippocampal levels of MAP2, PSD95, MTCO1, NRF1, and Pink1 were also reduced. At 3 months, levels of LC3B-II were reduced.

Conclusions: The cognitive decline associated with estrogen deficiency is preceded by mitochondrial dysfunction, abnormal mitochondrial biogenesis, irregular mitochondrial dynamics, and decreased mitophagy. Thus, mitochondrial damage may contribute to cognitive impairment associated with estrogen deficiency.

Keywords: estrogen, ovariectomy, cognitive function, mitochondrial biogenesis, mitochondrial dynamics, mitophagy, hippocampus

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease affecting over 6 million individuals globally, which is projected to increase to 14 million by 2060 (Matthews et al., 2019). AD is characterized by progressive memory loss, cognitive impairment, and abnormal behavior (Selkoe, 2001). Commonly recognized histopathological features of AD include intracellular neurofibrillary tangles (NFTs), extracellular amyloid-β (Aβ) deposition, neuron loss, synaptic damage, as well as changes in mitochondrial structure and function (Bloom, 2014; Fu et al., 2017; Wang et al., 2017; Reddy et al., 2018).

Many studies have indicated that abnormal mitochondrial bioenergetics is an important pathological feature of several neurodegenerative diseases (Swerdlow, 2018; Fišar et al., 2019). In order to maintain their function, neurons require substantial energy. As the main energy producer, mitochondria are crucial to the survival and proper function of neurons. Mitochondria produce ATP to maintain a variety of basic synaptic functions including ion gradients across the cell membrane, the release and circulation of synaptic vesicles, and the plasticity of synapses (Attwell and Laughlin, 2001; Li et al., 2004; Lee and Peng, 2008; Sun et al., 2013; Rangaraju et al., 2014). Mitochondria are highly dynamic organelles that constantly fluctuate with the changing metabolic and physiological needs of cells. The morphology and number of mitochondria are controlled through fusion and fission (Karbowski and Youle, 2003; Santel and Frank, 2008).

Since mitochondria play a crucial role in neuronal health, it is tempting to hypothesize that mitochondrial abnormality may contribute to early pathological changes in AD and ultimately to the characteristic cognitive impairments. Indeed, in the 3xTg mouse model of AD, mitochondrial bioenergy deficiency appears earlier than the pathological features of AD such as abnormal cognitive behavior, NFTs, and Aβ (Yao et al., 2009). Similarly, a mitochondrial abnormality is one of the earliest and most prominent features in the hippocampus of AD patients, occurring even before the appearance of Aβ and NFTs (Manczak et al., 2004). Whether estrogen deficiency leads to mitochondrial defects that precede and, therefore, help cause later AD pathological changes in postmenopausal women is unclear.

Epidemiological studies have shown that two-thirds of AD patients are women, which has been attributed to their longer average lifespan (Farrer et al., 1997). Increasing evidence shows that estrogen deficiency after menopause is associated with an increased risk of AD (Paganini-Hill and Henderson, 1994; Yue et al., 2005; Yao et al., 2012; Villa et al., 2016). Ovariectomy of premenopausal women increases the risk of AD by 40% (Rocca et al., 2010). The average woman experiences menopause around age 50 (Zhu et al., 2019), and the average age of AD onset in females is around 80 years (Beam et al., 2018). Several studies have demonstrated that estrogen replacement therapy can reduce or delay the onset of AD (Tang et al., 1996; Yaffe et al., 1998; van Duijn, 1999). A recent multimodal brain imaging study showed that a decrease in circulating estrogen is the main risk factor for female-specific brain abnormalities in AD (Rahman et al., 2020).

How estrogen deficiency leads to AD remains unclear, and it may involve perturbation of mitochondrial activity. Many estrogen-regulated signaling pathways are concentrated in mitochondria (Nilsen and Diaz Brinton, 2003; Brinton, 2008). Ovarian-produced 17β-estradiol (E2) is the most commonly circulating estrogen in women and can increase the activity and expression of several mitochondrial proteins involved in cellular respiration by acting on estrogen receptors in the mitochondria, namely complex Iβ subunit 8, complex IV, and complex V (Nilsen et al., 2007; Irwin et al., 2012). E2 stimulates the skeletal muscle to promote mitochondrial biogenesis, proliferation, and oxidative capacity (Capllonch-Amer et al., 2014). In the 3xTg AD mouse model, E2 can promote mitochondrial biogenesis, prevent free radical damage, and upregulate Aβ-degrading enzymes to reduce Aβ deposition (Brinton, 2009; Simpkins et al., 2010; Zhao et al., 2011). The function of estrogen is usually regulated by nuclear receptors, estrogen receptor alpha (ERα), and estrogen receptor beta (ERβ; Katzenellenbogen, 1996; Veenman, 2020), as well as membrane receptors, G protein-binding estrogen receptor-1 (GPER-1; Liu et al., 2009). ERα and ERβ are widely distributed in the central nervous system (Pérez et al., 2003). Many studies have found the presence of mitochondrial estrogen receptors (Yager and Chen, 2007; Mitterling et al., 2010; Irwin et al., 2012). ERα and ERβ were shown to directly bind mitochondrial DNA *in vitro* through mitochondrial estrogen response elements, and the binding response was increased with exposure to E2 (Chen et al., 2004). Estrogen through ERβ-mediated regulation of mtDNA transcription to regulate mitochondrial function (Yang et al., 2004; Irwin et al., 2012).

Using a postmenopausal mouse model, we explored here whether and how estrogen deficiency affects mitochondria, and whether this, in turn, relates to neuronal damage and cognitive impairment. Understanding the estrogen-dependent role of dynamic changes of hippocampal mitochondria in postmenopausal AD pathology will develop targeted drugs to better prevent or delay the occurrence of AD.

MATERIALS AND METHODS

Animals

Seventy-two adult female C57BL/6J mice at 3 months of age, weighing 20–25 g (Shandong Skobas Biotechnology, Jinan, China) were used in the experiments. Animals were housed in plastic cages with controlled temperature (24 ± 2°C) and humidity (40–50%), and kept on a 12-h light/dark cycle. Mice had free access to food and water. Experimental protocols were approved by the Committee of Animal Experimental Ethics of Shandong First Medical University and conducted in accordance with the US National Institutes of Health "Guide for the Care and Use of Laboratory Animals."

Experimental Design

Mice were randomly divided into two groups (n = 36 per group): sham operation (Sham) and ovariectomy operation (OVX). After a 7-day adaptation period, sham operation or bilateral ovariectomy was performed under anesthesia with 0.3% sodium

pentobarbital (0.1 ml/10 g) administered by intraperitoneal (IP) injection. A longitudinal incision was made inferior to the rib cage on the dorsolateral body wall. Mice subjected to the sham surgical procedures had a piece of fat excised from the body wall. In the OVX group, the bilateral ovaries were exteriorized, ligated, and excised. Eight mice from each group were sacrificed at 2 weeks, 1, 2, and 3 months after surgery.

Behavioral testing was performed 6 days before mice were sacrificed. After sacrifice, blood samples were collected from the orbital sinus, and serum estradiol was measured by enzyme-linked immunosorbent assay (ELISA). Neurons were identified based on immunofluorescence of microtubule-associated protein 2 (MAP2) on sections of the hippocampus. Total protein was extracted from hippocampal tissue upon sacrifice and analyzed by immunoblotting to assess synapses based on synaptic protein postsynaptic density-95 (PSD95); mitochondrial function, based on NADH: ubiquinone oxidoreductase subunit B8 (NDUFB8), Succinate dehydrogenase B (SDHB), Ubiquinol-cytochrome c reductase core protein 2 (UQCRC2), Mitochondrion cytochrome c oxidase subunit 1 (MTCO1), and ATP synthetase F1 complex α subunit (ATP5A1); mitochondrial biogenesis, based on peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α), nuclear respiratory factor 1 (NRF1), and mitochondrial transcription factor A (mtTFA); mitochondrial dynamics, based on dynamin-related protein 1 (Drp1), optic atrophy 1 (OPA1), and mitofusin 2 (Mfn2); and mitophagy, based on Phosphatase and Tensin Homolog -induced putative kinase 1 (PINK1) and microtubule-associated protein B (LC3B).

Morris Water Maze (MWM) Test

The cognitive function of mice was evaluated by the Morris water maze (MWM) test as previously described (Bromley-Brits et al., 2011). Briefly, mice were trained by successively placing them in the water at a location equidistant from the target platform in each quadrant for 5 days. During each trial, the mouse was allowed 60 s to locate the target platform by itself. If it failed to find the platform within 60 s, the experimenter placed the mouse on the platform for 10 s and the time required to reach the platform (escape latency) was recorded as 60 s. On day 6, the target platform was removed and the time spent by the mouse in the target quadrant where the platform had been and the number of times the mice crossed into the target quadrant was recorded for 60 s. In order to exclude variations caused by the circadian rhythm, animals were trained and tested each day between 10:00 AM and 5:00 PM. The tracking information was processed by the Topscan Package (Clever Sys Inc.).

Serum Estradiol ELISA

Blood samples were collected from the orbital sinus and serum was separated by centrifugation at 1,500× *g* for 15 min at 4°C. Serum estradiol was quantified using a mouse E2 ELISA kit (ml063198, Shanghai Enzyme-linked Biotechnology, Shanghai, China) according to the manufacturer's instructions. Absorbance at 450 nm was recorded with a multifunctional microplate reader (TECAN, Switzerland).

Immunofluorescence Analysis

Whole brains were collected, post-fixed in buffered 4% paraformaldehyde overnight at 4°C, dehydrated in a graded ethanol series, and embedded in paraffin. The tissue was sectioned to a thickness of 4 μm and then mounted on glass slides. The sections were dried in an oven at 60°C and stored at room temperature. The sections were deparaffinized, rehydrated, and subjected to antigen retrieval, then rinsed in distilled water. The sections were blocked in 3% bovine serum albumin (BSA; G5001, Servicebio, Wuhan, China) for 30 min, and then incubated with anti-MAP2 antibody (GB11128-2, 1:200, Servicebio) overnight at 4°C. The next morning, the sections were washed in distilled water and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (GB23303, 1:200, Servicebio) for 1 h in darkness at room temperature, then incubated with 4′,6-diamidino-2-phenylindole (DAPI, G1012, Servicebio) for 10 min. The sections were washed in phosphate-buffered saline (PBS), incubated with spontaneous fluorescence quenching reagent (GB1221, Servicebio) for 5 min, washed again in PBS, and mounted on microscope slides with an anti-fade mounting medium. Sections were observed under a fluorescence microscope (Axioscope 5, Carl Zeiss, Jena, Germany), and images were captured with a slice scanner (Pannoramic MIDI, Danjier, Jjinan, China) and processed with Image-Pro Plus software version 6.0 (Media Cybernetics Corp., Bethesda, MD).

Immunoblotting Analysis

Proteins were isolated from brain tissue and analyzed *via* Western blotting by standard methods (Zhou et al., 2021). Briefly, the hippocampus was homogenized in the presence of protease and phosphatase inhibitors (P1206, Solarbio, Shanghai, China), then centrifuged at 14,500× *g* for 10 min at 4°C. The supernatants were collected and the total protein concentration was measured by BCA assay (PC0020, Solarbio). Samples were run on a 10 or 12% SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane (IPVH00010, Merck Millipore, Darmstadt, Germany). Membranes were blocked in 5% milk for 2 h at room temperature and stained sequentially for proteins of interest. For each protein, membranes were incubated with a primary antibody overnight at 4°C, washed in tris buffered saline with 0.1% Tween 20, and incubated with the relevant secondary antibody for 1 h at room temperature before a final wash.

Primary antibodies were against the following proteins: PSD95 (ab18258, 1:1,000, Abcam, Cambridge, UK), NDUFB8 (ab192878, 1:4,000, Abcam), SDHB (ab14714, 1:1,000, Abcam), UQCRC2 (ab203832, 1:1,000, Abcam), MTCO1 (ab203912, 1:2,000, Abcam), ATP5A1 (ab14748, 1:1,000, Abcam), PGC-1α (ab54481, 1:2,000, Abcam), NRF1 (46743, 1:1,000, Cell Signaling Technology, Danvers, MA, USA), mtTFA (ab252432, 1:2,000, Abcam), Mfn2 (ab56889, 1:1,000, Abcam), OPA1 (ab157457, 1:1,000, Abcam), Drp1 (ab184247, 1:1,000, Abcam), p-Drp1 (ser 637; ab193216, 1:1,000, Abcam), LC3B (ab192890, 1:1,000, Abcam), PINK1(ab23707, 1:1,000, Abcam), β-actin (TA-09, 1:2,000, Zhongshan Golden Bridge, Beijing, China), and GAPDH (TA-08, 1:2,000, Zhongshan Golden Bridge). The

horseradish peroxidase-conjugated secondary antibodies were goat anti-mouse (ZB-2305, 1:3,000, Zhongshan Golden Bridge) and goat anti-rabbit (ZB-2301, 1:3,000, Zhongshan Golden Bridge).

Immunoreactivity was visualized using Enhanced Chemiluminescence reagents (BL520A, Biosharp, Beijing, China) according to the manufacturer's instructions, and images were acquired with the AI-600 System (GE, USA). Densitometry was performed using the Image-Pro Plus software version 6.0 and proteins of interest were normalized to GAPDH or β-actin. Normalized protein levels were expressed relative to those in the 2 weeks Sham group.

Statistical Analysis

All data were presented as mean ± standard error of the mean (SEM) and analyzed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA). Before the significance test, the Shapiro-Wilk test is used to verify the normal distribution, and all data are consistent with the normal distribution. Data were analyzed with two-way analysis of variance (ANOVA), followed by Dunnett or Bonferroni *post hoc* tests for pairwise comparison. Statistical significance was set at $p < 0.05$.

RESULTS

Estradiol Levels in Ovariectomized Mice

At 2 weeks after the surgery, serum E2 levels in OVX mice were not significantly different from those in the Sham mice. However, OVX mice exhibited significantly lower serum E2 levels than the Sham mice at 1 ($p < 0.05$), 2 ($p < 0.001$), and 3 months ($p < 0.001$) after surgery. Serum E2 levels in the OVX mice were significantly lower at 1, 2, and 3 months than at 2 weeks (all $p < 0.001$; **Figure 1**).

Estrogen Deficiency Contributes to Cognitive Impairment

At 2 weeks, 1, and 2 months after surgery, the Sham and OVX mice showed a similar reduction in escape latency during MWM training (**Figures 2A,D,G**). In the trial test on day 6 (with the platform removed), the OVX mice spent a similar amount of time in the target quadrant as the Sham mice at 2 weeks, 1, and 2 months after surgery (**Figures 2B,C,E,F,H,I**).

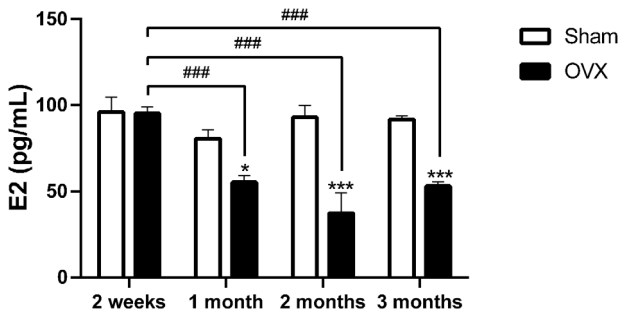

FIGURE 1 | Serum estradiol (E2) level in mice after sham surgery (Sham) or ovariectomy (OVX). Bars represent mean values ± standard error of the mean (SEM). $^{*}p<0.05$, $^{***}p<0.001$ compared with the Sham mice at the same timepoint; $^{###}p<0.001$ compared with the 2 weeks OVX mice ($n = 6$).

At 3 months after surgery, the OVX and Sham mice showed a reduced escape latency during the training process up to day 4 (**Figure 2J**, $p < 0.05$). On day 5, the OVX mice showed longer escape latency than the Sham mice ($p < 0.01$). Similarly, in the trial test (with the platform removed), the Sham mice spent significantly more time in the target quadrant than the OVX mice (**Figures 2K,L**, $p < 0.01$).

Effects of Estrogen Deficiency on Hippocampal Neurons and Synapses

MAP2 is considered an important component of the cytoskeleton of neurons (Sánchez et al., 2000). In the hippocampal CA1 region, the expression of MAP2 in the OVX mice was significantly reduced compared with the Sham mice of the same age 2 months after surgery ($p < 0.01$), which persisted to 3 months ($p < 0.001$; **Figure 3A**). Within the OVX mice, MAP2 expression decreased significantly and continuously from 2 weeks until 3 months after surgery and was significantly lower at 2 ($p < 0.05$) and 3 months ($p < 0.01$) than at 2 weeks after surgery.

PSD95 is a scaffold protein related to the assembly and function of the postsynaptic compact complex (Cao et al., 2005). Expression of PSD95 was significantly lower in the OVX mice than in the Sham mice of the same age at 2 ($p < 0.01$) and 3 months ($P < 0.01$) after surgery (**Figure 3B**). In fact, PSD95 expression within OVX mice continuously decreased from 2 weeks to 2 months after surgery, whereas expression in the Sham mice increased or remained constant over the same period and was significantly increased at 2 months ($p < 0.05$) than at 2 weeks after surgery.

Effect of Estrogen Deficiency on Mitochondrial Function in Hippocampus

Mitochondrial function was assessed by measuring the protein expression of mitochondrial respiratory chain enzymes complex I (NDUFB8), complex II (SDHB), complex III (UQCRC2), complex IV (MTCO1), and complex V (ATP5A1) in the hippocampus. Compared to the Sham mice, the OVX mice showed significantly lower levels of NDUFB8 (**Figure 4A**), SDHB (**Figure 4B**) and MTCO1 (**Figure 4D**) at 1 ($p < 0.05$ for NDUFB8 and SDHB), 2 ($p < 0.05$; $p < 0.01$; $p < 0.05$), and 3 months ($p < 0.001$; $p < 0.01$; $p < 0.01$). NDUFB8 and MTCO1 levels in the OVX mice continuously decreased from 2 weeks to 3 months and were significantly lower at 3 months ($p < 0.05$; $p < 0.01$) than at 2 weeks after surgery. In contrast, UQCRC2 and ATP5A1 levels did not differ significantly between the OVX and Sham mice at any timepoint, nor did levels vary significantly within either group over time (**Figures 4C–E**).

Effect of Estrogen Deficiency on Mitochondrial Biogenesis in Hippocampus

PGC-1α is considered the master regulator of mitochondrial biogenesis (Wu et al., 1999). The expression of PGC-1α was significantly lower in the OVX mice than in the Sham mice

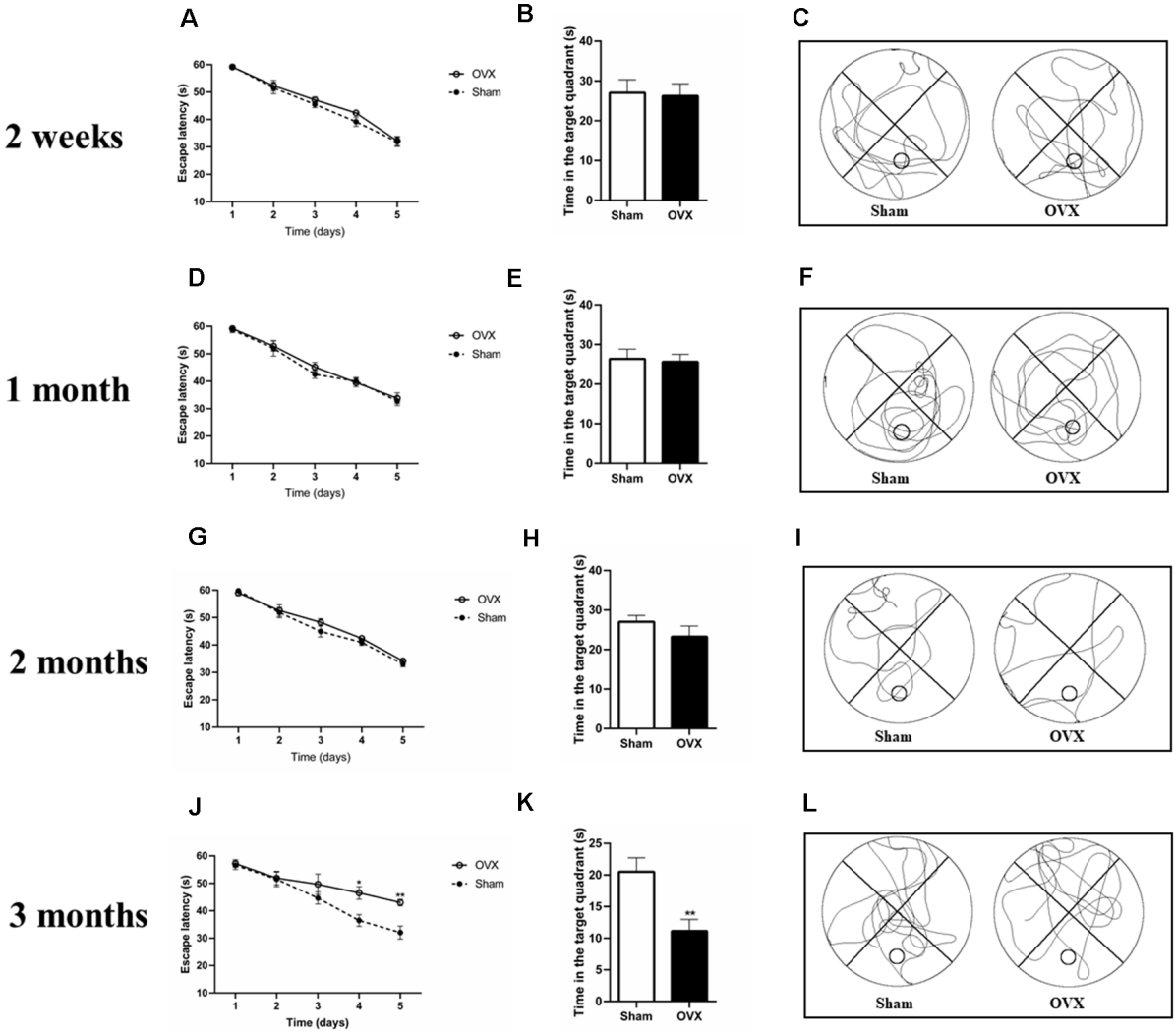

FIGURE 2 | Spatial cognitive impairments in ovariectomized mice (OVX) as measured in the Morris water maze test. **(A,D,G,J)** Escape latency. **(B,E,H,K)** Time spent in the target quadrant. **(C,F,I,L)** Representative animal trajectories during 60 s on day six. The circle in a lower quadrant marks the original placement on the platform. Bars represent mean values ± standard error of the mean (SEM). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the Sham group at the same timepoint ($n = 8$).

at 1 ($p < 0.01$), 2 ($p < 0.01$), and 3 months ($p < 0.001$) after surgery (**Figure 5A**). In fact, levels continuously decreased in the OVX mice from 2 weeks to 3 months and were significantly lower at 3 months ($p < 0.001$) than at 2 weeks after surgery, whereas levels increased in the Sham mice from 2 weeks to 1 month and were significantly increased at 1 ($p < 0.01$) and 2 months ($p < 0.01$) than at 2 weeks after surgery.

PGC-1α regulates mitochondrial biogenesis by activating multiple transcription factors, including NRF1 and mtTFA (Wu et al., 1999). Compared to the Sham mice, the OVX mice showed significantly lower levels of NRF1 (**Figure 5B**) and mtTFA (**Figure 5C**) at 1 ($p < 0.05$ for mtTFA), 2 ($p < 0.01$; $P < 0.01$), and 3 months ($p < 0.01$; $p < 0.001$). The decrease in mtTFA expression in OVX mice mirrored the PGC-1α trend (**Figures 5A–C**). NRF1 and mtTFA levels in the OVX mice continuously decreased from 2 weeks to 3 months and was significantly lower at 3 months ($p < 0.05$ for mtTFA) than at 2 weeks after surgery, whereas the levels in the Sham mice increased or remained constant over the same period and was significantly increased at 1 ($p < 0.05$ for mtTFA), 2 ($p < 0.01$; $p < 0.05$) and 3 months ($p < 0.05$; $p < 0.05$) than at 2 weeks after surgery.

Effect of Estrogen Deficiency on Mitochondrial Dynamics in Hippocampus

Levels of the mitochondrial fusion proteins OPA1 (**Figure 6A**) and Mfn2 (**Figure 6B**) were significantly lower in the hippocampus of the OVX mice than in the Sham mice at 1 (all $p < 0.05$), 2 ($p < 0.001$ for OPA1; $p < 0.05$ for Mfn2), and 3 months after surgery ($p < 0.001$ for OPA1; $p < 0.01$ for Mfn2). OPA1 and Mfn2 expression decreased

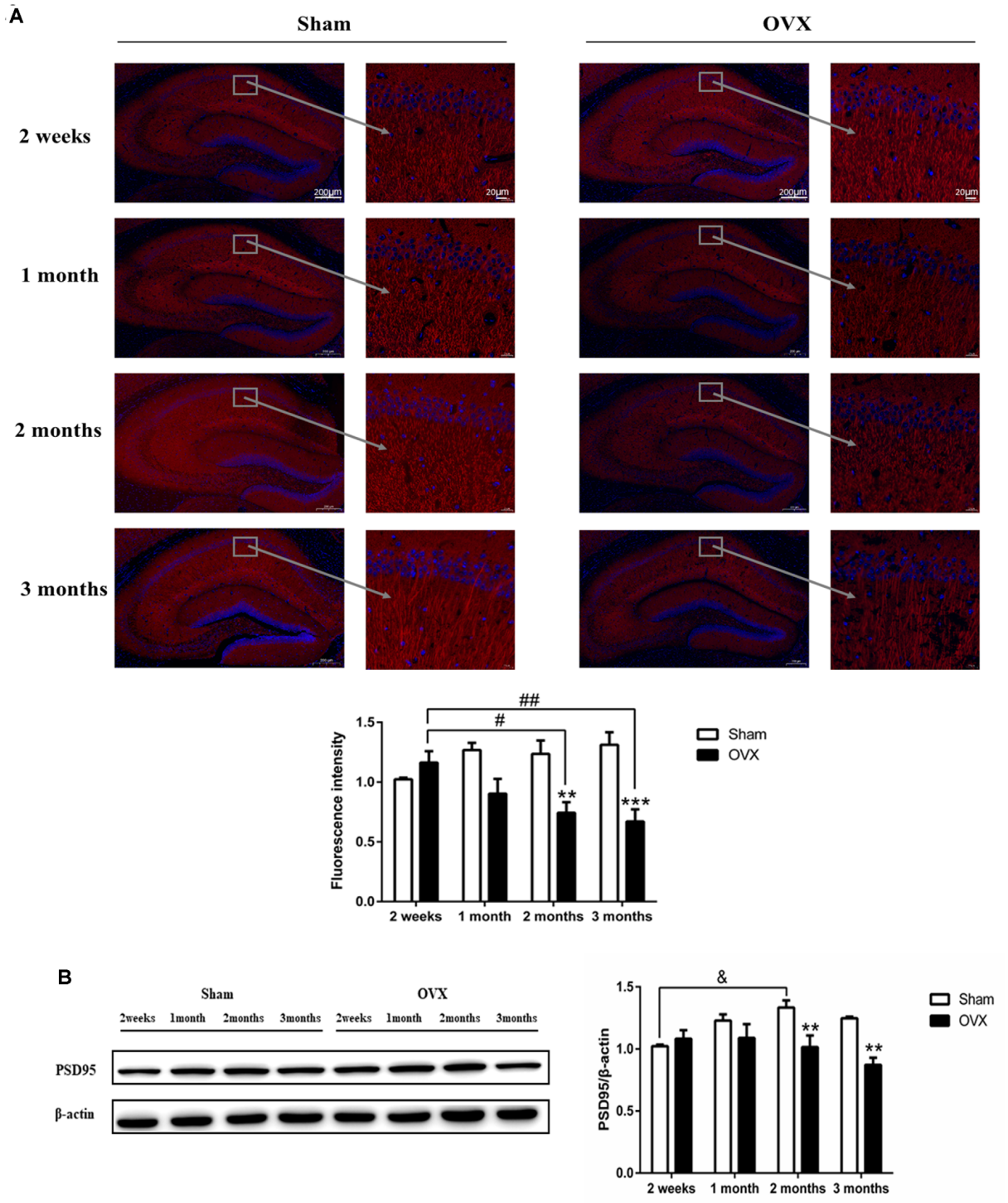

FIGURE 3 | The neuronal structure in the hippocampus CA1 region is damaged in response to ovariectomy (OVX). **(A)** MAP2 immunofluorescence and quantitation. **(B)** Immunoblotting analysis of PSD95 and quantitation. Bars represent mean values ± standard error of the mean (SEM). $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the Sham mice at the same timepoint; $^{\&}p < 0.05$ compared with the 2-week Sham mice; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the 2 weeks OVX mice ($n = 4$).

over time in the OVX mice, with the level at 1 ($p < 0.05$ for Mfn2), 2 ($p < 0.01$ for Mfn2), and 3 months ($p < 0.01$; $p < 0.01$) significantly lower than the level at 2 weeks. Levels of Drp1 phosphorylation at Ser637 were significantly lower in the OVX mice than in the Sham mice at all timepoints (**Figure 6C**).

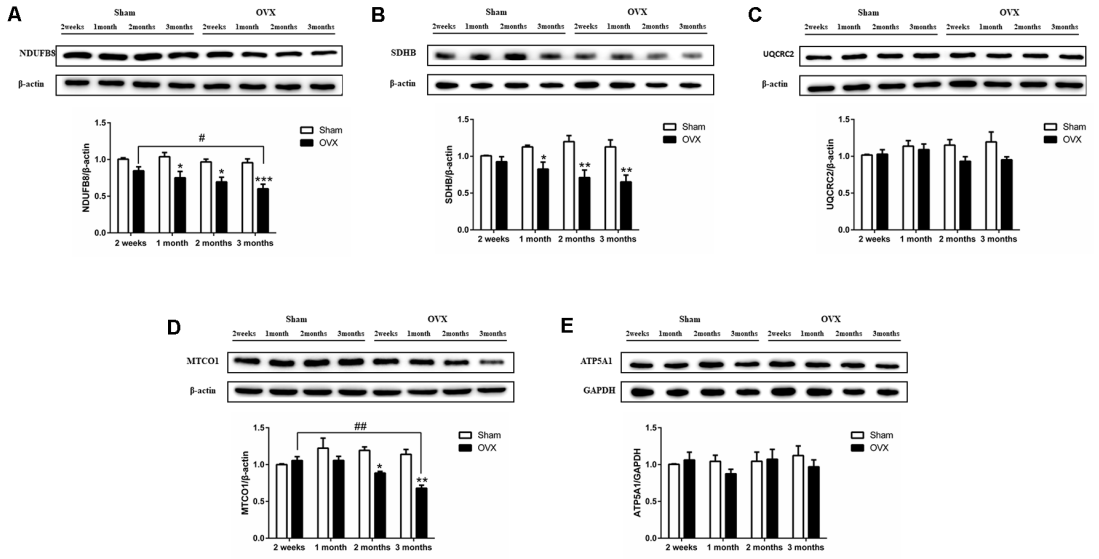

FIGURE 4 | Levels of the mitochondrial function proteins **(A)** NDUFB8, **(B)** SDHB, **(C)** UQCRC2, **(D)** MTCO1, **(E)** ATP5A1 in the hippocampus of sham-operated (Sham) and ovariectomized (OVX) mice. Bars represent mean values ± standard error of the mean (SEM). $^{*}p < 0.05$ $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the Sham mice at the same timepoint; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the 2 weeks OVX mice ($n = 4$).

Effects of Estrogen Deficiency on Hippocampal Mitophagy

Levels of LC3B-II were initially higher in the OVX mice than in the Sham mice, then they gradually became lower than in the Sham mice at later times, with the difference becoming significant at 3 months after surgery (**Figure 7A**, $p < 0.01$). Pink1 expression (**Figure 7B**) was significantly lower in the OVX mice than in the Sham mice at 2 months after surgery ($p < 0.01$), which persisted to 3 months ($p < 0.01$). Within the OVX mice, LC3B-II protein expression was significantly increased at 1 month than at 2 weeks after surgery ($p < 0.05$) and continuously decreased from 1 month to 3 months. Pink1 levels in the OVX mice continuously decreased from 1 month to 3 months and were significantly lower at 3 months ($p < 0.01$) than at 2 weeks after surgery.

DISCUSSION

Sex differences in neurodegenerative diseases suggest that sex hormones may play an important role regardless of age (Zárate et al., 2017; Scheyer et al., 2018). For the purpose of understanding the dynamic changes in the brain and clarifying the etiology of estrogen-related AD, we surgically removed the ovaries of mice to imitate menopause. Behavioral and histopathological observations at 2 weeks, 1, 2, and 3 months after ovariectomy allowed us to characterize the progression of AD in our postmenopausal mouse model. We can better understand the dynamic changes of hippocampal mitochondria after estrogen deficiency.

We found that the serum E2 levels of ovariectomized mice did not change significantly in the first 2 weeks, but they significantly decreased at all later timepoints (**Figure 1**). This may be related to stress compensation of estrogen levels in the short–term after ovariectomy. Ovariectomy can induce androstenedione and androstanol to produce estrogen by aromatase in adipose tissue, but this stress-dependent effect is only temporary, and estrogen levels later decrease (Aiman et al., 1978; Nelson and Bulun, 2001).

Ovariectomized young rodents with chronic estrogen deficiency show deficits in spatial learning tasks, including radial arm mazes and MWMs (Heikkinen et al., 2004; Gibbs et al., 2011). Consistent with previous studies, our OVX mice showed significantly lower memory and problem-solving ability in the MWM test than Sham animals at 3 months after surgery (**Figures 2K–M**). Postmenopausal women receiving estrogen replacement therapy may have improved cognitive function (Duarte et al., 2016; Merlo et al., 2017). Estrogen prevents aberrant hippocampal neuronal and cognitive deficits (Sales et al., 2010; Yazğan and Nazıroğlu, 2017).

Synapses are formed by connections between two neurons, allowing one neuron to transmit signals to another. Synaptic abnormalities may be related to a variety of nervous system diseases (Fu and Ip, 2017). PSD95 plays an important role in the formation and maturation of excitatory synapses during the development of hippocampal neurons (Gerrow et al., 2006; Bustos et al., 2017). MAP2 is a mature neuron marker, located in the cytoskeleton of neurons and plays an important role in the growth of dendrites (Heidemann, 1996). Our data showed that the hippocampal expression of PSD95 and MAP2 decreased 2 months after ovariectomy, with these decreases persisting until at least 3 months. The study found that the spines and synaptic boutons in the hippocampal CA1 area of ovariectomized rats were reduced, which was reversed after estrogen treatment (Woolley and McEwen, 1992). Consistent

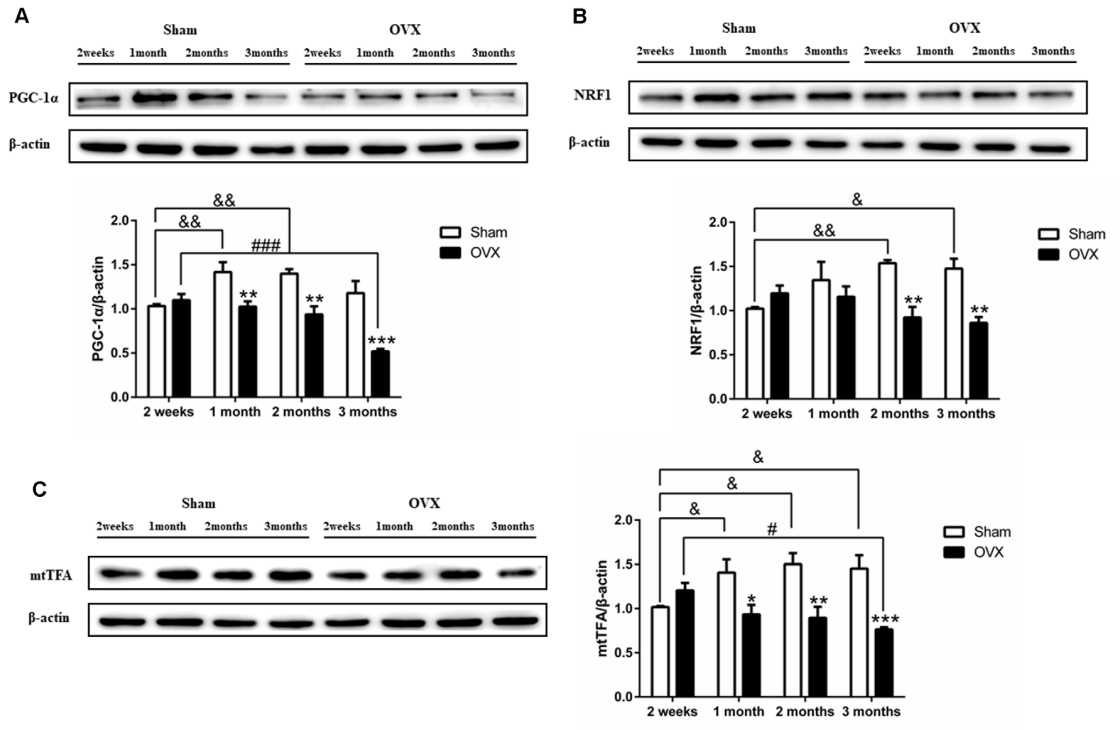

FIGURE 5 | Levels of the mitochondrial biogenesis proteins **(A)** PGC-1α, **(B)** NRF1, and **(C)** mtTFA in the hippocampus of sham-operated (Sham) and ovariectomized (OVX) mice. Bars represent mean values ± standard error of the mean (SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the Sham mice at the same timepoint; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ compared with the 2 weeks Sham mice; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared with the 2-week OVX group ($n = 4$).

with our results, estrogen deficiency can lead to the loss of synapses.

Mitochondrial dysfunction is involved in the pathological process of neurodegenerative diseases (Schon and Przedborski, 2011). Estradiol participates in the regulation of the activity and expression of brain mitochondrial respiratory chain enzymes (Nilsen et al., 2007; Irwin et al., 2012). Our data showed that 1 month after ovariectomy, the expression of hippocampal mitochondrial complex proteins NDUFB8 and SDHB decreased and 2 months after ovariectomy, the expression of hippocampal MTCO1 decreased. Decreased expression of mitochondrial oxidative phosphorylation protein could reduce the mitochondrial metabolism efficiency and trigger a series of dynamic changes in mitochondria. In this study, we further investigated the impact of E2 deficiency on mitochondrial bioenergetics, dynamics, and mitophagy over time. Our results suggest that ovarian hormone deficiency induced by ovariectomy causes a significant decrease in mitochondrial function in the hippocampus before cognitive impairment. Specifically, the hippocampus of OVX mice showed decreased mitochondrial biogenesis, mitophagy, and mitochondrial fusion, as well as an increase in mitochondrial fission. These data support the hypothesis that ovarian hormone deficiency compromises mitochondrial function (Yao et al., 2012).

Mitochondrial biogenesis is the process of the formation of new mitochondria by the growth and division of pre-existing mitochondria, which increases the mitochondrial mass in cells (Li et al., 2017). PGC-1α is a major regulator of mitochondrial biogenesis. It interacts with several DNA-binding transcription factors to regulate mitochondrial biogenesis and dynamic changes, thus maintaining mitochondrial pool (Wu et al., 1999; Dorn et al., 2015). Levels of mRNAs encoding PGC-1α, NRF1, and mtTFA are decreased in the brains of 1-month-old 3xTg AD mice (Singulani et al., 2020). The Swedish mutation (APPswe) reduces the expression of PGC-1α and impairs mitochondrial biogenesis in cellular models of AD (Sheng et al., 2012). Our data showed that the hippocampal expression of PGC-1α and mtTFA decreased 1 month after ovariectomy, and the hippocampal expression of NRF1 decreased 2 months after surgery, with these decreases persisting until at least 3 months (**Figure 5**). Since estrogen deficiency leads to a mitochondrial biogenesis defect, overexpression of PGC-1α to increase mitochondrial biogenesis is a potential strategy to treat mitochondrial diseases (Srivastava et al., 2009). PGC-1α can interact with other transcription factors, such as estrogen receptors, peroxisome proliferator-activated receptors (PPARs), and antioxidant proteins (Ventura-Clapier et al., 2008). This study found that estrogen deficiency can lead to mitochondrial biogenesis damage 1 month after ovariectomy. And considering that mitochondrial biogenesis is an early symptom of AD, and PGC-1α interacts with estrogen receptors. It was attractive to investigate estrogen regulate PGC-1α for improving brain mitochondria biogenesis in postmenopausal women with AD.

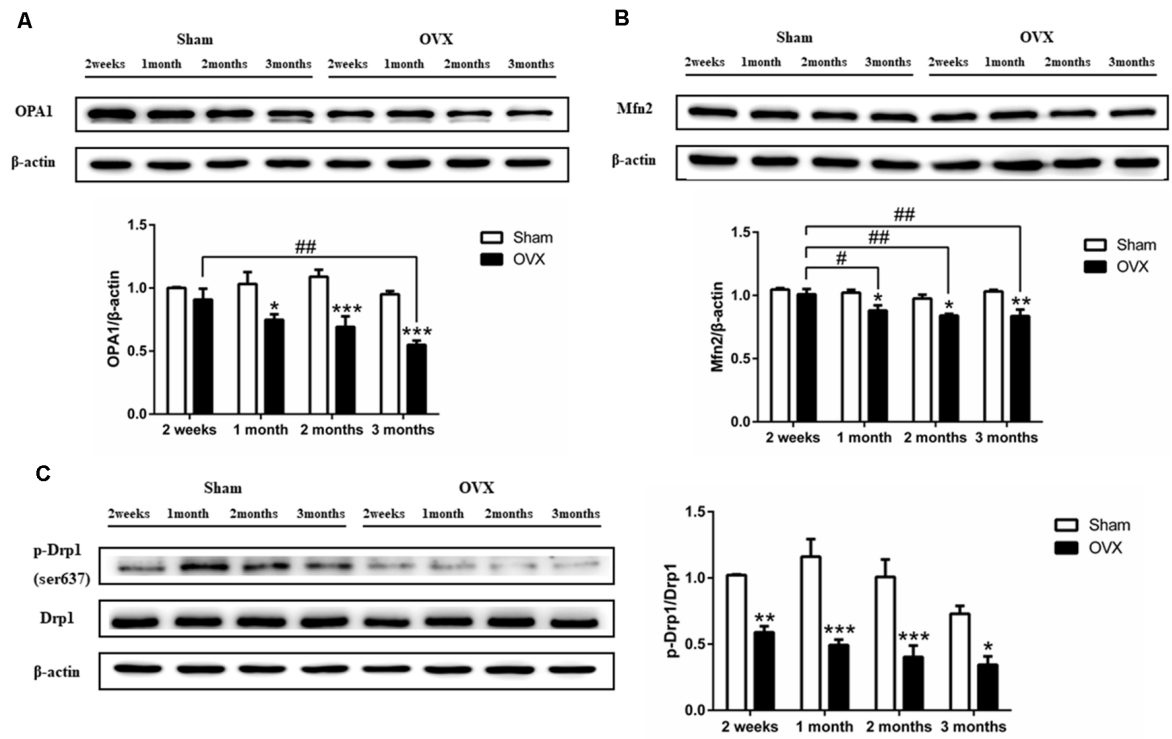

FIGURE 6 | Levels of the mitochondrial dynamics proteins **(A)** OPA1, **(B)** Mfn2 **(B)**, and **(C)** phosphorylated Drp1 (p-Drp1) in the hippocampus of mice after sham surgery (Sham) or ovariectomy (OVX). Bars represent mean values ± standard error of the mean (SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the Sham mice at the same timepoint; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the 2-week OVX group ($n = 4$).

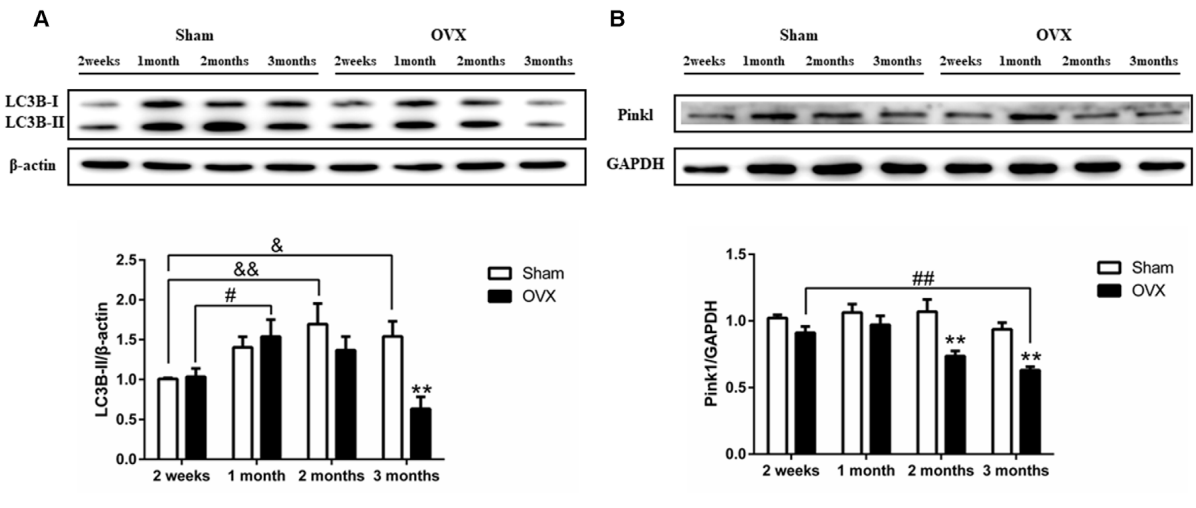

FIGURE 7 | Levels of the autophagy and mitophagy proteins **(A)** LC3B-II and **(B)** Pink1 in the hippocampus of mice after sham surgery (Sham) or ovariectomy (OVX). Bars represent mean values ± standard error of the mean (SEM). $^{**}p < 0.01$ compared with the Sham mice at the same timepoint; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ compared with the 2 weeks Sham mice; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the 2 weeks OVX mice ($n = 4$).

Mitochondria are highly dynamic organelles that undergo continuous fusion and fission in the cytoplasm to maintain the mitochondrial pool (Zhu et al., 2013). OPA1 on the inner mitochondrial membrane and Mfn2 on the outer mitochondrial membrane regulates mitochondrial fusion. At 1 month after ovariectomy, we found that OPA1 and Mfn2 were significantly decreased, indicating impaired mitochondrial fusion and, therefore, an increased mitochondrial pool. Drp1 regulates the fission of mitochondria, and its phosphorylation at Ser637 inhibits fission (Knott et al., 2008). Our data show that

at 2 weeks after ovariectomy, levels of phosphorylated Drp1 (Ser637) significantly increased, indicating impaired fission and a smaller mitochondrial pool. Thus, by 1 month after ovariectomy, estrogen deficiency leads to an imbalance of fission and fusion, causing mitochondrial fragmentation, which has been linked to nervous system disorders (Mishra and Chan, 2014). Studies have found that estrogen deficiency can damage the mitochondrial dynamics of the heart and skeletal muscle, which can be reversed by estrogen replacement therapy (Capllonch-Amer et al., 2014; Garvin et al., 2017; Minta et al., 2018). Our study extends the literature by providing evidence that mitochondrial dysregulation triggered by estrogen deficiency contributes to cognitive impairment in AD.

Impaired mitophagy may be an important cause of AD in menopausal women. Mitophagy refers to the selective removal of mitochondria through the autophagy mechanism, which is one way that the cell degrades dysfunctional mitochondria. Previous studies have found that estrogen promotes mitochondrial autophagy in a number of diseases, including Osteoarthritis and myocardial ischemia/reperfusion injury (Feng et al., 2017; Fan et al., 2018; Sun et al., 2018; Mei et al., 2020). Defective mitophagy contributes to the pathology of neural degeneration since it prevents the destruction of damaged mitochondria (Ni et al., 2015; Franco-Iborra et al., 2018). Damage to mitochondria can alter the mitochondrial membrane potential, activating Pink1 in the outer mitochondrial membrane, which initiates autophagy (Nguyen et al., 2016). In mouse models of AD, the decrease of Pink can aggravate synapse loss and cognitive dysfunction (Rodríguez-Navarro et al., 2008), which can be mitigated by promoting mitophagy (Du et al., 2017; Fang et al., 2019). Our data show that hippocampal mitophagy in mice was inhibited from 2 months after ovariectomy through at least 3 months. Our results imply that estrogen replacement therapy in early postmenopausal women may be a method to preserve mitophagy and slow the progression of AD.

Estrogen replacement therapy (ERT) can effectively reduce the cognitive impairment of some but not all postmenopausal women (Espeland et al., 2004; Vedder et al., 2014). Many evidence show that the effectiveness of ERT depends on its application in a critical period (Zandi et al., 2002; Qin et al., 2020). However, large clinical trials have indicated that HRT increases the risk of breast cancer and stroke (Beral, 2003; Stahlberg et al., 2004; Henderson and Lobo, 2012). Selective estrogen receptor modulators (SERM) by selectively affecting certain types of estrogen receptors as partial agonists, also acts as an antagonist of other types of signaling systems related to natural estrogen (Jenkins et al., 2021). SERM has the advantage of reducing the risk of estrogen-dependent tumors (Coman et al., 2017). Considering that estrogen regulates mitochondrial bioenergetics by regulating the transcription of mitochondrial DNA through estrogen receptors located on the mitochondria (Rettberg et al., 2014). Our study found that estrogen deficiency can lead to a series of mitochondrial damage and then affect cognitive function, which provides an important insight for SERMs as a treatment for cognitive and neurodegenerative diseases.

Understanding the impact of estrogen deficiency on the brain can further advance our understanding of AD pathogenesis in postmenopausal women. Our study in ovariectomized mice suggests that disruption of mitochondria function precedes cognitive impairment, which may help explain the subsequent damage to hippocampal neurons, synapses, and cognitive function that is characteristic of AD. This may have interesting implications for treating or preventing AD symptoms in women.

CONCLUSIONS

Estrogen deficiency appears to contribute to the cognitive defects of AD, through a mechanism that may involve disruption of mitochondrial biogenesis, fusion/fission, and function. These findings in mice justify further work to explore the possibility of improving mitochondrial function in postmenopausal women in order to delay or prevent AD symptoms.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee of Animal Experimental Ethics of Shandong First Medical University.

AUTHOR CONTRIBUTIONS

WZ: conceptualization and writing. YH, XS, and LW: methodology and data curation. FZ and HZ: investigation. HY and YZ: conceptualization and writing. All authors contributed to the article and approved the submitted version.

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Hemodynamic Responses Link Individual Differences in Informational Masking to the Vicinity of Superior Temporal Gyrus

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Suppressing unwanted background sound is crucial for aural communication. A particularly disruptive type of background sound, informational masking (IM), often interferes in social settings. However, IM mechanisms are incompletely understood. At present, IM is identified operationally: when a target should be audible, based on suprathreshold target/masker energy ratios, yet cannot be heard because target-like background sound interferes. We here confirm that speech identification thresholds differ dramatically between low- vs. high-IM background sound. However, speech detection thresholds are comparable across the two conditions. Moreover, functional near infrared spectroscopy recordings show that task-evoked blood oxygenation changes near the superior temporal gyrus (STG) covary with behavioral speech detection performance for high-IM but not low-IM background sound, suggesting that the STG is part of an IM-dependent network. Moreover, listeners who are more vulnerable to IM show increased hemodynamic recruitment near STG, an effect that cannot be explained based on differences in task difficulty across low- vs. high-IM. In contrast, task-evoked responses near another auditory region of cortex, the caudal inferior frontal sulcus (cIFS), do not predict behavioral sensitivity, suggesting that the cIFS belongs to an IM-independent network. Results are consistent with the idea that cortical gating shapes individual vulnerability to IM.

Keywords: informational masking, masking, auditory perception, functional near infrared spectrocopy, cochlear implant, hearing

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1. INTRODUCTION

Perceptual interference from background sound, also called auditory masking, has long been known to impair the recognition of aurally presented speech through a combination of at least two mechanisms. Energetic masking (EM) occurs when target and masker have energy at the same time and frequency, such that the masker swamps or suppresses the auditory nerve activity evoked by the target (Young and Barta, 1986; Delgutte, 1990). Informational masking (IM) is presently defined operationally. IM occurs when a target is expected to be audible based on EM mechanisms, yet cannot be dissociated from the background sound. Listeners experience IM when the masker is target-like (e.g., hearing two women talk at the same time vs. hearing out a female in the background of a male voice; Brungart, 2001b) or when the listener is uncertain about perceptual features of the

target or masker [e.g., trying to hear out a target with known vs. unexpected temporal patterning, cf. Lutfi et al. (2013)].

Unlike EM, IM is associated with striking variation in individual vulnerability (Neff and Dethlefs, 1995; Durlach et al., 2003). Moreover, an individual's susceptibility to IM is largely refractory to training (Neff et al., 1993; Oxenham et al., 2003). Identifying brain regions where IM-evoked activation patterns covary with individual differences in behavioral vulnerability to IM may thus hold a key for defining the neural mechanisms underlying IM.

Neuroimaging studies have greatly advanced our understanding of the neural mechanisms of masking. Converging evidence links both EM and IM to recruitment of superior temporal gyrus (STG) and frontal cortex (Davis and Johnsrude, 2003, 2007; Scott et al., 2004, 2006, 2009; Mesgarani and Chang, 2012; Lee et al., 2013; Michalka et al., 2015). For instance, the predominantly activated STG hemisphere can shift depending on the amount of IM in the background sound (Scott et al., 2009). Moreover, for speech that was either spectrally degraded or had impoverished amplitude cues, spanning the range from unintelligible to fully intelligible, activation near STG can account for approximately 40 to 50% of the variance in speech intelligibility (Pollonini et al., 2014; Lawrence et al., 2018).

In addition, lateral frontal cortex engages more strongly with increasing listening effort or increasing recruitment of higher-order semantic processes (Davis and Johnsrude, 2003; Scott et al., 2004; Wild et al., 2012; Wijayasiri et al., 2017). Parts of lateral frontal cortex, including the caudal inferior frontal sulcus (cIFS), are also sensitive to auditory short-term memory load in situations with IM (Michalka et al., 2015; Noyce et al., 2017). Using functional near-infrared spectroscopy (fNIRS), we previously confirmed that the cIFS region engages more strongly when listeners actively attend to speech in IM vs. listen passively (Zhang et al., 2018), making the STG and cIFS promising regions of interest (ROIs) for the current study.

Widening an established IM paradigm (Arbogast et al., 2002), we here compare hemodynamic responses to low vs. high IM speech. We test two hypotheses. H1: Individual differences in vulnerability to IM are mediated through processing limitations in the vicinity of STG. H2: Individual differences in vulnerability to IM arise near cIFS. Both hypotheses predict that for a given task difficulty, hemodynamic response strength in STG (H1) or cIFS (H2) accounts for behavioral sensitivity in situations where the background sound is target-like, but should not correlate with behavioral performance when the background sound is unlike the target.

To study how cortical responses shape individual differences in behavioral speech comprehension, our goal is to differentiate between brain areas with IM independence (task-evoked responses do not predict vulnerability to IM) vs. areas with IM dependence (task-evoked responses predict IM vulnerability). Using psychometric testing and fNIRS, we simultaneously quantify behavioral sensitivity and hemodynamic responses in the vicinity of STG and cIFS. In experiment 1, we contrast hemodynamic responses to speech detection in presence of combined target-unlike background noise ("low-IM") vs. target-like background speech ("high-IM"). In both conditions, target and background sound are presented to both ears, resulting in same-ear masking. Low-IM vs. high-IM maskers have similar long-term spectral densities. Therefore, the amount of energetic masking is comparable across those conditions. To elucidate the role of EM, in experiment 2, we then contrast high-IM with same-ear vs. opposite-ear masking. The same-ear high-IM condition is similar to that of experiment 1. The two experiments serve as their own control, confirming test-retest reliability of the measured cortical traces. However, in the opposite-ear condition, target and high-IM never excite the same cochlea and therefore EM cannot occur. Our results support H1 but not H2.

2. RESULTS

2.1. Experiment 1

Using the setup shown in **Figure 1A**, we recorded hemodynamic responses near cIFS and STG bilaterally, from normal-hearing young individuals. Target and masker were presented at equal broadband intensities to both ears. However, due to the presence of ITDs, listeners perceived the target as sounding from the left and the masker as sounding from the right. Listeners were instructed to detect when the target voice on the left uttered color keywords while SPEECH vs. NOISE maskers interfered from the right side (**Figure 1B**). Behavioral pilot testing confirmed that these spectrally sparse maskers produced high-IM (SPEECH) vs. low-IM (NOISE, **Supplemental Information 1**).

Accounting for approximately half of the variance in the recorded traces ($R^2 = 0.45$), a single Linear Mixed Effects Model (LMEM; see **Supplemental Information 2**) was then used to predict task-evoked hemodynamic responses, by regressing out reference channels (β_6 and β_7), block number (β_5), and pure-tone audiometric detection thresholds (PTA; β_{11} and β_{12}) from the full response (**Supplemental Information 2**). Note that the reference channels comprise 44.6% of the total activation levels in the LMEM fits, as calculated via the area under the fitted curve with vs. without β_6 and β_7. Task-evoked responses were modeled by a canonical hemodynamic response function (HRF) and that function's first derivative (HRF') to improve temporal accuracy in the fit. Indeed, unlike the full hemodynamic response, the LMEM-estimated task-evoked hemodynamic response aligns well with the task-onset (compare onset of darker shaded area and dashed line throughout **Figure 1D**).

Our main interest was to determine the weights of the LMEM factors modeling cortical hemisphere, cortical structure, and masker configuration. LMEM fits reveal significant task-evoked responses at all four ROIs (**Table 1**; $\beta_{1-4} > 0$, $p < 0.0001$; see **Figure 1D** for HbO (top row) and HbR traces (bottom row). Moreover, all ROIs were sensitive to IM. Activation was stronger in the SPEECH as compared to the NOISE configuration ($\beta_{10} > 0$). The size of the difference between SPEECH (black lines in **Figure 1D**) vs. NOISE (red lines) activation varied across ROIs, but these interactions with ROI were small compared to the overall effect size (interaction between masker configuration and cortical structure: $\beta_{13} < 0$; interaction between masker configuration and hemisphere: $\beta_{14} < 0$; see **Supplemental Information 3**).

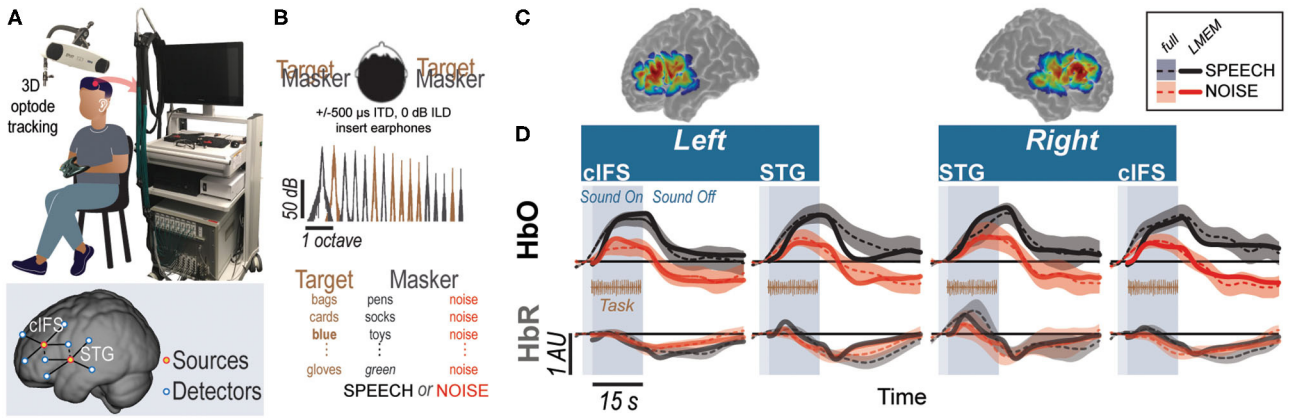

FIGURE 1 | High-IM elicits stronger task-evoked responses than low-IM across all tested ROIs in experiment 1. **(A)** Experimental apparatus and setup and optode placement for a representative listener. Blue circles show placements of detector optodes, red circles of source optodes. Optodes are geometrically arranged into two types of source-detector channels: (1) deep recording channels and (2) shallow reference channels. Source-detector pairs of deep recording channels are separated by 3 cm (solid lines in the bottom insert). Source-detector pairs of reference channels are separated by 1.5 cm (dashed lines in the bottom insert). **(B)** Task design for SPEECH vs. NOISE. Both target (left-leading interaural time difference [ITD] of –500 μs) and masker (right-leading ITD of 500 μs) were presented binaurally. Spectral densities for target vs. masker show mutually flanking, sharply tuned component bands. **(C)** Sensitivity maps for optodes placed in the vicinity of STG and cIFS. Warmer colors denote increased likelihood that photons will be recorded from these areas. **(D)** HbO (top) and HbR (bottom) traces. Full hemodynamic responses are denoted by solid lines and error ribbons. Here and elsewhere, ribbons show one standard error of the mean across listeners. Task-evoked hemodynamic responses predicted from the linear mixed effects model (LMEM) are shown as dashed lines. Shaded areas mark the task duration.

2.2. Experiment 2

The sharply tuned, mutually flanking bands of target and masker in experiment 1 were presented to both ears, and were designed to produce high- vs. low IM, with little EM. However, IM can also occur when target and masker are presented to opposite ears. It is unclear whether the neural mechanisms underlying IM are similar when target and masker are presented to the same vs. opposite ears. Thus, we next wished to examine whether the pattern of STG and cIFS recruitment would generalize to a dichotic IM configuration.

Testing a new group of 14 listeners, experiment 2 contrasted SPEECH with SPEECH-oppo, a stimulus configuration that was identical to SPEECH, except that target and masker were now presented to opposite ears (**Figure 2**). Mirroring results from experiment 1, a single LMEM fitting all HbO and HbR traces from experiment 2 accounted for approximately half of the variance in the recorded data ($R^2 = 0.52$), with 60.2% of the full hemodynamic activation attributed to reference channels. Moreover, LMEM fits confirmed that task-evoked responses in all four ROIs occurred in both masker configurations, even when target and masker were presented to opposite ears (**Table 2**; $\beta_{1-4} > 0$, $p < 0.0001$). All ROIs engaged more strongly in the SPEECH as compared to the SPEECH-oppo configuration ($\beta_{10} > 0$), with effect size depending somewhat on ROI (see **Supplemental Information 3**).

2.3. Vulnerability to Masking and Hemodynamic Responses

To test the core hypotheses, we next examined STG and cIFS for IM-dependence. We reasoned that in an IM-dependent ROI, the hemodynamic activation strength should predict behavioral sensitivity. Specifically, should hemodynamic activation near an ROI predict behavioral sensitivity for high-IM but not low-IM this would support the idea that brain regions in the vicinity of that ROI are IM-dependent (H1: STG, H2: ciFS).

For each ROI, planned adjusted coefficients of determination, R^2, between behavioral speech detection sensitivity and the peak of the HbO response were calculated. In experiment 1, individual behavioral thresholds were significantly anti-correlated with peak HbO only in the SPEECH configuration in the vicinity of left or right STG, where hemodynamic responses explained 23% (left STG) and 31% (right STG) of the behavioral variance (black square symbols in **Figure 3A**). In contrast, behavioral NOISE thresholds were uncorrelated with hemodynamic responses (**Figure 3B**). Note that these differences in hemodynamic activation patterns were observed despite the fact that the behavioral speech detection performance, measured during the fNIRS recordings, was comparable between NOISE and SPEECH [paired t-test: $t(13) = -1.14$, $p = 0.27$]. Furthermore, activity levels near cIFS (**Figure 1C**) were not correlated with behavioral thresholds in SPEECH or NOISE.

Testing a different group of listeners, experiment 2 confirmed the finding from experiment 1 that HbO peaks near left or right STG were significantly anti-correlated with behavioral sensitivity for the SPEECH configuration. Moreover, activity levels in cIFS were again uncorrelated with behavioral thresholds. Identical SPEECH configurations were assessed in experiments 1 and 2. Therefore, the converging results across two groups of listeners confirm high test-retest reliability of the current fNIRS approach. Specifically, in experiment 2, STG HbO peak activation explained 43 and 34% of the behavioral variance in left and right STG, respectively, (blue square symbols in **Figure 3A**). In contrast,

TABLE 1 | Results of LMEM, experiment 1.

	Term		Estimate	S.E.	*t*	*p*	
β_0	Intercept		–0.35	0.092	–3.8	0.0001	***
β_1	HRF_{HbO}		0.55	0.004	138.3	<0.0001	***
β_2	HRF'_{HbO}		0.17	0.004	39.4	<0.0001	***
β_3	HRF_{HbR}		0.02	0.004	5.8	<0.0001	***
β_4	HRF'_{HbR}		0.11	0.043	26.8	<0.0001	***
β_5	Block number		0.01	0.000	76.6	<0.0001	***
β_6	Reference $channel_{HbO}$		0.42	0.000	1342.0	<0.0001	***
β_7	Reference $channel_{HbR}$		0.44	0.001	580.8	<0.0001	***
β_8	Hemisphere		0.04	0.028	1.5	0.14	
β_9	Cortical structure		0.08	0.026	3.0	0.003	**
β_{10}	Masker		0.14	0.061	2.2	0.025	*
β_{11}	R ear PTA		0.02	0.008	1.8	0.08	.
β_{12}	L ear PTA		–0.01	0.005	–0.9	0.38	
β_{13}	Masker configuration	: Cortical structure	–0.03	0.003	–12.8	<0.0001	***
β_{14}	Masker configuration	: Hemisphere	–0.05	0.003	–21.1	<0.0001	***
β_{15}	Cortical structure	: Hemisphere	–0.01	0.003	–5.4	<0.0001	***
β_{16}	HRF_{HbO}	: Masker configuration	–0.19	0.004	–46.5	<0.0001	***
β_{17}	HRF_{HbO}	: Cortical structure	0.17	0.004	41.6	<0.0001	***
β_{18}	HRF_{HbO}	: Hemisphere	-0.43	0.004	–10.8	<0.0001	***
β_{19}	HRF'_{HbO}	: Masker configuration	0.02	0.004	5.6	<0.0001	***
β_{20}	HRF'_{HbO}	: Cortical structure	–0.22	0.004	–51.6	<0.0001	***
β_{21}	HRF'_{HbO}	: Hemisphere	–0.04	0.004	–9.5	<0.0001	***
β_{22}	HRF_{HbR}	: Masker configuration	–0.12	0.004	–30.2	<0.0001	***
β_{23}	HRF_{HbR}	: Cortical structure	–0.01	0.004	–1.0	0.3	
β_{24}	HRF_{HbR}	: Hemisphere	0.05	0.004	11.9	<0.0001	***
β_{25}	HRF'_{HbR}	: Masker configuration	-0.10	0.004	–22.4	<0.0001	***
β_{26}	HRF'_{HbR}	: Cortical structure	0.16	0.004	36.6	<0.0001	***
β_{27}	HRF'_{HbR}	: Hemisphere	0.04	0.004	9.3	<0.0001	***

Source: Zhang et al., 2021.

All estimates are referenced to a default condition in left cIFS for SPEECH.

*Significance codes: ***p < 0.001, **p < 0.01, *p < 0.05, · p < 0.1, p 0.1. Int, intercept; S.E., standard error of the mean.*

hemodynamic responses for SPEECH-oppo did not predict behavioral sensitivity (**Figure 3C**).

A caveat, unlike in experiment 1, in experiment 2, task difficulty differed across masking conditions. Specifically, behavioral speech detection thresholds were better for SPEECH-oppo than SPEECH [paired *t*-test: $t_{(13)} = -3.13$, $p = 0.008$; compare green symbols in **Figure 3C** falling to the right of the red, blue and black symbols in **Figures 3A,B**]. However, even for the more poorly performing listeners in experiment 2, no obvious trend links behavioral sensitivity to peak HbO levels in left or right STG.

Of note, behavioral responses were not predicted from HbR activity levels, across any of the tested conditions, in either of the two experiments. As expected, task-evoked HbO and HbR responses were robustly anti-correlated (in **Figures 1D**, **2**, compare dark dashed lines in the top row to the lighter dashed lines of the same color in the bottom row). This anti-correlation would predict that HbR responses mirror the correlation patterns between HbO peaks and behavioral sensitivity. However, in general, HbR response magnitudes were very small, approximately 20% of HbO magnitudes, hinting that here, the HbR responses may have been contaminated by the noise floor of the recording system.

3. DISCUSSION

The goal of the current work was to identify brain regions where individual differences in IM vulnerability emerge. To that end, we sought to differentiate between IM-independent parts of the brain whose activation levels are equivalently driven by low- or high-IM, vs. IM-dependent regions whose activation levels correlate with individual IM-vulnerability.

3.1. Hemodynamic Correlates of IM

The current data confirm that cortical regions at or near STG and cIFS engage during masked speech comprehension tasks (Scott et al., 2004, 2006, 2009; Kerlin et al., 2010; Ding and Simon, 2012; Mesgarani and Chang, 2012; Michalka et al., 2015; Noyce et al., 2017; Rowland et al., 2018; Zhang et al., 2018). For both high- and low-IM background sound, when a listener engaged in speech detection, robust task-evoked hemodynamic responses in STG and cIFS occurred in both brain hemispheres. Task-evoked bilateral responses in STG and cIFS were even observed when target and high-IM masker were presented to opposite ears (SPEECH-oppo in experiment 2).

SPEECH masking recruited a stronger task-evoked response than NOISE masking in both left and right STG, consistent with prior work (Scott et al., 2004). Activation levels during SPEECH masking consistently predicted a moderate 30% of variability of individual differences in vulnerability in left or right STG, in both experiments. Moreover, STG recruitment did not predict vulnerability to masking for the low-IM masker (NOISE condition in experiment 1). Together, these results show that recruitment in the vicinity of STG was IM-dependent. In contrast, while cIFS also showed task-evoked responses that were stronger in SPEECH than in NOISE, cIFS activation strength did not significantly correlate with individual vulnerability in any tested masking configuration, suggesting that the vicinity of cIFS was IM-independent. The observed association between hemodynamic response recruitment near STG was somewhat greater in experiment 2 than in experiment 1, and more variable in left than right STG, hinting that an uncontrolled source of variance contributed. It is important to note that here, we did not systematically control for across-participant variability

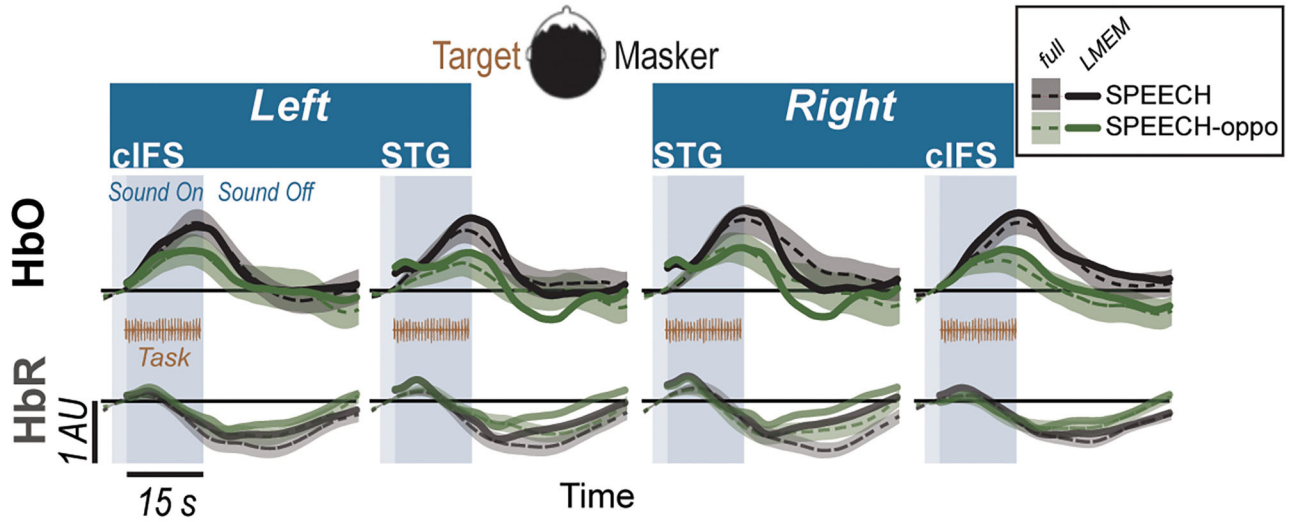

FIGURE 2 | Hemodynamic responses for SPEECH (Black) vs. SPEECH-oppo (green) show robust task-evoked recruitment of all ROIs in experiment 2, even when target and masker are presented to opposite ears. Solid lines and error ribbons denote raw recordings; dashed lines show LMEM fits.

in skull curvature, skin pigmentation or hair coarseness across participants.

IM is thought to be a central auditory mechanism. However, IM generally interferes much more strongly when target and masker are presented to the same ear(s), as compared to being presented to opposite ears (Brungart and Simpson, 2002, 2007; Kidd Jr et al., 2003; Gallun et al., 2005; Wightman and Kistler, 2005). It is unclear whether these mechanisms are similar for same-ear vs. opposite ear IM. Even when background sound enters a non-target ear, behavioral evidence suggests that IM interference can be attributed to a combination of a failure to attend to the target ear as well as increased listening effort (Gallun et al., 2007), whereas same-ear masking adds the possibility that energetic masking shapes IM through interactions with attention and across-time streaming (Ihlefeld and Shinn-Cunningham, 2008).

Here, SPEECH-oppo evoked bilateral responses in STG and cIFS. If identical STG-based networks were activated for same-ear-IM (SPEECH) and opposite-ear-IM (SPEECH-oppo), STG activity should have been a negative predictor of behavioral SPEECH-oppo sensitivity, but this was not observed. Behavioral sensitivity in this task was derived by calculating the d' difference between the rate of correct button-press responses vs. the rate of false-alarm button-press responses one would have obtained had the participant pushed the response button equally often but randomly (see Methods and Materials), resulting in a theoretical maximum d' of 3.25. Note that speech identification thresholds in SPEECH-oppo were at or close to this psychometric ceiling for a few of the listeners (note clustering of five green points at the right of **Figure 3C**), biasing the regression fits toward zero slope. However, ignoring these high-performing listeners, even for poorly performing listeners, no trend emerged linking the peak HbO response and behavioral sensitivity (**Figure 3C**). Moreover, the interpretation that contralateral IM recruits different brain networks than ipsilateral IM is also supported by prior evidence from research in children, where the ability to suppress a masker ipsilateral to the target matures more slowly than the ability to suppress a masker on the contralateral side (Wightman et al., 2010).

For same-ear IM, listeners reached comparable speech detection thresholds in low-IM and high-IM, but had marked individual difference during IM speech identification during behavioral pilot testing. This observation is consistent with the idea that more IM-vulnerable listeners exerted more listening effort (Pichora-Fuller et al., 2016). A cortical marker for listening effort was previously located in lateral inferior frontal gyrus, a brain area which shows attention-dependent increase in frontal brain activation during listening to degraded speech (Wild et al., 2012; Wijayasiri et al., 2017). The current study did not target the lateral inferior frontal gyrus, nor did we record alternative measures of listening effort, such as pupilometry (Zekveld and Kramer, 2014; Parthasarathy et al., 2020), precluding any direct test of this possibility.

Together, the results show that even with comparable behavioral sensitivities and similar long-term acoustic energy, high-IM in the same ear increased HbO peaks near STG and cIFS, as compared to low-IM. This effect was observed separately for same-ear as well as opposite-ear IM. Moreover, the observed anti-correlation between HbO peak levels and individual task performance in same-ear high-IM is consistent with the interpretation that left and right STG are part of a same-ear-IM-dependent network. In contrast, the vicinity of cIFS engaged in an IM-independent manner.

3.2. Emergence of IM

Listeners with higher cognitive abilities comprehend masked speech better (Rönnberg et al., 2008; Mattys et al., 2012), but prior work shows no evidence that cognitive ability contributes

TABLE 2 | Results of LMEM, experiment 2.

	Term		Estimate	S.E.	*t*	*p*	
β_0	Intercept		0.02	0.065	0.3	0.75	
β_1	HRF_{HbO}		0.29	0.003	89.4	<0.0001	***
β_2	HRF'_{HbO}		0.07	0.003	19.5	<0.0001	***
β_3	HRF_{HbR}		–0.04	0.003	–10.9	<0.0001	***
β_4	HRF'_{HbR}		0.07	0.004	20.8	<0.0001	***
β_5	Block number		0.01	0.000	39.1	<0.0001	***
β_6	Reference $channel_{HbO}$		0.67	0.001	1490.4	<0.0001	***
β_7	Reference $channel_{HbR}$		0.73	0.001	802.0	<0.0001	***
β_8	Hemisphere		–0.02	0.025	–0.7	0.46	
β_9	Cortical structure		0.04	0.034	1.2	0.23	
β_{10}	Masker		0.00	0.025	0.04	0.97	
β_{11}	R ear PTA		–0.01	0.011	–0.97	0.33	
β_{12}	L ear PTA		0.00	0.009	0.3	0.79	
β_{13}	Masker configuration	: Cortical structure	0.06	0.002	26.3	<0.0001	***
β_{14}	Masker configuration	: Hemisphere	–0.03	0.00	–14.5	<0.0001	***
β_{15}	Cortical structure	: Hemisphere	0.08	0.002	40.3	<0.0001	***
β_{16}	HRF_{HbO}	: Masker configuration	–0.1	0.003	–31.8	<0.0001	***
β_{17}	HRF_{HbO}	: Cortical structure	0.04	0.003	11.1	<0.0001	***
β_{18}	HRF_{HbO}	: Hemisphere	0.03	0.003	8.5	<0.0001	***
β_{19}	HRF'_{HbO}	: Masker configuration	–0.01	0.003	–1.8	0.072	.
β_{20}	HRF'_{HbO}	: Cortical structure	–0.19	0.003	–53.9	<0.0001	***
β_{21}	HRF'_{HbO}	: Hemisphere	–0.06	0.003	–16.63	<0.0001	***
β_{22}	HRF_{HbR}	: Masker configuration	0.003	0.003	1.1	0.29	
β_{23}	HRF_{HbR}	: Cortical structure	–0.05	0.003	–14.4	<0.0001	***
β_{24}	HRF_{HbR}	: Hemisphere	–0.04	0.003	–11.9	<0.0001	***
β_{25}	HRF'_{HbR}	: Masker configuration	0.01	0.003	3.0	0.0031	**
β_{26}	HRF'_{HbR}	: Cortical structure	0.06	0.003	17.8	<0.0001	***
β_{27}	HRF'_{HbR}	: Hemisphere	–0.01	0.003	–3.5	0.0006	***

Source: Zhang et al., 2021.

*All estimates are referenced to a default condition in left cIFS for SPEECH. Significance codes: ***p < 0.001, **p < 0.01, and · p < 0.1, p 0.1. Int, intercept; S.E., standard error of the mean. Int, intercept; S.E., standard error of the mean.*

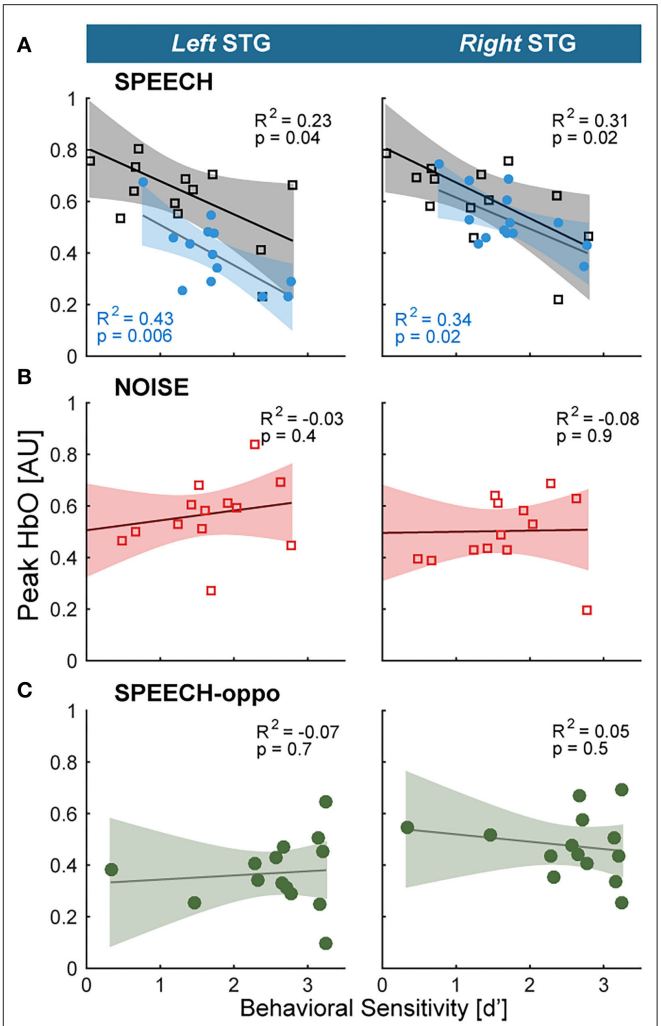

FIGURE 3 | Hemodynamic responses link individual differences in vulnerability toward IM to the vicinity of STG **(A)** STG activity and behavioral vulnerability to the high-IM SPEECH condition are robustly anti-correlated, across both hemispheres in experiments 1 and 2 (black vs. blue symbols, respectively). **(B)** There was no appreciable association between HbO peaks and the low-IM NOISE condition. **(C)** When target and masker were presented to opposite ears in the SPEECH-oppo configuration, HbO peaks did not predict psychophysical thresholds.

differently to IM vs. EM. For instance, cognitive scores poorly predict how well an individual can utilize an auditory scene analysis cue to suppress IM (Füllgrabe et al., 2015). Consistent with this, here, task-evoked responses near cIFS were IM-independent, unlike in the vicinity of STG.

Inded, prior work hints that IM emerges at the level of auditory cortex, a part of the STG (Gutschalk et al., 2008). We here tested maskers that were spectrally interleaved with the target, designed to produce either high IM (SPEECH) or low IM (NOISE). EM, when present, was limited to spectral regions outside the frequency bands that comprised most of the target energy. Consistent with this, for speech *detection*, behavioral thresholds were comparable between SPEECH and NOISE. However, our behavioral pilot results also confirmed that speech *identification* was much more difficult in the presence of SPEECH than NOISE (Freyman et al., 1999; Arbogast et al., 2002; Brungart et al., 2006; Wightman et al., 2010).

This behavioral pattern parallels a behavioral phenomenon in vision—called Crowding. In Crowding, the presence of visual target identification is severely impaired by nearby clutter or

"flankers" (Bouma, 1970; Rosen et al., 2014). In the current IM design, the spectrally sparse masker and target can be conceptualized as mutually flanking each other. Moreover, analogous to the current behavioral results, flankers that Crowd target identification do not affect target detection (Pelli et al., 2001). Furthermore, using a behavioral paradigm that is comparable to the current speech identification task, prior work shows that IM can occur even when the masker is softer than the target (Brungart, 2001a; Ihlefeld and Shinn-Cunningham, 2008). Analogously, Crowding can occur even when the flankers are smaller than the target (Pelli et al., 2001). Of importance to the current work, there is good evidence that the Crowding effect occurs in the visual cortex (Millin et al., 2014; Zhou et al., 2018a). In particular, flankers presented through one eye crowd a target presented through the other eye (Flom et al., 1963; Taylor and Brown, 1972; Tripathy and Levi, 1994). These striking similarities of IM and Crowding suggest that they result from analogous sensory processes, further supporting the prior notion that IM arises at the level of cortex.

3.3. Cortical Mechanisms of IM

The current results show that for similar behavioral sensitivities and similar long-term acoustic energy, individual differences in vulnerability to high-IM in the same ear correlated with increased need for supply of oxygen in the vicinity of STG, as compared to low-IM. However, converging evidence from prior work with electroencephalography (EEG) recordings also shows that the temporal fidelity by which cortical local field potentials encode sound, as opposed to their absolute response strength, correlates with task demands and predicts masked speech intelligibility (Choi et al., 2014; O'Sullivan et al., 2015; Viswanathan et al., 2019). Note that unlike with hemodynamic responses recorded with fNIRS, which emerge within proximity of the recording sensors at STG, it is generally more difficult to pinpoint where in the brain the EEG traces originate. In addition, even listeners with audiologically normally hearing can vary dramatically in their ability to resolve and utilize temporal fine structure cues (Ruggles et al., 2011; Bharadwaj et al., 2019). Moreover, an individual's sensitivity to monaural or binaural temporal fine structure predicts masked speech intelligibility, especially in temporally fluctuating background sound (Lorenzi et al., 2006; Papesh et al., 2017). Intriguingly, the neural mechanisms shaping temporal fidelity are thought to be of *sub*cortical origin (Parthasarathy et al., 2020). Furthermore, prior work with MEG indicates that a thalamo-cortical loop gates temporal signatures of sound to the cortical processing level (Bharadwaj et al., 2016). Consistent with this, recent cortical recordings in humans also demonstrate that neural tuning properties of the STG rapidly and flexibly shift in gain, temporal sensitivy and spectrotemporal tuning, depending on the stimulus (Khalighinejad et al., 2019; Keshishian et al., 2020).

Together, these findings raise the possibility that an individual's need for gating or adapting the neural code in STG should increase with decreasing temporal fidelity of subcortical information, as they need to work harder to overcome poor subcortical encoding of the target. Increased inhibitory activity in STG associated with stronger modulation or gating of subcortical temporal fidelity in vulnerable listeners should therefore increase the amplitude of hemodynamic responses (Stefanovic et al., 2004; Vazquez et al., 2018). Broadly increased inhibition would not necessarily be picked up via EEG analysis looking for temporal coherence and/or EEG recordings summing neural activity farther from STG. Thus, the current results are consistent the idea that increased gating or modulation of subcortical information via STG may be a potential mechanism contributing for individual variability in IM vulnerability. Future work is needed to explore how metabolic need and the fidelity of cortical temporal coding interact.

3.4. Spatial Specificity

The spacing of fNIRS optodes determines both the depth of the brain where recorded traces originate, as well as their spatial resolution along the surface of the skull. Here, optode sources and detectors were spaced 3 cm apart and arranged cross-wise around the center of each ROI (**Figure 1A**). To estimate the hemodynamic activity in each ROI, we averaged across the four channels of each ROI. This averaging greatly improved test-retest reliability of each ROI's activation trace during pilot testing, both here and in our prior work (Zhang et al., 2018). A caveat of this approach is that it reduces the spatial resolution of the recordings. Thus, it is unclear whether increased hemodynamic activity near STG is due to increased STG recruitment, or due to a more broadly activated brain network in the vicinity of STG. For instance, there is precedence for activation of additional brain regions as a compensatory strategy for coping with age-related cognitive decline (Presacco et al., 2016; Jamadar, 2020). Listeners who are more vulnerable may use either a broadened brain network or increase STG recruitment, two possibilities that the current data cannot differentiate. However, either interpretations is consistent with the idea that a central processing limitation exists that includes STG and shapes vulnerability to IM.

3.5. Diagnostic Utility

The current results bear clinical relevance. A technique we here used to design our stimuli, vocoding, is a core principle of speech processing with current cochlear implants. A pressing issue for the majority of cochlear implant users is that they cannot hear well in situations with masking, an impairment in part attributed to cortical dysfunction (Anderson et al., 2017; Zhou et al., 2018b). Sending target and masker sound to opposite ears can improve target speech identification in some, but not all, bilateral cochlear implant users of comparable etiology, suggesting that central auditory processing contributes to clinical performance outcomes (Goupell et al., 2016). This makes it desirable to assess auditory brain health in cochlear implant users. However, a challenge for imaging central auditory function in cochlear implant users is that cochlear implants are ferromagnetic devices. Thus, cochlear implants often either unsafe for use in magnetic resonance imaging (MRI) scanners and/or cause sizeable artifacts when imaged with MRI or EEG (Hofmann and Wouters, 2010). Moreover, when imaged under anesthesia, cochlear implant stimulation can fail to elicit cortical responses, making it potentially impractical to record cortical responses during CI surgeries (Nourski et al., 2013). In contrast, fNIRS,

a quiet and light-based technology, is safe to use with cochlear implants. Albeit limited to a small number of participants, the current paradigm demonstrates feasibility: fNIRS-recorded cortical responses to masked speech with impoverished, cochlear-implant-like qualities, can explain approximately a third of the variance in individual vulnerability to IM–an approach that, it is hoped, may prove useful in future clinical practice.

4. METHODS AND MATERIALS

4.1. Participants

Our sample size (14 participants for each of the two fNIRS experiments and 11 participants for a behavioral pilot control) was selected *a priori* using effect size estimates from prior work on IM (Arbogast et al., 2002; Zhang et al., 2018). Briefly, using prior psychometric functions of IM sensitivity, a sample size of 8 participants suffices to demonstrate behavioral differences in the task conditions tested here (Arbogast et al., 2002; Brungart and Simpson, 2007; Ihlefeld and Shinn-Cunningham, 2008). For the fNRIS recordings, where prior data with the specific recording system and auditory task did not exist, we ran a bootstrapping analysis, sampling with replacement our prior recordings on a related task (Zhang et al., 2018). We needed at least 12 participants to reliably arrive at the effect size that we previously observed with 10% tolerance (Zhang et al., 2018). We then conservatively chose slightly more participants than we had estimated. In total, we recruited 40 paid listeners, who were right-handed native speakers of English, and between 19 and 25 years old (17 females). Assessment of pure-tone audiometric detection thresholds (PTAs) at all octave frequencies from 250 to 8 kHz of 20 dB HL or better verified that all listeners had normal hearing. Specifically, the across-ear differences in pure tone thresholds was 10 dB or less, at all of the audiometric frequencies. All listeners gave written informed consent prior to participating in the study. All testing was administered according to the guidelines of the Institutional Review Board of the New Jersey Institute of Technology.

4.2. Speech Stimuli

There were 16 possible English words, each utterance recorded without co-articulation by each of two male talkers (Kidd Jr et al., 2008). The words consisted of the colors <red, white, blue, and green> and the objects <hats, bags, cards, chairs, desks, gloves, pens, shoes, socks, spoons, tables, and toys>. The colors were designated as keywords. Target word sequences were generated by picking a total of 25 random words from the overall set of 16, including between three and five target words, and concatenating them in random order with replacement (a set of more than 10^{26} possible permutations for the target sequence, $\binom{27}{3} \cdot 12^{22} \cdot 4^3 + \binom{28}{4} \cdot 12^{21} \cdot 4^4 + \binom{29}{5} \cdot 12^{20} \cdot 4^5 > 1.6 \cdot 10^{16}$). Similarly, masker sequences were made by picking 25 random words from the overall set of 16, constrained such that target and masker words always differed from each other, for any given word position in the target and masker sequence. One talker was used for the target, the other for the masker. Prior to concatenation, each utterance was initially time-scaled to a duration of 300 ms (Hejna and Musicus, 1991). In addition, 300 ms silences were included between consecutive words, such that the total duration of each target sequence equaled 15 s.

4.3. Vocoding

Next, the target word sequences were vocoded through an analysis-, followed by a synthesis-filtering stage. For the analysis stage, each word sequence was filtered into 16 adjacent spectral bands, with center frequencies from 300 to 10 kHz. These spectral bands were spaced linearly along the cochlea according to Greenwood's scale, with a distance of more than one equivalent rectangular cochlear bandwidth between neighboring filters (Greenwood, 1990; Chen et al., 2011). Analysis filters had a simulated spectral width of 0.37 mm along the cochlea (Greenwood, 1990) or approximately 1/10th octave bandwidth, had a 72 dB/octave frequency roll-off and were implemented via time reversal filtering, resulting in zero-phase distortion. In each narrow speech band, the temporal envelope of that band was then extracted using Hilbert transform. Broadband uniformly distributed white noise carriers were multiplied by these envelopes. For the synthesis stage, these amplitude-modulated noises were then processed by the same filters that were used in the analysis stage. Depending on the experimental condition, a subset of these 16 bands was then added, generating an intelligible, spectrally sparse, vocoded target sequence.

4.4. Target/Masker Configurations

A target sequence was always presented simultaneously with a masker sequence. Analogous to an established behavioral paradigm for assessing IM, we used two different masker configurations, consisting of different-band-speech or different-band-noise (Arbogast et al., 2002). In the SPEECH condition, the masker sequence was designed similarly to the target except that it was constrained such that (1) the target and masker words were never equal at the same time and (2) the masker was constructed by adding the remaining seven spectral bands not used to build the target sequence. In the NOISE condition, the masker sequence consisted of 300-ms long narrowband noise bursts that were centered at the seven spectral bands not used to build the target sequence. All processing steps were identical to the SPEECH condition, expect that, instead of being multiplied with the Hilbert envelopes of the masker words, the noise carriers were multiplied by 300-ms long constant-amplitude envelopes that were ramped on and off with the target words (10 ms cosine squared ramps). **Figure 1A** shows a representative spectral energy profile for a mixture of target (brown) and SPEECH (black) sequences. Note that the spectrum of a mixture of target and NOISE samples comprised of similar frequency bands would look visually indistinguishable from target in SPEECH and is thus not shown here (c.f. Arbogast et al., 2002).

In experiment 1, target and either a different-band speech or a different-band-noise masker were presented binaurally (**Figure 1B**). The target had a left-leading interaural time difference (ITD) of -500 μs. The masker sequence had a right-leading 500 μs ITD, resulting in two possible target/masker configurations, called SPEECH (different-band-speech with 500

μs ITD) vs. NOISE (different-band-noise with 500 μs ITD). The target and masker were each presented at 59 dBA, as calibrated with a 1-kHz tone that was presented at the same root mean square as the target and masker and recorded with KEMAR microphones (Knowles Electronics model KEMAR 45BB). As a result, the broadband Target-to-masker energy ratio (TMR) equaled 0 dB. However, at each of the center frequencies of the nine vocoded spectral bands that made up the target, the TMR equaled 93 dB or more.

In experiment 2, the masker always consisted of a different-band-speech sequence. Target and masker sequences were presented in two possible configurations. The first configuration was identical to the SPEECH condition of experiment 1, with the target presented binaurally with a –500 μs ITD and a SPEECH masker at 500 μs ITD. In the second "SPEECH-oppo" configuration, a target and different-band-speech masker were presented to opposite ears, with the target presented monaurally to the left, and a different-band-speech masker monaurally to the right ear (**Figure 2**).

4.5. Behavioral Task

The auditory task consisted of 12 45-s long blocks. To familiarize the listener with the target voice, at the beginning of each block, we presented a 3-s long cue sentence with the target talker's voice and instructed the listeners to direct their attention to this talker. The cue sentence was "Bob found five small cards," and was processed identically to the target speech for that block (same spectral bands, same binaural configuration). Each block then consisted of a 15-s long acoustic mixture of one randomly generated target and one randomly generated masker sequence, followed by a rest period of 30 s of silence. Moreover, at the end of each auditory task block, we added a random silent interval (mean: 3.8 s, variance: 0.23 s, uniform distribution). In experiment 1, we randomly interleaved six SPEECH blocks with six NOISE blocks, whereas in experiment 2, we randomly interleaved six SPEECH blocks with six SPEECH-oppo blocks. The spectral bands of the vocoded target and masker were fixed within each block and randomly interleaved across blocks.

Listeners were instructed to press a button each time the target talker to their left side uttered any of the four color keywords, while ignoring all other words from both the target and the masker. A random number (between three and five) of color words in the target voice would appear during each block. No response feedback was provided to the listener.

4.6. Behavioral Detection Threshold

Throughout each block we counted N_B, the number of intervals that the listener pushed the button of the response interface. If a button push occurred within 200–600 ms after the onset of a target keyword, the response was scored as a hit. Absence of any button push response in the same time period was scored as a miss. The observed percent correct was calculated by dividing the number of hits by the total number of target keywords during that block.

The baseline guessing rate was estimated via a bootstrapping analysis that calculated the chance percent correct that a simulated listener would have obtained by randomly pushing a button N times throughout that block. Specifically, to estimate the chance percent of keywords guessed correctly via random button push, for each particular listener and block, we randomly shuffled N_B button push intervals across the duration of that particular block's target sequence and counted the number of keywords guessed correctly, then repeated the process by randomly shuffling again for a total of 100 repetitions. To correct for bias, the observed vs. chance percent correct scores were then converted to d'-scores, by calculating the difference in z-scores of observed percent correct vs. chance percent correct (Klein, 2001). To prevent infinite d' values, hit and guessing rates were bracketed such that they could not fall below 0.001 and could not exceed 0.999.

4.7. Behavioral Pilot Control

Behavioral pilot testing established the presence of IM in our stimuli, while also verifying that the high- vs. low-IM conditions tested *via* fNIRS resulted in comparable speech intelligibility. Inside a double-walled sound-attenuating booth (Industrial Acoustic Company), we tested 11 normal-hearing listeners using the same auditory testing equipment and the same speech detection task that we used during the fNIRS recordings, except that listeners had their eyes open during this pilot testing.

In addition, using vocoded stimuli that were recorded by the same talkers as the stimuli used for the speech detection task, we assessed speech identification thresholds by using the coordinate response measure task (Brungart, 2001b; Kidd Jr et al., 2008). Briefly, this task presents listeners with the following sentence structure: "Ready [call sign] go to [color] [number] now." There were eight possible call signs < Arrow, Baron, Charlie, Eagle, Hopper, Laker, Ringo, Tiger>, the same four colors as in the detection task <red, blue, white, green>, and seven numbers (numbers one through eight, except "seven" because, unlike the other numbers, it consists of two syllables). The target sentence was spoken by the same talker for every trial and always had "Baron" as call sign; the masker was either SPEECH or NOISE from a different talker, and using a different call sign than "Baron." Listeners were instructed to answer the question "Where did Baron go?" by identifying the color in the target sentence. The masker was held fixed at 65 dB SPL, whereas the target level varied randomly from trial to trial from 45 to 85 dB SPL, resulting in five possible TMRs from –20, –10, 0, 10, and 20 dB. The target levels were randomized such that all five TMRs were tested in random order before all of them were repeated in different random order. Listeners competed 20 trials per TMR, both in SPEECH and in NOISE. In addition, to verify that all listeners could understand the vocoded speech in quiet at the softest target level, prior to testing masked thresholds, listeners completed 20 trials in quiet at 45 dB SPL.

In quiet, all listeners scored at or near ceiling in the identification task (**Figure 4A**), consistent with previous results that nine-band speech stimuli remain highly intelligible despite vocoding (Shannon et al., 1995). Speech identification thresholds were much worse in SPEECH than NOISE thresholds (**Figure 4B**), confirming that the current stimulus processing produces IM (Arbogast et al., 2002). Using Bayesian inference, each listener's SPEECH and NOISE percent correct speech

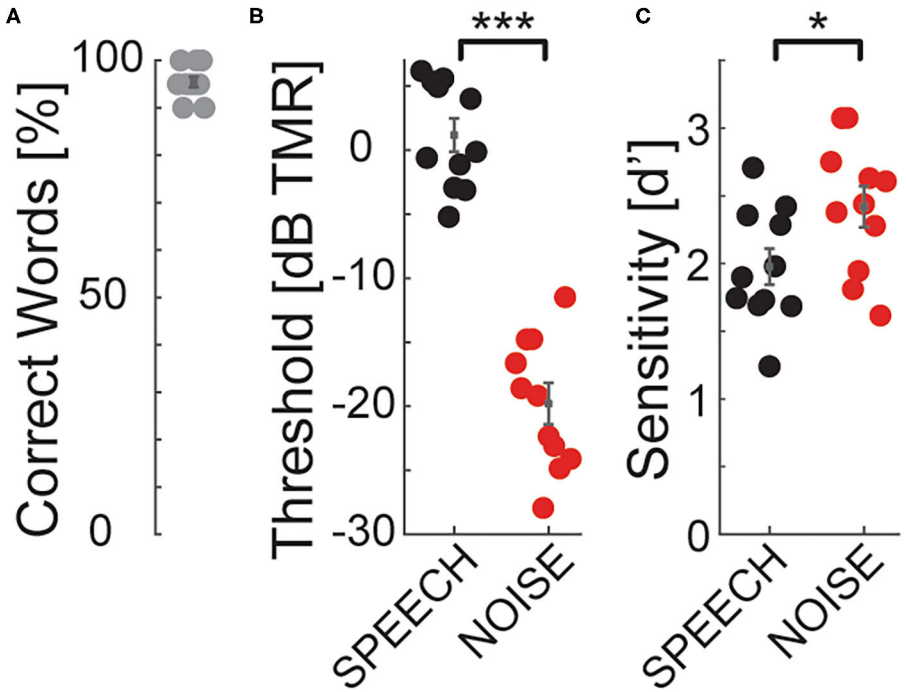

FIGURE 4 | Speech identification and detection performance during pilot testing for SPEECH vs. NOISE confirm that the SPEECH masker causes IM. The target had a left-leading ITD of -500's; the masker a right-leading ITD of 500 μs. **(A)** Quiet thresholds. Percent correct keywords identified without masker. **(B)** Speech identification task. Percent correct keywords identified with SPEECH (black) or NOISE (red) masking. **(C)** Speech detection task. Sensitivity to keywords with SPEECH (black) or NOISE (red) masking. Significance codes: $^{***}p < 0.001$, $^{*}p < 0.05$.

identification curves were fitted with sigmoidally shaped psychometric functions, as a function of TMR (Matlab toolbox: psignifit; Wichmann and Hill, 2001). Identification thresholds were defined as the TMR at 50% correct of these fitted functions. Paired *t*-tests comparing speech identification thresholds between SPEECH and NOISE found that performance was significantly worse in SPEECH [paired *t*-test, $t_{(10)} = 25.4$, $p < 0.001$]. The effect size, calculated as the Cohen's d ratio of the difference in SPEECH and NOISE thresholds divided by the pooled standard deviation across listeners, equaled 4.6. Similarly, speech keyword detectability was better in NOISE than SPEECH, by an average 0.4 d'-units [**Figure 4C**; paired *t*-test, $t_{(10)} = -2.6$, $p = 0.027$]. Cohen's d equaled 1.0.

We wished to eliminate the possibility of artifacts from eye movements and visual attention in our hemodynamic traces. Moreover, we wished to have comparable task difficulty across the tested conditions with fNIRS. Therefore, we next selected the keyword detection task for neuroimaging, because listeners could perform it with minimal body movement and closed eyes. Moreover, task performance was more comparable across maskers for speech detection vs. the identification task.

4.8. Neuroimaging Procedure

For both experiments, each listener completed one session of behavioral testing while we simultaneously recorded bilateral hemodynamic traces in the vicinity of STG and cIFS, using fNIRS. Throughout testing listeners held their eyes closed. Traces were acquired in 23-min sessions, consisting of 11 blocks of a controlled breathing task (9 min), followed by a brief break (ca. 2 min) and twelve blocks of auditory assessment (12 min). The controlled breathing task was identical to our prior methods [see details in Zhang et al. (2018)]. Briefly, the task consisted of 11 45-s-long blocks. In each block, listeners were instructed to breathe in for 5 se breathe out again for 5 s. This breathe-in-breathe-out pattern repeated for 6 times (30 s in total) before the listeners were instructed to hold breath for 15 s. The hemodynamic traces collected during this task establish a baseline dynamic range, from baseline to saturation, over which the optical recordings could vary for each particular listener, recording day and ROI. The auditory assessment was the behavioral detection task described above (see Behavioral Pilot Control).

4.9. Recording Setup for fNIRS

The listener wore insert earphones (Etymotic Research ER-2) and a custom-made fNIRS head-cap and held a wireless response interface in the lap (Microsoft Xbox 360 Wireless Controller; **Figure 1A**). Acoustic stimuli were generated on a laptop (Lenovo ThinkPad T440P) with Matlab (Release R2016a, The Mathworks, Inc., Natick, MA, USA), D/A converted with a sound card (Emotiva Stealth DC-1; 16 bit resolution, 44.1 kHz sampling frequency) and presented over the insert earphones. This acoustic setup was calibrated with a 2-cc coupler, 1/2" pressure-field microphone and a sound level meter (Bruel&Kjaer 2250-G4). The testing suite had intermittent

background sound level with peak levels of 44 dBA (moderately quiet university hallway with noise from staff walking by). Together with the ER-2 insert earphones, which provide approximately 30 dB attenuation, the effective background noise level reaching the listener's tympanic membrane was 14 dB A, i.e., moderately quiet.

A camera-based 3D-location tracking and pointer tool system (Brainsight 2.0 software and hardware by Rogue Research Inc., Canada) was used to place the optodes above the left and right cIFS and STG, referenced to standardized brain coordinates (Talairach Atlas; Lancaster et al., 2000). A custom-built head cap, fitted to the listener's head via adjustable straps, embedded the optodes and held them in place.

Hemodynamic traces were recorded with a 4-source and 16-detector continuous-wave fNIRS system (690 and 830 nm optical wavelengths, 50 Hz sampling frequency; CW6, TechEn Inc.). The system therefore limited us to 2 sources and 8 detectors on each side of the head. The spatial layout of the optical source-detector pairs was custom-designed to cover each of the four ROIs cross-wise using deep channels with source-detector distances of 3 cm (solid lines in the bottom insert in **Figure 1A**) and one short separation channel with a source-detector distance of 1.5 cm (dashed lines in bottom insert of **Figure 1A**). Specifically, on each side of the head, leveraging time-multiplexing, two of the detectors were used for both sources—alternating between serving as a short vs. a deep channel (denoted by the blue dots near the center of the bottom of **Figure 1A**). For each of the resulting 16 deep and 4 shallow source-detector pairs, we then used simulated photon paths to estimate a sensitivity map across the surface of brain by mapping the light paths through a standardized head (**Figure 1C**, AtlasViewer; Aasted et al., 2015).

4.10. Signal Processing of the fNIRS Traces

Raw fNIRS traces were processed to estimate hemodynamic activation strength (**Figure 1A** and **Supplemental Information 2**). We first used HOMER2 to process the raw recordings during both the breath holding and auditory tasks, at each of the 16 deep and four shallow source-detector channels (Huppert et al., 2009). Specifically, the raw recordings were band-pass filtered between 0.01 and 0.1 Hz, using time-reversal filtering with a fifth order zero-phase Butterworth filter for high pass filtering and time-reversal filtering with a third order zero-phase Butterworth filter for low pass filtering (commands *filtfilt* and *butter* in Matlab 2016). Next, we removed slow temporal drifts in the band-pass filtered traces by de-trending each trace with a 20th-degree polynomial (Pei et al., 2007). To suppress artifacts due to sudden head movement, these de-trended traces were then transformed with Daubechies-2 base wavelet functions. Wavelet coefficients outside the one interquartile range were removed, before the remaining coefficients were inversely transformed (Molavi and Dumont, 2012). We then applied a modified Beer-Lambert law to these processed traces, resulting in the estimated oxygenated hemoglobin (HbO) and deoxygenated hemoglobin (HbR) concentrations for each channel (Cope and Delpy, 1988; Kocsis et al., 2006). To obtain hemoglobin changes relative to the maximum dynamic recording range for each individual listener and recording site, we then applied a normalization step. Specifically, for each listener and each of the 20 source-detector channels, we divided the HbO and HbR concentration from the task conditions by the peak of the HbO concentration change during the controlled breathing task, resulting in normalized HbO and HbR traces for each channel. Finally, we averaged the four deep channels at each ROI, resulting in a total of four task-evoked raw hemoglobin traces per ROI and listener (deep and shallow, HbO and HbR). We previously found that this dynamic range normalization step helps reduce across-listener variability in our listener population with a diverse range of skin pigmentations, hair consistencies and skull thicknesses (Zhang et al., 2018).

4.11. Hemodynamic Activation

To estimate auditory-task-evoked neural activity predicted by fixed effects of high- vs. low-IM, for each of the two experiments, we next fitted a linear mixed effect model (LMEM) to the pre-processed deep HbO and HbR traces (see **Supplemental Information 2** for details on the equations). The LMEM model assumes that three main sources of variance shape the HbO and HbR traces: (1) a task-evoked response with IM independence (significant task-evoked activation that does not covary with IM vulnerability), (2) a task-evoked response with IM dependence (significant task-evoked activation that covaries with IM vulnerability), and (3) nuisance signals, deemed to be unlikely of neural origin. In addition, the LMEM includes the following factors that are known to drive neural response changes in STG and cIFS: audibility as modeled through left and right across-frequency average PTAs, and plasticity as modeled through change in output attributed to block number. To allow direct comparison of the masker evoked responses across different ROIs, all β_i were referenced relative to the SPEECH recordings in left cIFS.

To estimate whether a neural response captures behavioral phenotypes for vulnerability to IM, for each listener, masker configuration and ROI, we calculated the predicted total HbO and HbR responses from the LMEM weights, ignoring nuisance signals, PTA and plasticity. We next identified when the reconstructed HbO or HbR traces reached their maxima during the task interval, and measured the amplitudes at those single time points. Using these peak height of the reconstructed HbO or HbR traces as a measure of that ROI's neural recruitment for that masker, we then evaluated whether that ROI's hemodynamic recruitment correlated with the listener's behavioral d' sensitivity to IM.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the New Jersey

Institute of Technology. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AI and MZ designed the experiments. MZ, AI, and NA implemented the experiment. MZ collected the data. MZ, AI, and NA analyzed the data. AI wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.675326/full#supplementary-material

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REVIEW
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Mental Resilience and Coping With Stress: A Comprehensive, Multi-level Model of Cognitive Processing, Decision Making, and Behavior

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Aversive events can evoke strong emotions that trigger cerebral neuroactivity to facilitate behavioral and cognitive shifts to secure physiological stability. However, upon intense and/or chronic exposure to such events, the neural coping processes can be maladaptive and disrupt mental well-being. This maladaptation denotes a pivotal point when psychological stress occurs, which can trigger subconscious, "automatic" neuroreactivity as a defence mechanism to protect the individual from potential danger including overwhelming unpleasant feelings and disturbing or threatening thoughts.The outcomes of maladaptive neural activity are cognitive dysfunctions such as altered memory, decision making, and behavior that impose a risk for mental disorders. Although the neurocognitive phenomena associated with psychological stress are well documented, the complex neural activity and pathways related to stressor detection and stress coping have not been outlined in detail. Accordingly, we define acute and chronic stress-induced pathways, phases, and stages in relation to novel/unpredicted, uncontrollable, and ambiguous stressors. We offer a comprehensive model of the stress-induced alterations associated with multifaceted pathophysiology related to cognitive appraisal and executive functioning in stress.

Keywords: cognitive appraisal, cerebral functional activity, coping, decision making, executive functioning, psychological stress

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INTRODUCTION

The impact of minor and major stressors on psychological and physical health is well documented. It is clear from this literature that stressors are salient stimuli, including events and behavior, that can evoke strong negative emotions and feelings such as fear, betrayal, confusion, and powerlessness (i.e., psychological stress), which in turn, can lead to significant morbidity including depression, PTSD, coronary heart disease, and ischemic stroke (e.g., Stansfeld and Candy, 2006; Hamer et al., 2012; Richardson et al., 2012; Brainin and Dachenhausen, 2013; Henderson et al., 2013; Wei et al., 2014a). Psychological stress is an appropriately evoked biological reaction intended to recalibrate and optimize executive functions to stay focused on the stressor at hand, and thus mitigate the potential harm to the organism. Although this mechanism is intended to be adaptive, it is not perfect, particularly in the case

of intense and/or chronic stress. In this context, the neuroactivity can constrain cognition and increase the risk of mental and social dysfunction, as well as neural and systemic inflammation (e.g., Shin and Handwerger, 2009; Hassija et al., 2012; Latack et al., 2017; Auxéméry, 2018; Mills et al., 2019; Quinones et al., 2020; Slavich, 2020; Vaillancourt and Palamarchuk, 2021). The origin of this type of stress-associated cognitive maladjustment belongs to attentional tunneling (i.e., stressor preoccupation, e.g., Chajut and Algom, 2003; Roelofs et al., 2007; Pilgrim et al., 2010; Tsumura and Shimada, 2012; Shields et al., 2019), which restricts cognitive flexibility (e.g., Alexander et al., 2007; Shields et al., 2016; Marko and Riečanský, 2018), and distorts memory because aversive information is prioritized over neutral or positive information (e.g., de Quervain et al., 2009, 2017; Palamarchuk and Vaillancourt, under review; Vaillancourt and Palamarchuk, 2021). Moreover, despite the shift in cognitive defence mechanism to liberate the emotional burden *via* the downplaying of aversive feelings and thoughts, the attempted suppression of the stressor's influence can still affect mental health. For instance, internalizing can lead to dysphoria or anhedonia (Salmon and Bryant, 2002), core symptoms of major depressive disorder (American Psychiatric Association, 2013).

The effect of a psychological stressor is primarily related to the level of perceived stress severity, i.e., cognitive appraisal/interpretation of the stressor. Stressors can represent various aversive events regardless of their proximity (i.e., direct or remote such as in witnessing or learning), which commonly disrupt emotional integrity (**Figure 1**). This mechanism and development have not been described comprehensively in one integrated model. In this review, we outline the central neural dynamics and highlight the main phases of stress development. We define a neuropathophysiological mechanism of psychological stress that represents a complex cognitive construct beyond the classic fear-conditioning model. We detail neural dynamics in stress, and in doing so, propose a multi-level model to describe the accumulated neuronal alteration of cognitive dysfunctions. Our review highlights the importance of ameliorating psychological assessment, clinical screening, prevention, and treatment of altered adaptive-learning abilities of psychologically distressed and depressed individuals.

STRESSOR DETECTION AND AROUSAL

Psychological stress is a challenge, but the nervous system stands its homeostatic ground. First, it facilitates the detection of a stressor with noradrenergic signaling *via* the locus coeruleus-norepinephrine (LC-NE) system (e.g., Sara and Bouret, 2012; Bari et al., 2020; Poe et al., 2020). The LC-NE system is formed by the LC in the brainstem, which is a cluster of neurons encompassing NE. The axons of the LC neurons are organized in the several modules that project across the brain and format a noradrenergic system with extensive collateralization. Thus, LC activation results in a diffuse NE surge in the cerebral networks (e.g., Sara and Bouret, 2012; Szabadi, 2013; Schwarz et al., 2015; Bari et al., 2020; Poe et al., 2020), which is linked to cognitive (e.g., attention and flexibility) and behavioral outcomes (e.g., Skosnik et al., 2000; Morilak et al., 2005; Alexander et al., 2007; **Figure 2**).

The LC neurons can be subconsciously activated in response to fear, which is likely linked to the corticotropin-releasing factor (CRF) afferents from the amygdala (e.g., Pacak et al., 1995; Dunn et al., 2004; Valentino and Van Bockstaele, 2008; Sara and Bouret, 2012; Szabadi, 2013; Godoy et al., 2018; Reyes et al., 2019). The amygdala is principally associated with a fear response (e.g., Etkin and Wager, 2007; Godoy et al., 2018; Palamarchuk and Vaillancourt, under review). Chronic psychological stress strengthens the functional connectivity between the LC and amygdala that relates to fear learning. Specifically, *via* hypothalamic orexin, LC activity facilitates amygdala-dependent aversive/fear memory (e.g., Sears et al., 2013), with early retrieval (up to 6 h) associated with activated prelimbic prefrontal cortex (PFC) → basolateral amygdala circuits and later retrieval (up to 28 days) associated with activated prelimbic PFC → thalamic paraventricular nucleus → central amygdala circuits (rat model, Do-Monte et al., 2015). At the same time, prolonged severe stress has been found to impair amygdalar inhibition, seen in reduced PFC → basolateral amygdala connectivity that hyperactivated the amygdala and ensued aggressive behavior (Wei et al., 2018). That is, in chronic stress, the amygdala is relaxed from the PFC, yet thalamic pathways reconnect the pair, at least for fear memory retrieval.

The LC-amygdala connectivity is reciprocal as the amygdala can phasically activate LC neurons as well (e.g., Bouret et al., 2003). Liddell et al. (2005) showed that subliminal fear stimuli (i.e., fearful faces) coactivate the LC, amygdala, pulvinar, and frontotemporal areas related to orienting an "alarm system" (hereafter referred to as *cognitive defence* that is induced by "alarmed" LC-NE system; see **Figure 2**). Leuchs et al. (2017) validated previous findings that phasic pupil dilations, which are related to the LC activity (e.g., Murphy et al., 2014) in response to aversive (e.g., Wiemer et al., 2014) and emotionally arousing stimuli (e.g., Bradley et al., 2008), are a physiological marker of fear learning/conditioning. Fear learning is associated with a functional coactivity between the amygdala, anterior cingulate cortex (ACC), insula, thalamus, and PFC (e.g., Etkin and Wager, 2007; Fullana et al., 2016; see **Figure 2**. At the same time, almost all of the neocortex (e.g., the PFC related to cognitive appraisal and stress controllability; and the ACC together with the insula related to social monitoring/pain network; Palamarchuk and Vaillancourt, under review) can modulate LC activity *via* passing already processed/encoded information about the salient sensory and behavioral stimuli (e.g., Sara and Bouret, 2012; Szabadi, 2013; Schwarz et al., 2015).

The LC neuronal activity is a bimodal—tonic (sensory-orientated) and phasic (action-orientated)—firing that regulates attention and ongoing behavior. Specifically, the levels of tonic activity relate to drowsiness and disengagement (low), arousal (moderate), and hyperarousal (high; Sara and Bouret, 2012; Hofmeister and Sterpenich, 2015; Bari et al., 2020). Hyperarousal has been found to be associated with an increased effort to face challenges (Varazzani et al., 2015). The phasic activity increases in response to relevant behavior and hence prioritizes a goal-directed attentional processing over a stimulus-driven

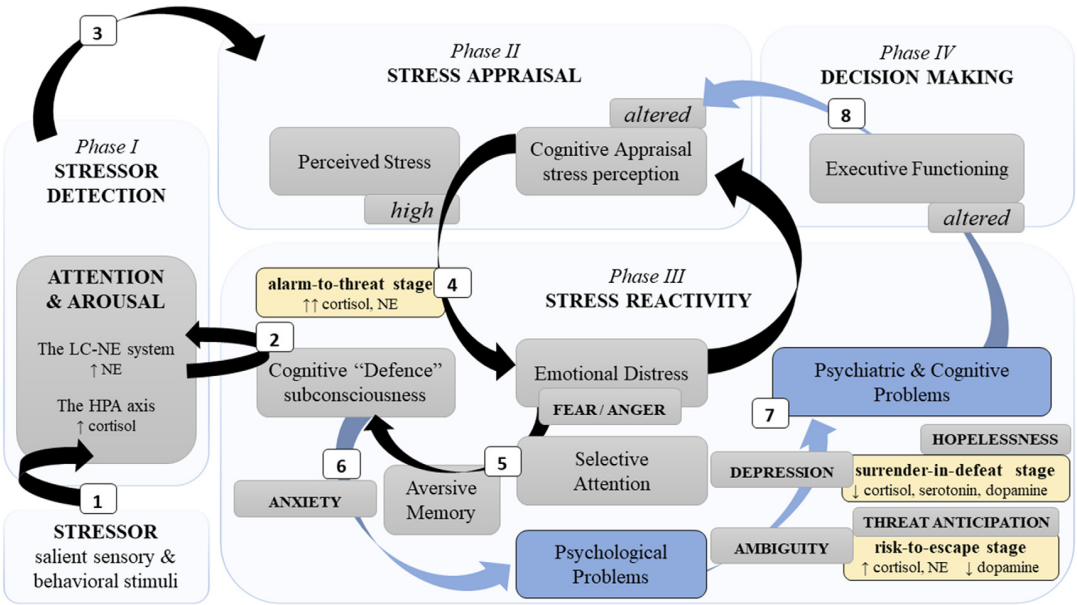

FIGURE 1 | A simplified schema of the neurocognitive reactivity to a psychological stressor. Note. This schema presents major neurocognitive dynamics during stress development phases (light blue blocks) and stages (yellow blocks). Neurocognitive stress reactivity is facilitated by two principal neural limbs, the LC-NE system and the HPA axis. *Phase I*: (1) The LC-NE system detects a challenging stimulus (i.e., stressor) and "informs" the neocortex related to cognition. (2) Automatically, it triggers subconscious cognitive defence mechanisms to activate the HPA axis. *Phase II*: (3) Further engagement of cognitive appraisal defines the severity of a stressor. *Phase III*: (4) Severe stress perception distresses emotions. (5) Fear promotes selective attention and aversive memory which aggravates cognitive defence and (6) can result in psychological problems. (7) Insufficient fear downregulation in chronic and/or intense stress (alarm-to-threat stage), as well as chronic uncertainty (risk-to-escape stage) and/or losing hope (surrender-in-defeat stage) can lead to psychiatric disorders and cognitive alterations, e.g., poor memory and executive dysfunctions. *Phase IV*: (8) Consequently, poor neurocognitive functioning affects decision-making, as well as alters recognition (phase I), appraisal (phase II), and response (phase III) of/to a novel stressor. *Legend*: HPA—hypothalamic "pituitary" adrenal; LC-NE—locus coeruleus-norepinephrine; ↑: hyperactivity/increase; ↓: decrease; black arrows—adaptive path; blue arrows and blocks—maladaptive path.

attention, which serves adaptive behavioral performance (Sara and Bouret, 2012; Hofmeister and Sterpenich, 2015). The phasic activity also reacts to fear, nociception (e.g., Valentino and Van Bockstaele, 2008; Sara and Bouret, 2012), and motivation (i.e., anticipated reward size; Bouret and Richmond, 2015), that modulate behavioral performance. However, upon detecting a stressor, the LC drops its phasic activity and increases its tonic activity, which is seen in hyperarousal and hypersensitivity and relates to scanning attention and the analysis of behavior (Valentino and Van Bockstaele, 2008). That is, when facing a stressor, the LC puts goal-directed attentional processing (the dorsal frontoparietal network) on hold so the challenge can first be inspected (the ventral/mesial frontoparietal network, mainly the dextral part including the inferior frontal gyrus, frontal/insula regions, and basal ganglia; Corbetta and Shulman, 2002; Corbetta et al., 2008; Shulman et al., 2009; see also Godoy et al., 2018). Therefore, we define cognitive defence as the ventromedial fronto-temporo-parietal network driven by fear which can emerge when fearful stimuli (frontotemporal circuits) and novel/unexpected stimuli (frontoparietal circuits; **Figure 2**) are presented.

Unexpected novel stimuli that do not have predictive value will elicit larger event-related potential responses measured by electroencephalography and prolonged reaction time to the subsequent target (i.e., larger arousal), that in turn, will modulate behavior (Knight and Nakada, 1998). The findings in shocked rats are that, compared to expected stressors, unpredictable stressors evoke greater LC-NE reactivity seen in the higher levels of principal NE metabolite in the amygdala, hypothalamus, and thalamus, and higher levels of corticosterone in plasma. In contrast, predictable stressors do not elevate NE metabolite levels in the LC and thalamus, nor corticosterone levels in plasma, the way unpredictable stressors do, compared to non-shocked rats (Tsuda et al., 1989). The potential mechanism of the higher impact of unpredictable stress may relate to altered serotoninergic (5-HT) signaling that relates to preserve the β-adrenoreceptors' upregulation (e.g., Asakura et al., 2000; Yalcin et al., 2008), which is also seen in conditioned fear and inescapable stress (Kaehler et al., 2000). However, McDevitt et al. (2009) showed that although stress controllability modulates NE levels, it does not affect NE signaling in the LC neurons; whereas stressor controllability relates to the medial PFC function to downregulate the amygdalar hyperactivity associated with altered 5-HT signaling (e.g., Amat et al., 2005; see also Puig and Gulledge, 2011; Leiser et al., 2015; Garcia-Garcia et al., 2017; Palamarchuk and Vaillancourt, under review). The findings collectively highlight that neurocognitive stress reactivity is orchestrated by the LC-NE system, fueled by the fear-driven amygdala, and regulated by the PFC/5-HT circuits.

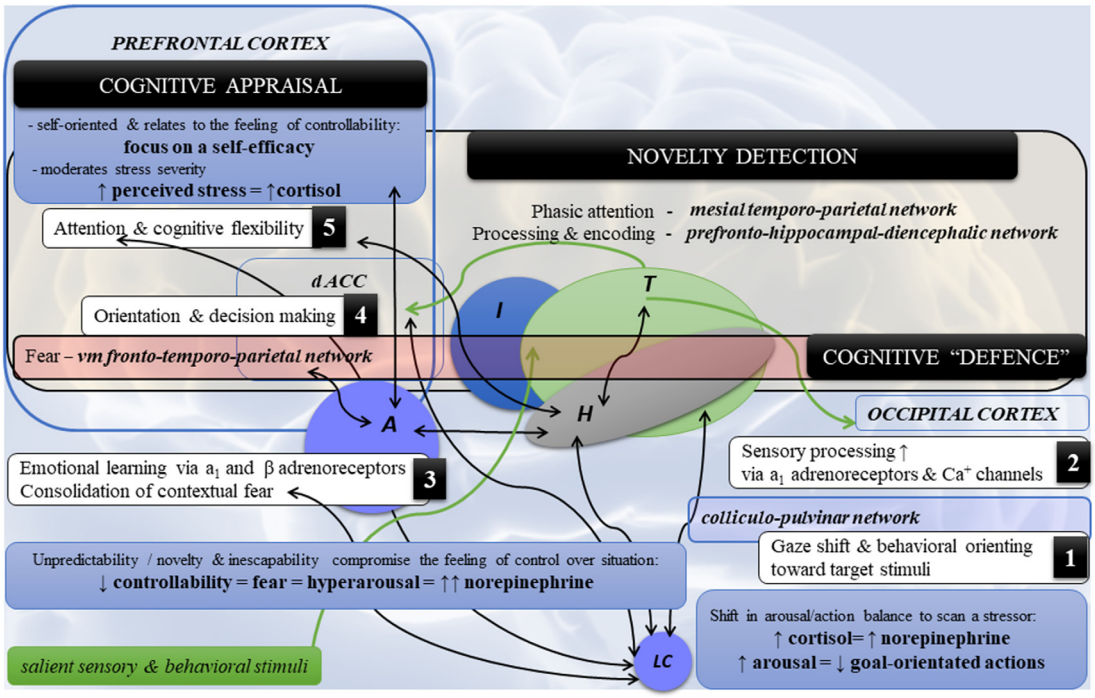

FIGURE 2 | Highlights of the neural dynamics and topology in neurocognitive stress reactivity. Note. Schematic diagram of the main co-occurrences (1–5) in neurocognitive reactivity and cerebral topology in psychological stress. (1) Detection of a threat by the LC-NE system and (2) its sensory processing triggers (3) the amygdala (fear), which in turn affects (4,5) cognition and behavior *via* the ventromedial fronto-temporoparietal network [cognitive defence] directed towards fearful stimuli (the fronto-temporal circuits) and novel/unexpected stimuli (the fronto-parietal circuits). Novelty detection encompasses the following circuits: (a) *mesial temporoparietal network* for phasic attention to the novel stimuli such as auditory and somatosensory, but to the lesser degree visual; (b) *the prefrontal-hippocampal-diencephalic network* (i.e., frontocentral hippocampal regions, adjacent fusiform, lingual gyri, fornix-mammilothalamic-cortical pathways and calcarine) for novelty processing and encoding. By contrast, the posterior hippocampal region is associated with spatial processing and encoding. *Legend*: A—amygdala; dACC—dorsal anterior cingulate cortex; H—hippocampus; I—insula; LC—locus coeruleus; NE—norepinephrine; T—thalamus; vm—ventromedial; ↑: hyperactivity/increase; ↓: decrease; ↔: functional coactivity.

COGNITIVE APPRAISAL OF STRESS SEVERITY

Elevation of cortisol levels in response to a stressor is associated with perceived stress severity (e.g., Sladek et al., 2016; Gabrys et al., 2018, 2019; Woody et al., 2018). That is, a psychological threat "exists" to the extend cognition "sees" it. Though cognitive capability may help with the avoiding of dangerous situations, it is the cognitive appraisal that helps reduce psychological stress *via* a self-appraisal perspective that conquers challenges, but not the challenging stimulus *per se*. Slattery et al. (2013) tested the associations between three neurocognitive variables, IQ, academic achievement, and verbal/visual short–term memory, which were measured at age 14, during a standardized psychosocial stress paradigm delivered at age 18. Results indicated that poor cognitive appraisal, but not cognitive skill, predicted stress responses. Specifically, stress-coping abilities during stress anticipation depended on "secondary" cognitive appraisal related to the perception of poor self-efficacy (we term this appraisal related to the perception of self-efficacy to deal with the stressor *self-appraisal*), but not on "primary" cognitive appraisal (greater threat/challenge-perception, which we term *stressor-appraisal*). Poor self-appraisal independently predicted lower cortisol reactivity during the test indicating an insufficient stress response in adolescents. At the same time, poor visual memory predicted cortisol hyperreactivity to stress, whereas internalizing disorders increased the links between verbal memory and cortisol reactivity. These results denote an important fact that intelligence alone is not likely a marker of emotion regulation that is sufficiently related to stress outcome. Rather, the outcome associated with stress is principally influenced by an individual's cognitive self-appraisal.

Other findings support the impact of self-appraisal on stress severity. In adolescents, Sladek et al. (2016) showed that higher levels of perceived daily stress severity were linked to elevated cortisol levels, compared to diurnal patterning, only in: (1) individuals with low self-appraisal; and (2) in situations with higher "engagement" coping (i.e., support seeking). The situational variation of cortisol reactivity likely indicates that engagement coping may be due to lower self-belief in coping capacity and thus lower self-appraisal. Coping efficacy related to self-belief in one's capacity to deal with a stressful situation has been found to be linked to psychological problems in children

of divorced parents (Sandler et al., 2000). In another study, compared to peers with high coping efficacy, adolescents with increased loneliness and low coping efficacy presented a flatter diurnal cortisol slopes, a marker of poor cortisol regulation, later on in college; while higher coping efficacy predicted lower levels of the cortisol awakening response in college (Drake et al., 2016). In their subsequent work, Sladek et al. (2017b) found that girls with an active engagement coping style in response to interpersonal stress had lower cortisol levels (measured by diurnal cortisol slope, total output across the day (AUCg), and cortisol awakening response). However, higher rates of using active coping related to higher cortisol awakening responses the next morning. For women with attentional avoidance of social threat cues, Sladek et al. (2017a) showed that increased use of social support coping predicted lower cortisol responses to social stress and flatter average diurnal cortisol slopes compared to women with attentional vigilance (i.e., a bias toward threat). Similar cortisol patterns were found in children who had more social problems compared to their peers, which was seen in flatter slopes of cortisol decline from wakening to bedtime; as well, children presented with higher cortisol at wakeup time the next morning after higher than usual rates of peer or academic problems at school (Bai et al., 2017; see **Figure 3**).

The impact of self-appraisal on stress response/severity is in keeping with meta-analytic results by Kammeyer-Mueller et al. (2009), which demonstrated that core self-evaluations (i.e., a stable personality trait that encompasses self-efficacy, locus of control, self-esteem, and neurotism) related to lower perceived stress, higher rates of problem-solving coping, reduced strain, and lower levels of engagement in avoidance coping. In this meta-analysis, self-appraisal was not significantly linked to emotion-focused coping and emotional stability moderated the association between stress and strain and was uniquely linked to the coping process and stress. A meta-analysis by Connor-Smith and Flachsbart (2007) adds to the idea that personality traits can predict higher rates of specific coping strategies, including problem-solving and cognitive restructuring (for extraversion and conscientiousness), support seeking (for extraversion), and wishful thinking (i.e., mental avoidance), withdrawal, and emotion-focused coping (for neuroticism).

The effect of self-appraisal may be related to the aforementioned sensory-driven shift in the LC firing in response to stress, that suppresses goal-orientated actions, which need to be balanced with the action-orientated switch (i.e., subconsciousness "cognitive defence task"). In other words, sufficient self-appraisal supports self-belief and reduces the "mental barriers", which in turn facilitates active, problem-solving coping. Further research is needed to lend more clarity on these associations (see **Figure 2**). A meta-analysis by Penley et al. (2002) showed problem-solving coping, but not emotion-focused coping, was associated with positive outcomes on general physical and psychological health. The nuances were that deliberate actions or analytical efforts and problem-focused coping were helpful only in acute interpersonal stress, correlating positively to psychological health outcomes. The effect was opposite in chronic stress, correlating negatively to psychological health outcomes. This highlights the fact that chronically distressed individuals do require social/psychological assistance. In contrast, seeking social support, confrontation, self-blame, mental or physical avoidance/distancing, self-control, and positive reappraisal in which emphasis is placed on a positive side of a situation, correlated with poor psychological self-reported outcomes in acute stress.

The major role of self-appraisal aligns with Social Self Preservation Theory (Gruenewald et al., 2004; see also Dickerson and Kemeny, 2004). For instance, in social evaluative stress, both acceptance threat and status threat can elicit a cortisol response (Smith and Jordan, 2015), and threats to the social self can induce shame and reduce self-esteem, which correlates with stress-induced cortisol levels (Gruenewald et al., 2004). It has also been demonstrated that high cortisol in social evaluative stress is accompanied by sympathetic activation (i.e., hyperarousal due to the NE surges), but not parasympathetic activation (i.e., measured by heart rate variability, can relate to affective responses; Bosch et al., 2009; Mackersie and Kearney, 2017; Poppelaars et al., 2019). Further, the magnitude of the stress response has been shown to increase in women with the size of the audience (Bosch et al., 2009), whereas sympathetic hyperreactivity was found to predict increased reactivity of the hypothalamic–pituitary–adrenal (HPA) axis, again in women (Poppelaars et al., 2019).

Stress perception also moderates the impact of a stressor on neurocognitive function. For instance, Jiang et al. (2017) showed that higher levels of stress perception correlated with poor episodic memory and frontal executive function in older adults free of mild cognitive impairment and dementia. Higher stress severity can be experienced in novel/unpredictable and inescapable conditions (e.g., Sauro et al., 2003; Lupien et al., 2007; Slattery et al., 2013) and is distinguished by hyperarousal. Tsuda et al.'s (1989) rodent studies, where these types of conditions, but not predictable stress, elevated NE in the LC and corticosterone in plasma. The apparent effect of the compromised feeling of control over unknown/novel challenges or in learned helplessness, aligns well with the self-appraisal influence discussed above. Dickerson et al.'s (2004) meta-analysis provides evidence that uncontrollable social threat relates to the highest levels of cortisol and adrenocorticotropin hormone responses to stress and the longest post-stress recovery.

Aversive emotions in both stress and stress anticipation that result in NE surge affect cortisol influence on attention, cognitive flexibility, memory, and learning, and thus aggravate the intensity of a stressor (Skosnik et al., 2000; Morilak et al., 2005; Alexander et al., 2007; Kvetnansky et al., 2009; Gray et al., 2017). That is, in intense stress, negative emotions enhance aversive memories and withdraw the cognitive focus from the "peripheral" details. Such selective attention is associated with poor working memory and memory retrieval (de Quervain et al., 1998, 2009; Roozendaal et al., 2006, 2008). The effect of emotional valence in stress involves concurrent activation of glucocorticoid receptors (GRs) and adrenoreceptors, specifically, central β-adrenergic receptors activation linked to long-term declarative memory for emotionally arousing information

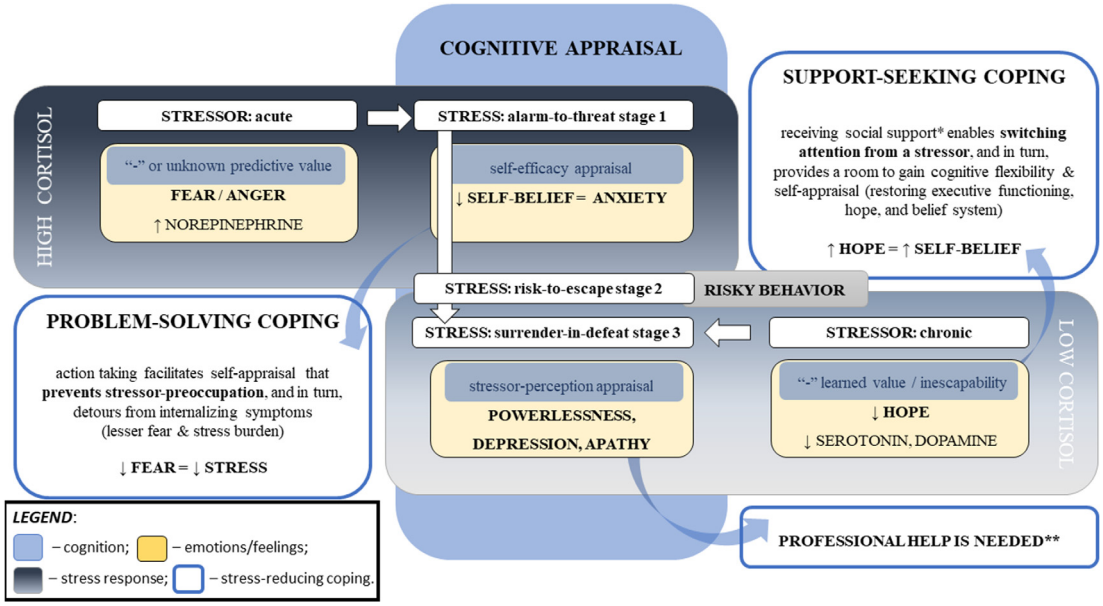

FIGURE 3 | Major cognitive determinants of the cortisol responses linked to stress psychopathology. Note. This diagram represents the major factors influencing cortisol response to stress that can lead to stress disorders. Stress responses depend on the particular challenge, one's perception of the stressor, and the ability to cope with the stressor. The stressor's intensity, acuity, and persistence relate to cortisol responses, which are moderated by cognitive appraisal that is associated with self-efficacy and coping abilities. The stressor's novelty (i.e., unknown predictive value) and inescapability (i.e., negative "learned" value) increase negative predictive values (i.e., fear and powerlessness, respectively), that hinder self-appraisal and aggravate stress severity. Repeated exposure to homotypic stressors resets the hypothalamic–pituitary–adrenal axis. Chronic stress can result in blunted cortisol responses to a stressor, flattened diurnal slops, and increased cortisol awakening responses. *Legend*: *—not limited to the emotional aspect that reduces stress perception (e.g., motivation, compassion[1], and sense of belonging[2–4]) but also social and physical aspects directed to a reduction in the stressor's influence (e.g., physical or financial help); **—risk of PTSD and suicidal ideation; ↑: increase; ↓: decrease, "-": negative; [1]Vaillancourt and Palamarchuk (2021); [2]Grobecker (2016); [3]Choenarom et al. (2005); [4]Stachl and Baranger (2020).

(e.g., Cahill et al., 1994; Cahill et al., 2004; Maheu et al., 2005a,b; see also Gibbs and Summers, 2000, 2002; Schwabe et al., 2009; Smeets et al., 2009; Lonergan et al., 2013) and activation of α_1-adrenoreceptors that were insensitive previously to NE in the medial entorhinal cortex, linked to hippocampal memory dysregulation (e.g., Carrion and Wong, 2012; Hartner and Schrader, 2018). As well, a deletion variant gene that encodes α_{2B} adrenoceptor, ADRA2B, contributes to the cognitive processing of emotional information (see meta-analytic review by Xie et al., 2018). Levels of hyperarousal and its proximity to the occurrence of stress modulate memory formation, whereas higher hyperarousal can be seen in children due to neurodevelopmental sensitivity (e.g., Palamarchuk and Vaillancourt, under review; Vaillancourt and Palamarchuk, 2021), and in women due to the LC-NE system specifics (e.g., Bangasser et al., 2016; Bangasser and Wicks, 2017; Bangasser et al., 2018, 2019; see also Mulvey et al., 2018). Additionally, the sex differences are that emotionally influenced memory relates to hyperactivated amygdala with a stronger effect in the left hemisphere for women and in the right hemisphere for men (e.g., Cahill et al., 2004). Animal studies on fear conditioning show that mild-to-low levels of hyperarousal can impair spatial recognition memory, yet moderate-to-strong levels of hyperarousal can enhance the memory (e.g., Baars and Gage, 2010; Conrad, 2010). Therefore, stress reactivity has inter-individual variations that can be mild or more pronounced depending upon the individual's stress appraisal and valence of aversive emotions, which are moderated by age and gender. Additionally, glucocorticoid stimulation followed hours earlier by NE secretion has been shown to inhibit arousal effect on memory (Osborne et al., 2015).

DECISION MAKING AND STRESS

The executive functioning facilitates adaptation with decision-making based on the evaluated external (environmental) and internal (sensory) information (e.g., De Kloet et al., 1998; Wager and Smith, 2003; Collins and Koechlin, 2012; Barbey et al., 2013; Dajani and Uddin, 2015). Executive functioning integrates memory, cognitive flexibility (such as rapid attention and task-shifting, as well behavioral adjustments, e.g., Palamarchuk and Vaillancourt, under review), learning fortification, reasoning, insecurity predictability, and monitoring behavioral strategies (e.g., Collins and Koechlin, 2012; see also Grissom and Reyes, 2019). The distinctions are that the ventromedial PFC integrates memory and emotional systems that are needed for decision-making, whereas the striatal and ACC inputs can affect it with bias (e.g., Gupta et al., 2011; Ho et al., 2012; Shimp et al., 2015; Goulet-Kennedy et al., 2016; Fitoussi et al., 2018; Hiser and Koenigs, 2018; Palamarchuk and Vaillancourt, under review). At the same time, the amygdala mediates emotional responses that engage the insula, which relates to social pain, empathy,

and anger (e.g., Palamarchuk and Vaillancourt, under review). In a social context, the medial PFC and amygdala, but not ventral striatum, moderate decision-making (Ho et al., 2012; see also Hiser and Koenigs, 2018); whereas high levels of fear or anger (i.e., the amygdalar hyper response to a stressor) can affect decision-making with impulsivity/immediate actions (e.g., Gupta et al., 2011). Conversely, the stress associated with uncertainty and unknown power over a situation involves the frontrostriatal circuits, where task-sets and actions are driven by the references of cognitive/behavioral strategies stored in the long–term memory as a *script* (relates to the dorsal striatum/left caudate nucleus engaged in reward and motivation). Thus, in the context of stress-related ambiguity, the choice depends on predicted outcome values (related to the ventral striatum/the nucleus accumbens and ventral putamen engaged in cognitive control) to maximize their utilization, i.e., reinforcement learning/instrumental conditioning (O'Doherty et al., 2004; see also Hollerman et al., 2000; Brovelli et al., 2011; Vogel et al., 2015, 2017). The strategy is selected if it is absolutely reliable (the ventral striatum, nucleus accumbens) among the assortment of scripts (the dorsal striatum, nucleus caudate); and if it is unavailable, a new task-set is created because the decision-making is binary when the stimulus is ambiguous (e.g., Collins and Koechlin, 2012).

Emotional state/mood can affect the interpretation of the stressor, i.e., the mood-incongruent effects. Anxiety can lead to attentional bias toward threat due to higher predicted negative outcome of the stressor (i.e., ambiguity (fear, e.g., Blanchette and Richards, 2003; Barazzone and Davey, 2009). An anxious state also increases speed in the detection of aversive changes on a subliminal level and increases attention and conscious awareness on a supraliminal level (Gregory and Lambert, 2012). For example, in adults with high trait anxiety, the anxious state lowers awareness thresholds. In particular, fearful faces or non-threat faces presented among threatening faces are detected faster (Ruderman and Lamy, 2012). Neurocognitive functioning in stress thus drops cognitive flexibility (i.e., reduced functions of the dorsolateral PFC) to stay focused on the stressors, this attentional tunneling during emotional arousal allows the individual to detach from the "peripheral" information unrelated to the stressor that might distract the individual who is under pressure (e.g., Palamarchuk and Vaillancourt, under review; see also Brosch et al., 2013; LeBlanc et al., 2015). However, attentional tunneling and enhanced memory for aversive experiences can lead to psychological maladjustment, for instance, emotion-focused coping, anxiety, and PTSD (e.g., Palamarchuk and Vaillancourt, under review).

DISCUSSION

Hypothesis: Coping Mechanisms Are Driven by the Stress Stages

We define coping styles as intra-individual neurocognitive variability moderated by stress development across three main stages: (1) *alarm-to-threat stage* → (2) *risk-to-escape stage* → (3) *surrender-in-defeat stage.* Potentially, the full development can be observed in chronic, intense, and homotypic stress associated with the HPA resetting and circulating cortisol decline. It is likely that these stress stages can be disrupted/attenuated, escalated, and/or distorted according to the level of perceived stress severity and neuropsychological status; whereas novel stressors can restart stress phases cycling (e.g., stress detection phase I; see **Figure 1**). Therefore, coping styles can fluctuate in a predictable intra-individual manner and recognizing the stress stage can expedite adequate interventions to prevent or treat maladaptive coping.

Alarm-to-Threat (Check) Stage

Acute intense stress triggers right amygdalar fear-related effects such as tunneling attention, anxiety, and impulsivity seen in a reactive aggression as a sympathetic *fight-or-flight* response that is driven by high cortisol and NE levels (e.g., Palamarchuk and Vaillancourt, under review). The core mechanism is that fear can initially serve adaptation by reducing risky behavior (e.g., Pabst et al., 2013a,b; Yu, 2016; Vogel and Schwabe, 2019), because, in contrast, positive emotions can increase the probability of risk-taking (e.g., LeBlanc et al., 2015). Specifically, aversive emotions during mild psychological stress can facilitate the most reliable cognitive strategy *via* the narrowed scope of attention (that can also be induced by the pre-goal desire, e.g., LeBlanc et al., 2015), reduced configural associative learning (i.e., reduction in tri-/biconditional discrimination), and enhanced binary (uniconditional as irrelevant vs relevant) discrimination (e.g., Byrom and Murphy, 2016). Of relevance, social stress has been shown to increase activity in the anterior PFC associated with parallel processing during decision-making performance (e.g., the Game of Dice Task, Gathmann et al., 2014; see also Schiebener and Brand, 2015; Shimp et al., 2015). However, stimuli associated with extreme/traumatic experiences can trigger inadequate responses and reduce responses to contextual cues such as focusing on aversive sound and disregarding the safety of the environment that promotes automatic retrieval of traumatic experiences (e.g., Cohen et al., 2009; Otgaar et al., 2017). This is an example of accentuated alarm-to-threat stage by rigid binary cognitive strategy, whereas improving cognitive flexibility by configural associative learning could be a key element in the psychotherapeutical approach. Another example is that strong fear can elicit avoidance behavior related to the left lateral amygdala and anterior hippocampal hyperactivity (Abivardi et al., 2020). In other words, "cold" executive functioning is set to prioritize the most reliable decision-making to avoid danger when confronting a threat, yet it limits attention and flexibility. The mechanism is facilitated by promoted dorsal striatum-dependent ("habit") learning and behavior over hippocampal-dependent ("cognitive") memory encoding and retrieval, which leads to stereotypical ideas and thus maladaptive functioning in chronic stress (e.g., Packard, 2009; Vogel and Schwabe, 2016; Vogel et al., 2017; Zerbes et al., 2020; see also Schiebener and Brand, 2015; Shimp et al., 2015; Fitoussi et al., 2018). In particular, poor consequences can be seen in attentional set-shifting deficits, poor memory,

anxiety, and depression (e.g., Palamarchuk and Vaillancourt, under review).

If acute stress subsides, attention can be improved with the decline of cortisol (e.g., Zandara et al., 2016). Conversely, intense stress can hyperactivate the LC that is associated with anxiety (Borodovitsyna et al., 2018; Morris et al., 2020) due to limbic dysregulation (e.g., Herman et al., 2005). In particular, it is related to the functional connectivity between the bed nucleus of the stria terminalis (BNST) and amygdala (e.g., Clauss, 2019; Knight and Depue, 2019; Hofmann and Straube, 2021). The nuances are that the amygdala is involved in explicit threat processing (i.e., threat confrontation), whereas the BNST is involved in ambiguous threat processing (i.e., threat anticipation; Herrmann et al., 2016; Klumpers et al., 2017; Naaz et al., 2019; see also Fox et al., 2015; Fox and Shackman, 2019; Luyck et al., 2019). As well, the BNST → central amygdala projections relate to cued-fear inhibition (Gungor et al., 2015; see also Clauss, 2019). The BNST plays a critical role in fear acquisition/expression, which relates to stress maladaptation and the development of stress-related disorders like PTSD (e.g., Miles and Maren, 2019) and involves CRH signaling (e.g., Hu et al., 2020). This functional interplay between the BNST and amygdala relates to the inter-individual differences in threat processing and trait anxiety (Brinkmann et al., 2018), which likely influences the development of the next stage in chronic intense stress.

Risk-to-Escape (Stalemate) Stage

The evidence is that stress, predominantly chronic, can increase risk-taking behavior (Starcke et al., 2008; Lighthall et al., 2009; Pabst et al., 2013c; Ceccato et al., 2016; see also Brand et al., 2006; Starcke and Brand, 2012; Yu, 2016). We predict that stress-induced risk-taking is largely driven by threat anticipation due to hyperactivated BNST. The BNST integrates limbic information and valence monitoring and plays a central role in the hippocampus-hypothalamic paraventricular nucleus circuit that activates the HPA axis and has a psychogenic effect (e.g., Lebow and Chen, 2016). The BNST is sexually dimorphic; its activity is heritable and relates to anxiety in ambiguous and sustained threat (e.g., Clauss, 2019). The neurophysiological background is that the BNST receives multiple signals, including, but not limited to, dopamine and 5-HT from the dorsal raphe and NE from the nucleus tractus solitarii (e.g., Glangetas and Georges, 2016). Moreover, increased impulsivity relates to alteration in the central amygdala → BNST dopaminergic projections that inhibit impulsive behavior (Kim et al., 2018).

We thus predict that in prolonged homotypic stress, hyperactivated BNST *covers a shift from the front-line stress-care* medial PFC-amygdalar circuits. This is likely a *now-or-never* response to escape the burden of anticipated threat, driven by dopamine reductions in uncertain conditions which recruit the dorsal PFC-striatal circuits related to impulsive and risky behavior. Our reasoning is that, in contrast to fear, ambiguity can be perceived as a *dormant threat* that increases approach behavior (the hippocampal rectivity, e.g., O'Neil et al., 2015) and risky behavior (the ventral striatal reactivity moderated by impulsivity traits, e.g., Mason et al., 2014; Goulet-Kennedy et al., 2016). As well, the activity of the ventral striatum is associated with a motivational control of performance and is regulated by the dorsolateral PFC (Hart et al., 2014). Therefore, it could be a part of an adaptive mechanism to confront the challenge although it requires adequate executive functioning, and by extension, goal-oriented actions. The pitfalls are that poor cognitive control and insular risk-processing can increase perceived stress, and in turn, risk-taking behavior (e.g., among adolescents, Maciejewski et al., 2018). In contarst, risk-taking behavior is inversely associated with a cortisol increase for boys/men but not girls/women (e.g., Daughters et al., 2013; Kluen et al., 2017). This effect relates to greater activity and novelty preferences due to higher sensation seeking in boys/men compared to girls/women who are more punishment sensitive (meta-analysis by Cross et al., 2011). The developmental moderation of stress-induced responses can also lead to impulsive errors in girls (e.g., Lukkes et al., 2016), which is also moderated by personality traits related to impulsivity (e.g., negative urgency that correlates to impulsivity, Berg et al., 2015; see also Cyders and Smith, 2008a,b; Herman et al., 2018). The levels of impulsivity in healthy young adults inversely correlate with the levels of released dopamine from the ventral striatum in low to moderate stress; yet high stress reduces dopamine responses (e.g., Oswald et al., 2007; see also Palamarchuk and Vaillancourt, under review).

In sum, poor cognitive functioning and cortisol decline can promote a burden of uncertainty (*stalemate*), and as dopamine drops, risk-taking ensues to which young men are more prone to than young women. The mechanism is that the striatal networks can serve decision-making with the learned behavior/"script" when facing explicit danger in acute stress. In contrast, when dealing with prolonged uncertainty, decision-making can be impulsive and risky due to poor risk-processing, and potentially, motivation/urge to terminate the *status quo* in chronic intense stress. Accordingly, improving cognitive control with proper risk-processing (psychological help) and facilitating adequate options to avoid predictable danger (social assistance) could be a key intervention to prevent poor outcomes. Although our hypothesis has yet to be tested, it sheds light on why stress can induce risk-taking behavior.

Surrender-in-Defeat (Checkmate) Stage

We interpret that in acute and extreme stress associated with a loss or defeat, as well as in chronic stress with a prolonged ambiguity, the executive functioning "surrenders" in the absence of absolutely reliable task-sets and incapacity to create new ones (i.e., defeat/*checkmate*), which is why serotonin levels drop and depression emerges. Of relevance, Yu et al.'s (2016) findings in rodent models demonstrate that repeated social defeats, but not social threats, increase cortisol and NE levels but decrease dopamine, its metabolites, and serotonin levels in the striatum and hippocampus (see also Palamarchuk and Vaillancourt, under review).

On a molecular level, stress adaptation relates to a negative feedback of the HPA axis seen in cortisol hyposynthesis as ACTH sensitivity declines (e.g., Juruena et al., 2003; McEwen, 2012;

Gray et al., 2017). In particular, the duration of exposure to a homotypic stressor displays a linear and inverted U-shaped dose-effect on a stress response: (1) a novel stressor can increase ACTH sensitivity; (2) a repeated stressor can initially desensitize ACTH; and (3) a chronic stressor relates to an unceasing ACTH sensitivity (Aguilera, 1994, 1998; Aguilera and Liu, 2012). Prior exposure to homotypic stressors can compromise stress response to a novel stressor (e.g., García et al., 2000), which in turn can expose a previous stress-induced latent behavioral sensitization that often surpasses the HPA axis sensitization (Belda et al., 2015; also see McCarty, 2016). Not surprisingly, intense stressor can facilitate certain cognitive functions and thus promote stress resilience (e.g., Ellis et al., 2017) although its chronic exposure is associated with mood disorders such as depression and anxiety (e.g., Juruena et al., 2020). According to the aforementioned findings on stress responses, we hypothesize that intra-stages expressions and inter-stage transitions in our model of stress development depend on the novelty, intensity, timing, and chronicity of the stressor. Stress stages can be desensitized in subchronic exposure to the same stressor (or homotypic stressors) but accelerated/exacerbated in chronic exposure to the homotypic stressors, which in turn can also hypersensitize stages toward a novel stressor.

We acknowledge that sex/gender may affect the coping-related neural pathways due to sex and stress hormones co-signaling. In particular, neurocognitive variability during stress development can be affected by the levels of circulating estradiol/estrogen. Estrogen signaling influences memory, social learning, and aggressive/defensive behavior associated with the hippocampal and medial PFC functioning (e.g., Milner et al., 2008; Luine and Frankfurt, 2012; Laredo et al., 2014; Almey et al., 2015) and thus contributes to sex differences in stress coping. In females, circulating estradiol levels mediate stress resilience (e.g., Wei et al., 2014b; Luine, 2016; Yuen et al., 2016) and facilitate cerebro- and cardio-protection (e.g., Guo et al., 2005; Murphy, 2011; Adlanmerini et al., 2014) in linear and inverted U-shaped dose-effect (e.g., Bayer et al., 2018), where high estrogen levels increase cognitive sensitivity to stress (e.g., Graham and Scott, 2018; Hokenson et al., 2021). On the one hand, this may help explain why the prevalence of PTSD—*surrender-in-defeat stage* in our model—is two times higher in women than in men (e.g., Breslau, 2002; Zlotnick et al., 2006; Pooley et al., 2018). On the other hand, the androgen effect may explain the findings of why men are inclined toward impulsive behavior (i.e., *risk-to-escape stage* in our model, e.g., Hernandez et al., 2020) and are more affected by stress magnitude, compared to women who are more affected by stress frequency (e.g., Grissom and Reyes, 2019; see also Hidalgo et al., 2019).

Our hypotheses need to be tested to further clarify the various interfering factors with stress reactivity and resilience, such as sex hormones and genetic polymorphism related to serotonin and dopamine signaling reviewed above, as well as stressor type and stress timing/continuity (single, repeated intermittent, or chronic) that can involve different neural pathways and different reactivity of the HPA axis and LC-NE system. Nevertheless, these hypotheses can help explain why active coping is negatively linked to psychological health as reviewed above (**Figure 1**). It also supports the fact that chronically stressed individuals with depression/anxiety and poor cognition require psychological and social assistance.

Concluding Remarks

Neurocognition plays a vital role in adaptation and monitors the severity of challenges faced. When cognitive appraisal assigns a negative value to the salient stimuli, it is the moment they become psychological stressors and stress arises. Thus, psychological stimuli can vary in nature because it is the level of cognitive "attention" that determines stress and its severity, that is the stress appraisal/interpretation, but not the stimuli *per se*.

To address the nuances underlining stress severity, we propose to update a dichotomy in the cognitive appraisal terminology—*self-appraisal* (i.e., the perception of self-efficacy to deal with the stressor) and *stressor-appraisal* (the perception of threat/challenge). This dichotomy is intended to facilitate cognitive behavioral therapy, as well as translational research on stress and mental resilience. Specifically, *self-appraisal* relates to successful emotional downregulation and enables cognitive flexibility vs. *stressor-appraisal* which can contribute to emotional dysregulation and attentional tunneling that restricts/alters executive functioning. Noted specifics of the cognitive appraisal duality are associated with the PFC and amygdala interplay during the processing of aversive emotions and fear, which is linked to stress sensitization and psychiatric consequences (e.g., Palamarchuk and Vaillancourt, under review).

To advance our understanding of mental resilience and stress development, we offer new insights to the scholarly literature on psychological stress coping with respect to previously published reviews. First, we differentiate the neurocognitive aspects in stress development with four key phases: (i) stressor detection, (ii) stress appraisal (assessment of stress severity), (iii) stress reactivity, and (iv) decision making. Clinical analysis of each phase may help with ruling out primary and secondary causes of behavioral maladaptation. For instance, it is important to keep in mind that sudden and inadequate behavioral reaction to an event (i.e., detection of a novel stressor) may be related to a totally different event that occurred chronically in the past that latently compromised psychological health (i.e., prior chronic exposure to homotypic stressors can trigger cognitive "defence," see **Figure 1**). Another example is that prolonged uncertainty increases the chances of risky/impulsive behavior.

Second, we model a complex concept of stress development that introduces an intra-individual variability factor in the stress reactivity phase, which is based on the neural dynamics in cognitive processing. In particular, we hypothesize that coping styles are influenced by intra-individual neurocognitive variability moderated by stress reactivity (phase iii) across three major stages: (1) alarm-to-threat [check] stage → (2) risk-to-escape [stalemate] stage → (3) surrender-in-defeat [checkmate] stage (**Figure 1**). *Alarm-to-threat stage* denoting the cortisol and NE surges in response to psychological stress must not be confused with the *alarm phase*, classically referred to triphasic allostasis process, which originated from the "general

adaptation syndrome" concept by Selye (1998), reprint of 1936) that described "typical syndrome" following "diverse nocuous agents." That is, the general alarm reaction within "6–48 h in rat models of acute nonspecific stress."

Finally, we emphasize that stress coping can fluctuate in a predictable intra-individual manner. Identifying the stressor's novelty/chronicity and stress stage/phase can help with early prevention and appropriate therapy of maladaptive stress coping, and in turn, prevent mental disorders.

AUTHOR CONTRIBUTIONS

TV encouraged, supported, and supervised ISP to investigate stress impact on cognition. ISP planned and carried out the project, the main conceptual ideas, developed the theoretical models and hypotheses, and designed the figures. ISP wrote the manuscript with support from TV. ISP and TV provided critical feedback, helped shape the manuscript, and contributed to the final version. The authors are accountable for the content of the work. All authors contributed to the article and approved the submitted version.

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Toward EEG-Based BCI Applications for Industry 4.0: Challenges and Possible Applications

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In the last few decades, Brain-Computer Interface (BCI) research has focused predominantly on clinical applications, notably to enable severely disabled people to interact with the environment. However, recent studies rely mostly on the use of non-invasive electroencephalographic (EEG) devices, suggesting that BCI might be ready to be used outside laboratories. In particular, Industry 4.0 is a rapidly evolving sector that aims to restructure traditional methods by deploying digital tools and cyber-physical systems. BCI-based solutions are attracting increasing attention in this field to support industrial performance by optimizing the cognitive load of industrial operators, facilitating human-robot interactions, and make operations in critical conditions more secure. Although these advancements seem promising, numerous aspects must be considered before developing any operational solutions. Indeed, the development of novel applications outside optimal laboratory conditions raises many challenges. In the current study, we carried out a detailed literature review to investigate the main challenges and present criteria relevant to the future deployment of BCI applications for Industry 4.0.

Keywords: EEG, BCI applications, BCI challenges, technology transfer, Industry 4.0

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1. INTRODUCTION

Recent advances in neuroscience and engineering led to the development of new applications interfacing minds with machines, known as Brain-Computer Interface (BCI) technology. The origins of BCI date back to the 1960s, with Delgado (1969) who notably developed an implantable chip used to both stimulate the brain by radio and send electrical signals of the brain by telemetry, allowing the subject to move about freely. A few years later, Vidal (1973) explored the use of scalp-recorded brain signals in humans to implement a simple non-invasive BCI based on "visually evoked potentials" (see Vidal, 1973). Those experiments paved the way for the development of non-invasive BCI paradigms that made use of neuroimaging techniques as electroencephalography (EEG), magnetoencephalography (MEG), functional magnetic resonance imaging (fMRI) and functional near-infrared spectroscopy (fNIRS) (see Rao, 2013 for a comprehensive review). Indeed, by translating the recorded neural activity into digital commands via mathematical and AI methods (see Wolpaw et al., 2002) (**Figure 1**), BCI enables controlling external devices with the brain (e.g., Padfield et al., 2019; Khan et al., 2020), such as a computer, a robot, or an exoskeleton (e.g., Nuyujukian et al., 2018; Benabid et al., 2019; Moses et al., 2019). This ability is particularly interesting in specific contexts where voice or motor commands cannot be used (e.g., Lin et al., 2014).

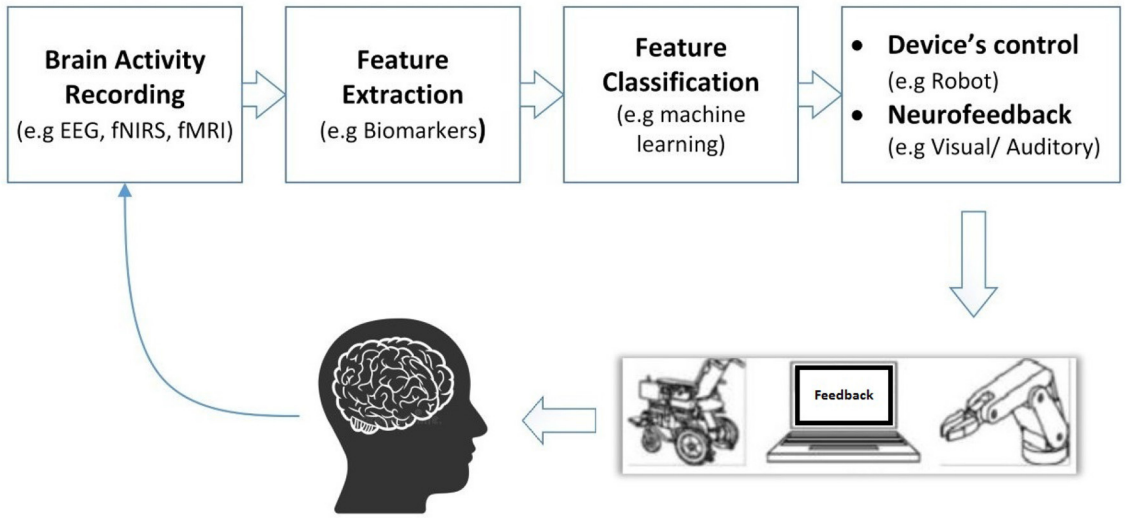

FIGURE 1 | Brain-computer interface scheme.

Regarding the user's task and the neural patterns of interest, we can distinguish three main categories of BCI, namely, active, reactive and passive BCI (see Kögel et al., 2019). Firstly, while using an active BCI, the agent must intentionally modulate their brain activity to bring out neural characteristics that will become identifiable after mathematical processing and classification, as Motor Imagery (MI) paradigm (e.g., Salvaris, 2014). Secondly, reactive BCI relies on neural activity that is typically triggered by an external stimulus—mostly visual or auditory—and that evokes brain responses, such as P300 event-related potential (ERP) (e.g., Jin et al., 2012) or Steady State Evoked Potentials (SSEP) (e.g., Chen et al., 2017). Thirdly, passive BCI relies on brain activity which is not voluntarily modulated by the user, in order to evaluate psychological states such as drowsiness (e.g., Hongfei et al., 2011; Dehais et al., 2018), frustration, or even cognitive load (e.g., Roy et al., 2013; Myrden and Chau, 2017). More details concerning the neurophysiological underpinnings, as well as the advantages and limitations of these three BCI methods are provided in section 3.1.

Thus, in the last two decades, many types of BCI techniques and applications have emerged, especially in the clinical field where it represents a promising technology for assisting or rehabilitating neurological patients and contribute to the faster reintegration of brain-injured patients (e.g., Chaudhary et al., 2016; Verplaetse et al., 2016). However, recent advances in neuroscience and technology, especially non-invasive and portable brain imaging techniques related to EEG, have encouraged the development of novel applications outside the medical and scientific areas (e.g., Abdulkader et al., 2015; Rashid et al., 2020). Notably, one might list the following fields of education (e.g., Wegemer, 2019), entertainment (e.g., Bonnet et al., 2013; Kerous et al., 2018; Ramchurn et al., 2018; Vasiljevic and Miranda, 2020), biometrics authentification (e.g., Alariki et al., 2018; Chan et al., 2018), or even civil and military aviation (e.g., Dehais et al., 2018).

Recently, the industrial sector has also shown a growing interest in BCI (e.g., Angrisani et al., 2018), where the societal, economic and commercial impacts of this technology could be important (e.g., Van Erp et al., 2012). Indeed, since modern times, Industry has continuously seized on emerging technologies to improve its efficiency and performance and each industrial revolution has entailed deep socioeconomic changes and challenges (see Morrar et al., 2017). The last industrial revolution, also known as Industry 4.0, specifically harnessed digital technologies such as AI, big data and analytics, or the Internet of things (see Chunguang et al., 2020), which led to a constantly evolving and intelligent automation of industrial processes. However, it also raises important questions regarding environmental, ethical and human factors (see Melnyk et al., 2019). Interestingly, similar technologies are presently required to develop sophisticated BCI, notably, to efficiently process brain signals and emit commands to the connected device. Then, regarding this technological compatibility, BCI applications could theoretically constitute a potential extension of the 4th industrial revolution. Moreover, "Human enhancement" provided by BCI techniques could represent a viable way to conciliate industrial and societal concerns in a near future.

2. RESEARCH GOALS

To this day, the use of BCI techniques in Industry 4.0 remains theoretical, being mainly explored in academic articles or exhibition demonstrators. In the following, we investigate the potential benefits of implementing BCI and how it could help to re-introduce humans within the industrial processes, by facilitating the operator's work and limiting potential risks and human errors (e.g., Jinjing et al., 2021). Indeed, technical and ethical limitations inherent in non-invasive BCI necessarily hamper the expansion of this technology within operational contexts (e.g., Rashid et al., 2020). Thus, the question of selecting

the most suitable and relevant BCI technique for the industrial sector arises, especially regarding its reliability, its generalizability or its ease of use. In this context, we sought to explore the criteria that will be decisive for the potential integration of BCI in industrial settings regarding the current maturity of BCI techniques. In the current review, we attempt to answer the following question:

- Which BCI techniques are most likely to be deployed in future industrial applications?

To this end, we carried out a detailed literature review of the databases (Science direct, PubMed, IEEE, Springer, ArXiv, ResearchGate, Google Scholar, MDPI, HAL) queried with the following inclusive keywords: *BCI, Brain-computer interface, BCI and Industry 4.0, EEG-based BCI, BCI applications, BCI challenges, Assistive technology* associated to scientific studies published between 2010 and 2021. Then, we extracted recent and relevant empirical and review articles, conference proceedings, research reports related to the usage of BCI in industrial environments. Note that the exclusion criteria include "Invasive BCI techniques, non-invasive BCI for both clinical applications and non-industrial fields." In addition, all the references that are provided in the following sections serve as recent examples for the identified BCI applications for Industry 4.0.

3. RESULTS AND DISCUSSION

3.1. BCI Applications for Industry 4.0

Theoretically, the deployment of BCI applications in Industry 4.0 could contribute to put the operator back at the center of industrial processes. The possible industrial applications could be categorized as follows: (1) safety at work, (2) adaptive training and (3) device's control (e.g., Tamburrini, 2014; Balderas et al., 2015; Oztemel and Gursev, 2018).

3.1.1. Safety at Work: *Passive BCI*

Recently, there has been a growing interest in using EEG-based BCI as a potential solution allowing to reduce safety risks, while enhancing productivity and improving decisions in operators and managers (e.g., Villalba-Diez et al., 2019). Regarding technical aspects, this application would rely on the use of passive BCI. Notably, the decomposition of EEG signal into frequency bands represents a convenient way to identify the user's neurocognitive condition. For instance, many studies have shown that the spectral power in alpha (8–13 Hz) and theta (4–7 Hz) bands increase when a person feels fatigued (e.g., Craig et al., 2012; Dehais et al., 2018). Such modulation can therefore constitute a first-rate indicator of the user's arousal state (e.g., Zhang et al., 2017). Changes in theta rhythm in frontal sites and alpha rhythm in parietal sites (e.g., Borghini et al., 2013), could also indicate a state of cognitive overload, which has been linked to reduced performances in complex tasks (e.g., Aricò et al., 2018). Thus, by allowing user-state monitoring, passive BCI could notably limit or prevent safety risks and human errors without requiring any particular effort from the user. Theoretically, this passive aspect makes it potentially usable in multitasking contexts and does not induce additional fatigue. However, passive BCI is subject to an important inter-individual variability. Moreover, EEG band frequencies must be carefully analyzed because similar spectral power patterns could be associated to several mental states (e.g., Aricò et al., 2018).

Then, the neurofeedback provided by passive BCIs could reinforce safety at work by preventing agents from committing dangerous errors due to drowsiness or cognitive overload for instance (e.g., Villalba-Diez et al., 2019). In fact, some industrial sectors—including among others, manufacturing, quality control or pharmaceutical industry—require operators to carry out a large number of repetitive and sensible actions that directly depend on the operator's neurocognitive states (e.g., Villalba-Diez et al., 2019). In this context, EEG-based BCI could allow to monitor operators' mental states like fatigue, stress, or loss of vigilance which can be critical during dangerous activities. In particular, fatigue monitoring is more considered as a valuable tool in repetitive and automatic tasks such as driving, piloting or quality control (e.g., Zhang et al., 2017; Huang et al., 2019). For this reason, some solutions that integrate EEG captors within worksite helmets (e.g., Li et al., 2014; Barkallah et al., 2015) or under headwears (e.g., Zhang et al., 2017) have already been proposed to warn users whenever a critical drowsiness threshold is reached.

3.1.2. Adaptive Training: *Passive BCI*

Another emerging BCI application—also relying on passive BCI technique—concerns adaptive training, which might reinforce the learning process of complex industrial procedures. This neurofeedback approach is already used in clinical settings, to support learning or rehabilitate attention in children with neurodevelopmental disorders (e.g., Papanastasiou et al., 2020). Indeed, such monitored training would allow boosting attentional processes while adapting task difficulty according to cognitive load or vigilance, to optimize learning and prevent frustration. In this context, Huang et al. (2019) proposed to combine BCI with other technologies such as Virtual Reality (VR) and/or Augmented Reality (AR) to make the learning task even more immersive and efficient. Similarly, Nisiforou (2013) proposed the use of eye tracking coupled with EEG to assess and evaluate students' cognitive dimensions.

3.1.3. Device's Control: *Reactive* and *Active BCI*

Besides monitoring applications, another potential industrial use case concerns cobots or machine' s control. Both active and reactive BCI paradigms could be relevant for this kind of application. Regarding active BCI, motor imagery (MI-BCI) is the most commonly used paradigm. During this task, the user is typically required to imagine specific movements (e.g., for limb), that allows controlling an external device in the same way (e.g., a robot, an exoskeleton or an avatar etc.). Indeed, imagining a movement typically produces neural activity that is spatiotemporally similar to the activity generated during actual movement, but smaller in magnitude (see Wolpaw et al., 1991; Pfurtscheller et al., 1997; McFarland et al., 2000). Although this method is particularly promising in the context of motor disability, its main drawbacks stem from some limitations of the EEG method itself. Notably, due to a low spatial

resolution, it is not possible to localize accurately activation sources within the same hemispheric sensorimotor cortex, which prevents the reliable identification of fine motor movements (e.g. distinguishing a movement of the whole arm from a movement of the sole hand). This generally limits the number of potential and reliable orders to 4 (e.g., Schlögl et al., 2005). Another disadvantage of the MI paradigm relies on the EEG low signal/noise ratio. Indeed, due to its non-invasive nature, EEG recordings also contain irrelevant, non-brain signals like environmental electromagnetic artifacts or peripheral nervous transmissions. In addition, this technique requires a long training phase to be properly mastered (e.g., Vidaurre et al., 2011) that can last several days or weeks and which is incompatible with many industrial contexts. Then, even after a substantial training phase, more than 30% of individuals would remain BCI illiterate (e.g., Ahn et al., 2014; Lee et al., 2019), by staying unable to control the device. Finally, motor imagery requires an intense concentration from the user and is incompatible with other "real" movements that would interfere with the thought command and ultimately, with the BCI accuracy. Thus, MI paradigms prevents the user from performing other tasks at the same time and necessarily generates fatigue (e.g., Talukdar et al., 2019) which makes its use rather inflexible. Despite all these limitations, MI training session design is of vital importance for clinicians planning to implement interventions adapted to participant health status, age and gender. For that, numerous studies are attempting to overpass these limitations—notably regarding the problematic user-training phase—by proposing some guidelines that could be useful to improve this critical dimension (e.g., Schuster et al., 2011; Jeunet et al., 2016)

Regarding reactive BCI, the two most widely used and reliable EEG markers are the P300 and SSVEP (steady-state evoked potentials). P300 is a positive event-related potential that is apparent whenever the user has noticed an unexpected or a rare visual or auditory event (e.g., Walter et al., 1964; Donchin and Smith, 1970). Although associated to a fast training, this technique remains very sensitive to surrounding noise and motor artifacts (e.g., Chamola et al., 2020), preventing its use in a noisy or multitasking context. In addition, a single command requires the user to focus their attention on several consecutive events, including non-relevant (non-rare) and relevant (rare and unexpected) ones, which necessarily decreases the system's speed (e.g., Lotte et al., 2015) while being costly in terms of attentional processes (fatigability).

As regards SSVEPs, the distinct potential commands are displayed via a visual interface (e.g., screen or AR glasses), icons that flicker at distinct frequencies (e.g., 10 Hz) represent different options. Then, while the user is focusing on one flickering option, visual neurons (i.e., from the primary visual cortex) are synchronously discharging at the same rate, which will ultimately allow the user's choice to be identified with classification algorithms (e.g., Middendorf et al., 2000; Faller et al., 2010). Interestingly, SSVEP-BCI is less prone to inter-individual differences, which enhances its accuracy (e.g., Lotte et al., 2015) and reduces its illiteracy rate (e.g., Lee et al., 2019). Moreover, it does not require a long training phase (e.g., Guger et al., 2012) while the latency between the neural command and the command execution can potentially be lower than in other BCI paradigms. However, similarly to the P300 paradigm, extended use can induce significant fatigue, due to the required active concentration on stimuli. Another disadvantage of reactive BCI is the need to use external stimuli to allow the agent to make a choice. The exerted control is therefore limited to the presented options and is not strictly endogenous. Moreover, it requires an additional interface, such as a screen, which decreases its portability (e.g., Cecotti et al., 2010). Regarding this last limitation, recent works have attempted to create new interface designs and stimuli that reduce fatigue and discomfort, to promote a daily and long-term use of this BCI paradigm (see Baek et al., 2019).

In industrial settings, an SSVEP-BCI combined with AR glasses could facilitate making certain tasks hands-free (and therefore, replace buttons/joysticks) for operators who control machines (e.g., Angrisani et al., 2018, 2020). Looking further ahead, one might also imagine that a MI-BCI could eventually allow to quickly take over control of transport vehicles in case of emergency braking. In this scenario, BCI must be sufficiently advanced to allow a reliable transmission of the "thought" braking command to the mobile device. It should also be faster than our peripheral nervous transmission to become valuable regarding accidents prevention, which is far from being the case at present (e.g., Royer et al., 2010; Kim and Lee, 2017; Georgescu et al., 2020).

3.2. Specifications and Limitations

Regardless the potential benefits that BCI could bring to Industry and besides the actual weaknesses of EEG-based BCI, it is also necessary to consider its current ethical, ergonomic and technical limitations, before any operational development or usage.

The first limitation relates to **ethics** and acceptability that must be further questioned and regulated regarding the individual and societal impacts that industrial BCI applications might have. Among other things, industrial BCI must ensure data confidentiality and security given the sensible and personal nature of recorded physiological signals (e.g., Burwell et al., 2017). In addition to the operator consent, individual data must be locally stored and processed. Then, the relevant extracted information must be accessible to the sole concerned operators. To be acceptable to the end users, the BCI system must provide a real improvement of work conditions and/or safety by limiting the risks in dangerous conditions, such as passive BCI. Presently, non-invasive BCI for device control (active and reactive BCI) remains too immature to get easily used and adopted by the agents. According to Burwell et al., 2017, the need for regular and challenging training sessions (e.g, Motor Imagery) may impose physical, emotional, and financial burdens on the user (e.g., Fenton and Alpert, 2008) and it may require more cognitive planning and attention than a user can achieve on a regular basis, leading to frustration (e.g., Glannon, 2014).

Another crucial requirement for an effective adoption by end-users concerns **ergonomics** (e.g., Li et al., 2014). More precisely, BCI solutions must be non-invasive; comfortable to wear; portable and not bulky to allow mobility in different

TABLE 1 | Levels of adequacy between industrial and technical BCI requirements based on the EEG, regarding potential industrial applications.

		Active BCI	**Reactive BCI**		**Passive BCI**	
		Motor imagery	**P300**	**SSVEP**	**Fatigue monitoring**	**Cognitive load monitoring**
	Industrial applications	Device control	Device control	Device control	Safety, training	Safety, training
Ethics	Acceptability	++	++	++	++	+
Ergonomics and user experience	Portability	+++	++	++	+++	+++
	Fatigability	+	++	++	+++	+++
	Multitasking	+	+	++	+++	+++
	Training/calibration	+	++	+++	++	+
Technical	Reliability	+	++	+++	++	++
	Rapidity	+	+	++	++	++
	Flexibility	+	+	++	+++	+++

areas; non-tiring for the user; multitasking-compatible to allow usual tasks without limitations; inexpensive in terms of training time and dedicated resources. On the one hand, passive BCIs are currently more suited to the criteria of portability, non-fatigability and multitasking since they do not require external stimuli and devices (e.g., AR glasses or monitor) and they do not require to perform a particular cognitive task, in comparison with active and reactive paradigms (see Wolpaw et al., 2002; Rao, 2013). On the other hand, the training cost is particularly important in active BCI, while it is less important in reactive and passive BCI (e.g., Cecotti, 2010; Jeunet et al., 2017; Myrden and Chau, 2017).

Moreover, BCI **technical** specificities must be considered to ensure: (1) *reliability*—which depends on classifications' *accuracy*—(2) *reactivity* in terms of response time and (3) *flexibility* to adapt to context and individual differences (e.g., Rashid et al., 2020). In other words, an ideal BCI solution must be able to interpret an operator's neural signal, by minimizing classification errors and training time, while increasing the information transfer rate (ITR) and its generalizability of use (e.g., Rashid et al., 2020). According to literature, reliability and flexibility appear to be higher in reactive BCI and particularly in SSVEP-based BCI (e.g., Chen et al., 2014), relative to active and passive paradigms. Flexibility appears lower in active paradigms, with a high illiteracy rate, in comparison with reactive and passive paradigms (e.g., Lee et al., 2019). Reliability depends on the quality of the collected signal and the relevance of AI algorithms applied. Besides, some neurophysiological markers used are more or less resistant to surrounding noise and should be carefully selected with a reduced latency. In addition, a large-scale deployment BCI requires adaptability and flexibility to make it usable and equally reliable for a large number of users.

Based on the advantages and disadvantages of each BCI application described previously (section 3.1), **Table 1** summarizes the estimated levels of adequacy between the main industrial criteria (section 3.2) and the actual state-of-the-art EEG-based BCI, in the light of potential applications. More specifically, we have compared the three main non-invasive BCI paradigms and their potential industrial applications considering the most important criteria related to ethics, ergonomics & UX, and technical.

The level of adequacy is ranked as follow: "+" rating represents a low level of match between the industrial requirement and the BCI technique, while "++" and "+++" means an intermediate and a high level of suitability, respectively.

4. CONCLUSIONS AND PERSPECTIVES

BCI is an emerging technology that enables to decode brain activity and translate it into a set of actions reflecting the user's intention, mental state, and even emotions. Numerous public and private actors are envisioning the deployment of BCI in industrial settings in a near future (e.g., Sujatha Ravindran et al., 2020). In the present paper, we summarized the potential applications, key success factors and the most advanced EEG-based BCI paradigms for Industry 4.0. Currently, none of the EEG-based BCI evaluated fits ideally to all the essential industrial criteria we have established based on ethical, ergonomic and technical factors. However, SSVEP-based BCI represents a highly promising technology for device control, while fatigue monitoring appears particularly interesting and appropriate to prevent damaging errors and safety risks in dangerous contexts, or optimize training upstream of these critical situations. With the dramatic rise of EEG-based BCI studies—regarding material, associated algorithms (machine learning, deep learning etc.) or even psychological aspects of BCI—we believe that the large-scale deployment of BCI applications in the Industry is a matter of years. Thus, the ethics and rules related to BCI applications in industrial settings need to be carefully defined to pave the way to effective use.

5. STUDY LIMITATIONS AND FURTHER WORK

The authors are aware that the present results should be interpreted with caution and some important limitations deserve to be mentioned. Firstly, the envisioned industrial applications of BCI are still at the first stages of research and development. Thus, the development of reliable and ethical BCI solutions

adapted to end-users in industrial contexts will necessarily take several years. Secondly, research focussing on BCI users' experience remains extremely rare and further studies must explore this important dimension. Though the users' experience is currently strongly related to technical specificities of BCIs solutions, a user-centered approach similar to the one proposed by Kubler et al. (2014) must be systematically used in future research before considering any large-scale deployment of such neurotechnology.

AUTHOR CONTRIBUTIONS

KD conceived the initial idea, conducted the research, and wrote the first draft with AL. GB reviewed the article's structure. SL contributed with BCI paradigms relevant to Industry 4.0, performed the synthesis, and perspectives with KD. RB and LN contributed, with other authors, to the final proofreading and commented on the draft. All authors have read and approved the final paper.

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REVIEW
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Neural Correlates of Trust in Automation: Considerations and Generalizability Between Technology Domains

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Investigations into physiological or neurological correlates of trust has increased in popularity due to the need for a continuous measure of trust, including for trust-sensitive or adaptive systems, measurements of trustworthiness or pain points of technology, or for human-in-the-loop cyber intrusion detection. Understanding the limitations and generalizability of the physiological responses between technology domains is important as the usefulness and relevance of results is impacted by fundamental characteristics of the technology domains, corresponding use cases, and socially acceptable behaviors of the technologies. While investigations into the neural correlates of trust in automation has grown in popularity, there is limited understanding of the neural correlates of trust, where the vast majority of current investigations are in cyber or decision aid technologies. Thus, the relevance of these correlates as a deployable measure for other domains and the robustness of the measures to varying use cases is unknown. As such, this manuscript discusses the current-state-of-knowledge in trust perceptions, factors that influence trust, and corresponding neural correlates of trust as generalizable between domains.

Keywords: interpersonal trust, gender difference, reliability, human-robot interaction, vehicle automation

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PROBLEM STATEMENT

Trust in automation is a rising concern in many safety-critical systems due to its influence on the utilization strategy and emergent complacency behaviors of the operators. As such, measuring trust in such complex systems is essential to improve system safety and collaborative performance. In collaborative human-automation teaming, the operators can choose when and how to rely on or utilize automation features, highly dependent on how well calibrated their trust is. When trust is lower than the system's capabilities, operators tend to underutilize automated features, either by turning them off or rejecting the technology completely (Lee and See, 2004; Mouloua and Hancock, 2019). When trust is higher, operators tend to over utilize or misuse the assistance, such as continuing to use the assistance despite signs of unreliability, utilizing it in situations that the assistance was not designed for, or easily becoming distracted or complacent when automation has taken over. Currently, the state-of-the-art method to capture trust states is through surveying or interviewing the operators (Lewis et al., 2018; Hopko et al., 2021b), which, due to the discrete nature of surveys, cannot be used as a continuous real-time measure for adaptive automation or trust-sensitive detection systems.

Moreover, these subjective measures are invasive and can be biased based on how the surveys are presented to a population; a worker, pilot, or driver can be biased to answer safety-related surveys following societal and employer expectations assuming they are able to stop work to answer the survey. In a research setting, surveys may also prime the participants to focus on trust, which may disrupt experimental manipulations. Due to these limitations, surveys alone cannot be readily applied to develop adaptive automation systems that can monitor and respond to trust levels real-time, nor used to mechanistically understand trust influencers' impact on perceptions and corresponding behaviors. Deployable continuous techniques, such as functional brain imaging, to objectively quantify trust in automation have promise in filling the need for an accurate and deployable measure of trust. This paper discusses the basis of neural activity as a corollary measure of trust and the potential risks of generalization between interpersonal and automation domains in addition to generalization between popular safety-critical domains such as aviation, vehicle automation, human-robot collaboration, cognitive decision aids, and automation in medical devices (Hopko et al., 2021a). This manuscript discusses the differences between interpersonal and technological domains for how trust is defined, the basis for trust perceptions, the factors that influence trust (including dispositional and gender difference), the neurological basis of trust through a review of trust-manipulated studies, and identifies the current gaps in knowledge.

TRUST DEFINITIONS IN AUTOMATION

Operator trust is a complex and dynamic human factor that is impacted by a myriad of cultural, environmental, and system factor influences (Hancock et al., 2011; Schaefer et al., 2014; Chiou and Lee, 2021). Because trust is complex and dynamic in its nature, it is difficult to comprehensively operationalize the definitions of trust, regardless of technology domain. The most commonly referenced definition in technology is by Lee and See (2004) that depends on the uncertainty and vulnerability of the operator given the automated agent's actions (Chiou and Lee, 2021). While Lee and See's definition is general, some definitions of trust have domain specific divisions. For example, Hald et al. (2019) defined trust as "the combination of feeling physically safe around [the robot] and being able to predict the robot's action in context of the shared task." While this is related to the vulnerability (safety) and uncertainty (predictability), it is a more specific application of Lee and See's definition as vulnerability is specified to physical safety, excluding potential fiscal loss, workload changes, or connected affect state. Similar specification of trust definitions has been observed in other safety critical domains, including cognitive aids and alarms that focus more on system reliability than safety (Madhavan et al., 2006; Parasuraman et al., 2008). While definitions are similar, the traditional survey measurements are influenced by the nuances in definitions. A literature survey by Lewis et al. (2018) found that the majority of utilized trust surveys are un validated, i.e., they are developed study-specific by the researchers. Given specializations of trust definitions across domains, and that many surveys are developed based on these definitions, there are nuanced differences between technology domains on what trust is, the importance of factors that influence it, and questions that are relevant to trust perceptions. In analyzing two validated trust surveys, one generic to trust in automation and another specific to trust in robotics, Kessler (2020) found that the surveys were not interchangeable and were capturing distinct trust perceptions. Given that many studies that investigate neural correlates of trust validate their response to subjective questionnaires, there is a need for the trust domain to unify trust definitions, trust models, and measurement techniques (i.e., surveys, physiological responses), or to acknowledge domain specific findings.

BASIS OF TRUST IN INTERPERSONAL VERSES TECHNOLOGY AUTOMATION

Comparison of trust between interpersonal collaborative domains and technologies is rare, especially with regard to the generalizability of neural signatures (Parasuraman et al., 2014). Madhavan and Wiegmann (2007) performed a literature review illustrating that the predominate bases of trust differ between interpersonal trust and human-technology trust. Interpersonal trust is based on three major rationales: (1) the integrity of the trustee (i.e., how lawful and of good moral), (2) the ability of the trustee (i.e., their capabilities to accomplish the desired interaction/task), and (3) the benevolence of the trustee (i.e., how Good, or altruistic, an individual is). Another investigation notes that interpersonal trust is also influenced by the familiarity between the trusting parties, shared experiences, shared goals, reciprocal discloser, and demonstration of non-exploitation, all expressed over long-durations (Dani et al., 2006). Interpersonal trust directly differs from trust in technology in two ways. The first is that technology lacks intentionality, unlike humans. Automation is based on scripts, system capabilities, and algorithms scoped to a specific use case. Thus, automation cannot truly develop its own intents unlike humans (Madhavan and Wiegmann, 2007; Charalambous et al., 2016), although its intents may be reflective of the designer's (i.e., human) biases. Moreover, the use of automation in safety critical systems are assumed to be designed such that it improves the system or a subcomponent of the system; users may assume automation is intended to work in support of them. Therefore, the reciprocal discloser, demonstration of non-exploitation, or other anthropomorphic traits tend to be less relevant than they are in interpersonal trust. As such, the perceived capability of the automated system has been deemed as the primary basis for trust in automation (Chen et al., 2018).

The second major difference between interpersonal trust and trust in automation is the lack of anthropomorphism (in many technologies) and accompanying societal expectations. While benevolence and integrity are not directly attributable to the technology, users are able to personify technology (Nass and Moon, 2000). Systems that parallel human-like characteristics and personas (i.e., humanoid robots, intelligent agents such as

Alexa) tend to have more trust than systems designed with the same capacities and purpose, but with non-anthropomorphized characteristics (Hancock et al., 2011; de Visser et al., 2017; Calhoun et al., 2019). However, there is a point where extreme similarity between a technology and human can result in a significant drop in trust levels, often referred to as the uncanny valley (Flemisch et al., 2017). Because of this interaction between trust and human-like characteristics in technology, there are observable differences in trusting behavior, founded on emotional connection to the system rather than system capability (Jensen et al., 2020). Given the spatial association of cognitive and emotional systems in the brain, it is conceivable that these trust differences are observable in neural activity between interpersonal, anthropomorphized technology, and non-anthropomorphized technology trust.

FACTORS INFLUENCING TRUST IN AUTOMATION

Trust is influenced by human factors, automation factors, and environmental factors (de Visser and Parasuraman, 2011; Hancock et al., 2011; Schaefer et al., 2014; Lewis et al., 2018). Example human factors include demographics and user characteristics (age, gender, culture, race, ethnicity, personality, etc.), situational factors (mood, fatigue, affect, vigilance, task engagement, etc.), and user attributes (mental workload capacity, capability, expertise, etc.). Example automation factors include the purpose of the system, the process in which the system completes its task, automation level, the system attributes (size, safety features, etc.), in addition to the capability, accuracy, reliability, or ease of use of the system (Hoff and Bashir, 2015). Environment factors depend on the scoped boundary of the system, but can include interaction with other systems, physical layout and proximity to the system, cultural, and societal factors, weather and others (Hancock et al., 2011). The following review will compare and contrast these factors in the broad sense of safety critical automation domains. Because the use cases, and thus goals, tasks, strategies, and perceptions, are different, we posit that the tendencies of trust perceptions differ between technologies, potentially changing the accompanying neural signatures.

Perceptual Basis of Trust

While the magnitude and variance may change between domains, trust is comprised of three, highly interrelated components: dispositional, situational, and learned (Hancock et al., 2011). Dispositional trust is the reasonably static underlying tendency to trust the automation in general, and can be influenced by several factors, such as demographics, culture, race, age, gender, etc. Situational trust is the trusting behavior based on both internal factors (i.e., mood, engagement, fatigue), and external situational factors (i.e., environment, system process, features). Learned trust is a dynamic mental model of the trustworthiness of a system as one gains familiarity, both through the reputation of the system and first-hand interaction. While these components are not completely separable, studies primarily focus on manipulating situational instances, such as reliability and environmental factors, or manipulate situational-learned components such as the influence of reliability (or unreliability) over time or user experience with a specific task. The neural activity associated with these manipulations relies heavily on cognitive ability to determine an associated mental model of the reliability and risk, such as Bayesian mental models (Adolphs, 2002). It is clear that emotional or affect factors also influence trust perceptions as anxiety, hostility, and negative attitudes are often measured alongside trust perceptions (Hopko et al., 2021b), although emotional studies of trust are scarce (Jensen et al., 2020).

Dispositional and Gender Differences in Trust Perceptions

The impact of dispositional trust on overall trust perceptions has been commonly overlooked, where most trust-manipulation studies focus on event-based trust breaches or system wide changes that manipulate trust (Parasuraman et al., 2008). Dispositional trust is key as the generalization of the importance of trust influencers (e.g., reliability, cyber security, accuracy) requires understanding of how users value trust influencers in individual domains. This is needed in order to build robust designs given the user population and predisposition to utilize the technology. For example, gender differences have been observed in both technology (Syrdal et al., 2007; Kuo et al., 2009; Strait et al., 2015) and interpersonal trusting behavior (Croson and Buchan, 1999). Furthermore, there have been gender differences in societal values, where women have historically been more implicated in healthcare and the well fair of the family than men, making ∼80% of decisions for their families (Matoff-Stepp et al., 2014). This has resulted in healthcare marketing strategies that target women more often than men. However, other domains, such as vehicle automation and industrial robotics, do not have similar marketing or training strategies, which may have implications for both safety and technology-focused workforce development. Because gender is a factor in trusting behavior, and gender has domain-specific characteristics and values, there may be rationale for dispositional trust differences between these technologies. This can be observed in a pilot survey conducted by the authors (Hopko et al., 2021a), where the traditional measure for dispositional trust, using a propensity to trust automation questionnaire (Jessup et al., 2019), was found to correlate with trust more strongly in cyber/cognitive aid technologies than any other investigated domain (e.g., medical, robots, vehicle).

Situational and Learned Differences in Trust Perceptions

The underlying trust behaviors are also likely to be different due to the familiarity and experience with a domain. The average knowledge of automation, given cultural standards, varies between applications; people are more familiar with vehicle automation than they are of collaborative robotics (Hopko et al., 2021a) likely because it is a more commercialized technology; moreover, aviation automation is a normality for pilots (Trösterer et al., 2017). Furthermore, as these domains have different working environments and different tasks, the severity of situational trust factors may not be

directly generalizable between domains; a reliability of 90% may be acceptable for diagnostic systems but unacceptable for vehicle automation (Wickens and Dixon, 2007). Because each technology domain is a different use case, the relevance of trust influencers (e.g., reliability) to operators and the socially acceptable behaviors (e.g., reliability thresholds) of the systems differ between domains. The importance of traditional trust scales, namely reliability, accuracy, or ease of use, have been shown to differ between technology domains, such as accuracy being most important in medical device automation, and reliability most important in vehicle and robots (Hopko et al., 2021a). Thus, while there is overlap in many of these technologies (e.g., vehicles can have cyber aid alarm systems), the magnitude and relevance of trust influencers, or trusting behaviors, between domains need independent considerations.

The three trust components of trust and their interdependency is likely to differ between domains. The extent to which these differences would influence physiological correlates is currently unknown. It is likely that physiological responses, such as dermal activity, heart rate activity, etc., lack the spatial resolution offered by neurological responses to capture these distinctions. For example, an increase in heart rate may signal increased physical workload (Roscoe, 1992; Garet et al., 2005), but also an anxious response (Perrotta, 2019), while distinct changes in the amygdala, the hippocampus and the prefrontal cortex may signal specific onset of anxiety (Perrotta, 2019). The initial biases of participants toward trusting certain technologies more than others and the grounds for which trust perceptions are founded hint that accompanying neural and physiological responses may differ. The magnitude of the neurological responses, and brain function-specific activation patterns, may also differ between domain due to these attitudes and emotional connection to certain technologies, which has been shown to influence neural activity (Larsen et al., 2008).

NEURAL BASIS OF TRUST

Introduction to Popular Brain Imaging Techniques

There is no better place to capture a subjective human state than at the source: the brain. The non-invasive brain imaging techniques discussed in this section are measurable corollary responses of trust. Functional magnetic resonance imaging (fMRI) is considered the gold-standard for mechanistic brain imaging due to its increased spatial resolutions; however, it has reduced temporal resolution, high cost, and is not ambulatory (Logothetis, 2008; **Table 1**). In contrast to fMRI, electroencephalogram (EEG) and functional near-infrared spectroscopy (fNIRS) are ambulatory measures; they can measure brain activity in real-time during a task with higher temporal resolution, although are limited to measuring cortical brain activity with lower spatial resolution (Ferrari and Quaresima, 2012; Mehta and Parasuraman, 2013).

TABLE 1 | Summary of popular brain imaging techniques.

Imaging Method	fMRI	fNIRS	EEG
Spatial Ranking	High	Mid-High	Mid
Temporal Ranking	Low	Mid	High
Cost	Expensive	Affordable	Upper-Affordable
Measurement	Hemoglobin Dynamics	Hemoglobin Dynamics	Electrical Activity
Ambulatory		✓	✓
Measurable Locations	Cortical + Subsurface	Cortical	Cortical

TABLE 2 | Search terms.

Group	Terms
Brain	neural correlates, neural signatures, neural patterns, neural features, brain, neuro, fMRI, EEG, fNIRS
Trust	trust, distrust, overtrust, undertrust, mistrust
Technology	robot, automation, technology, tech, machine, artificial intelligence, computer, vehicle, aviation, pilot, air traffic, operator

Review of Neural Correlates of Trust in Automation

Here we review studies that manipulate trust in automation and measure accompanying neural correlates. Studies were identified using the following three search term groups in the title, keywords, or abstract of the paper (**Table 2**) in addition to reviewing references of included papers. A total of eleven studies were included based on the criteria of (1) the study focused on trust in any type of automation, (2) the study must have manipulated trust and measured accompanying neural activity, (3) the study must have reported the locations that correlated with trust condition. Studies that applied machine learning techniques to all measured brain locations but did not provide which locations were significant contributors were excluded. And, (4) the studies were not focused on highly anthropomorphized technology. While anthropomorphism was allowed in the included studies, highly anthropomorphized technology (e.g., gendered humanoid robots, systems with emotions) were excluded from this synthesis because high anthropomorphism influences the bases for which trust is provided as discussed in section Basis of trust in interpersonal verses technology automation.

The locations that were found to correlate with trust are defined based on Brodmann area or, for cortical locations, the 10–20 international standard, whose locations are universally denoted as follows: Nasion (N), Frontal (F), Central (C), Parietal (P), Occipital (O), and Inion (I). **Table 3** summarizes studies' trust manipulation methods, imaging method, and locations that were significantly different between trust-manipulated conditions. **Figure 1** is the visualization of the identified locations, where each green location was found to correlate with trust in at least one study. The primary method to capture

TABLE 3 | Summary of neural correlates of trust in technology domains.

Paper	Trust manipulation method	Imaging method	Locations correlated with trust manipulation
Wang et al. (2018)	Autonomous agents are designated as "Trustworthy" or "Untrustworthy" each with different probabilities of getting a financial return.	EEG	AFP5, F7, F5, F3, AF7, AF5, AF3, F1, Fz, C1, Cz, AFP6, AF6, AF8, POz, O1, O2
Eun-Soo et al. (2019)	Human and robot faced agents with different risk-taking natures who provide advice with a set reliability rate.	EEG	Cz, FC1, FC2
Ajenaghughrure et al. (2019)	*Who Wants to be a Millionaire* game with different question difficulties and points per question with human or computer help line	EEG	C3, C4, P4, POz
Hu et al. (2016)	Vehicle obstacle detection with reliability of 100% or 50% that considers trust over time.	EEG	Cz, C4
de Visser et al. (2018)	Agent has credibility (expert, novice) with correct response reliability rate (90, 60)%	EEG	region centered at FCz
Dong et al. (2015)	Prisoner's dilemma type game against human or computer agent with collaborative or egoism strategies.	EEG	Fz, Cz, Pz, FC1, FC2
Goodyear et al. (2016, 2017)	X-ray luggage-screening task with human or computer agent advice each with 60% reliability at detecting a knife.	fMRI	right rostrolateral-PFC, right V1, right and left pre-SMA, right orbito-FC
Sanders et al. (2019)	Two levels each of reliability and credibility	EEG	Anterior cingulate cortex, centered at FCz
Pushparaj et al. (2019)	Aircraft conflict with accurate alerts at 5 levels of detection difficulty and shown videos of potential conflicts.	fMRI	Insular cortex, amygdala, putamen, nucleus accumbens, anterior and posterior cingulate cortex
Akash et al. (2018)	Obstacle detection alert with reliability of 100% or 50% where the operator must choose to take or ignore advice	EEG	POz, P3, Fz, C3, C4, Cz

neural activity was with EEG, where the remaining papers utilized fMRI. The majority of the studies included looked into artificial intelligence or computer algorithms with correct and incorrect responses at different reliability rates (Dong et al., 2015; Goodyear et al., 2016; Akash et al., 2018; de Visser et al., 2018; Wang et al., 2018; Ajenaghughrure et al., 2019; Eun-Soo et al., 2019; Pushparaj et al., 2019; Sanders et al., 2019). It was shown that humans react differently to correct and incorrect actions of the agent, and these reactions correlate with subjective trust scores, with accompanying neural activation.

Activation Features

Neural activation is the primary feature extracted from neural activity. It illustrates the strength to which a region, or location, is responding to a stimulus. The study conducted by de Visser et al. (2018) predicted that a mismatch between expected and actual outcomes in artificial intelligence agents would result in a growing negative potential in the anterior cingulate cortex, which can be measured in the front ocentral scalp region centered around FCz. The authors confirmed that correct vs. error responses have different neural signatures and that different levels of reliability, but not credibility, impact the magnitude of the activation; stronger activation occurs in the intermediate frontal cortex, supplementary motor area, and premotor cortex when there is an error in highly reliable situations. They also report that this activation negatively correlates with human trust; an increase in activation suggests lower trust. Validation of this experiment was perfomed by Sanders et al. (2019), who also observed increased activation of the region around FCz during unreliable conditions. All EEG studies that manipulated reliability report similar findings, where at least one of the locations Fz, F1, F2, FC1, FCz, FC2, C1, Cz, C2 are identified with identical activation direction (increased activity for lower trust). The fMRI study (Goodyear et al., 2016, 2017) also identified the left and right pre supplementary motor area, located around FCz, which overlaps with the regions identified in the aforementioned EEG-based trust studies. Time-domain features were predominately found significant for EEG features in the frontocentral region locations that are centered around FCz (Akash et al., 2018; Eun-Soo et al., 2019), similar to that identified in a literature review of EEG brain-computer-interface features (Lotte et al., 2007).

For studies that did not manipulate reliability, there was more variance in the locations that correlate with trust conditions. For example, in an investigation that had agents provide recommendations for a financial investment with three different underlying probabilities of return, the locations that were found to vary by probability were primarily located in the left, medial, and right frontal lobe and occipital lobe (Wang et al., 2018). The investigations into game theory strategies and game show tasks also identified the locations in the parental cortex and occipital cortex (Dong et al., 2015; Ajenaghughrure et al., 2019). While these investigations focus on cyber aid technology, the slight differences in the studies as they move farther away from detection systems (namely, game theory studies) illustrates the need to consider the generalizability of these correlates to different use cases and trust influencers. These correlates may only identify the influence of reliability to trust perceptions, rather than robust to any trust influencer, such as gender.

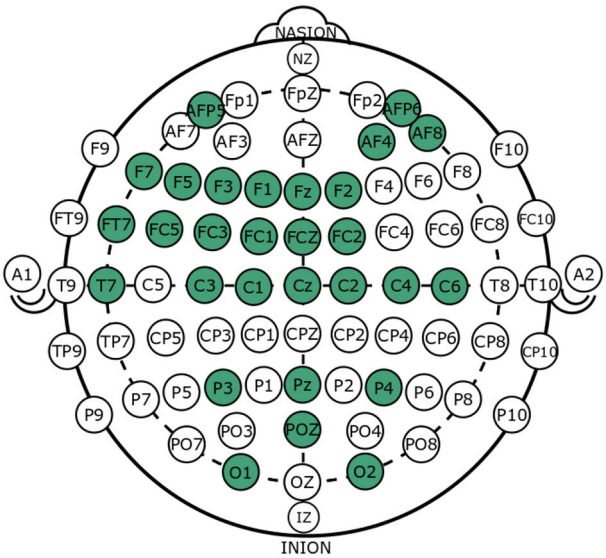

FIGURE 1 | Binary summary of neural correlates of trust in technology domain. All green locations have bee shown to vary by trust condition in at least one study (refer to **Table 3**).

Four of the studies also compared human or anthropomorphized agents to non-anthropomorphized agents (**Table 4**), all of which observed differences in trusting behaviors and neural correlates (Dong et al., 2015; Goodyear et al., 2016, 2017; Eun-Soo et al., 2019). In addition to distinct neural patterns between the reliable and unreliable conditions in the intermediate frontal cortex, Eun-Soo et al. (2019) reported that the strength of activation for unreliable conditions were more noticeable for human-faced assistive agents than for robot faced agents. Similarly, Goodyear observed higher activation for unreliable human-agents in the insular cortex, somatosensory association cortex/ visuo-motor coordination, agranular retrolimbic area, anterior prefrontal cortex, and superior temporal gyrus during the first trials of the task as compared to machine-agents. When participants observed feedback on their decision, increased activation for human-agents who provided good advice was observed in the left dorsomedial prefrontal cortex and the medial frontal gyrus (BA 9/10). Furthermore, their participants were more likely to follow the advice of a human-agent and perceived the machine-agent advice as more unreliable even though the objective reliability and subjective trust scores were similar between the two groups.

Dong et al. identified varying event-related potentials such as increased mean amplitudes associated with human-like cues and increased visual saliency affects that strongly correlated with the participants' perceived capability of the teammate. Similar cues were not identified in the conditions without human-like cues. These findings demonstrate the physical and behavioral anthropomorphism of a machine teammate impacts trust perceptions, where increased human-like features result in increased trust, measurable in neural correlates.

Connectivity Features

In addition to activation features, connectivity features can provide context into which regions are functionally working together or driving activations in other regions. Two main types of connectivity analysis are traditionally employed: functional connectivity and effective connectivity. Functional connectivity is the non-directional coupling of regions whereas effective connectivity is a directional coupling. Only three of the studies performed any type of connectivity analysis (**Table 5**). In addition to identifying significant locations, Goodyear et al. (2016, 2017) also used granger causality, a type of effective connectivity analysis. When comparing human and machine agents, they found that the left anterior precuneus and the posterior insula are drivers of the trust network with influences on all other significant regions of interest identified as correlates after FDR corrections (namely, right anterior precuneus, left posterior temporoparietal junction, posterior cingulate cortex, and left rostro lateral prefrontal cortex). They argue that the left anterior pre-cuneus and posterior insula jointly work together by integrating social and logical evaluations with internal interception responses. Granger causality was also used by Sanders et al. (2019), although no specific conclusions are reported due to the limited sample size of the study. They do suggest that the anterior and posterior cingulate cortex may be important nodes to consider in connectivity networks, and that the total flow and asymmetry of the network may be important features. The last study used seed-based connectivity, a functional connectivity analysis (Pushparaj et al., 2019). For all five levels of task difficulty, the anterior cingulate cortex was a strong network seed connected to the insular cortex, and in more difficult tasks, the putamen. The insular cortex was similarly a strong seed in the functional connectivity network connected to the anterior cingulate cortex, insular cortex network, putamen, and nucleus accumbens.

Considerations for Neural Correlates of Human-Automation Trust

There is consensus within the current studies that trust in automation can be monitored using neural signatures, frequently identified in the fronto-central region, including areas functionally named as the intermediate frontal cortex, primary motor cortex (MC), pre-MC, and supplementary motor area (SMA), potentially influenced by the anterior or posterior cingulate cortex. It is not surprising that the primary MC, pre-MC, and SMA were recognized in technology agent studies, as they are responsible for planning and executing motor actions based on internal and external cues. These areas also have overlap with the intermediate frontal cortex that is thought to be responsible for managing uncertainty (Ferng, 2020). These locations could potentially provide a continuous measurement of trust to be used for trust calibration, in addition to providing a mechanistic understanding of trust influencers.

While the prefrontal cortex (PFC) was not found significant in most of the papers, our review identified its relevance. It has been previously observed that trust and workload act a co-varying entities, where an increase in workload results in a

TABLE 4 | Summary of activation features for trust-agent type interaction.

Paper	Agent types investigated	Imaging methods	Trust-agent type interaction
Dong et al. (2015)	Human verses computer agent	EEG	Increased mean amplitudes ERPs is associated with human-like cues. And, increased visual saliency affects strongly correlated with perceived capability of the teammate.
Eun-Soo et al. (2019)	Human verses robot faced agents	EEG	Strength of activation for theta frequency band in unreliable conditions are more noticeable for human-faced agents.
Goodyear et al. (2016, 2017)	Human verses machine agent	fMRI	Higher activation early on for human agents in the insular cortex, somatosensory association cortex, agranular retrolimbic area, anterior PFC, superior temporal gyrus. When receiving feedback, Increased activation for human agents who provided good feedback in the left dorsomedial PFC and medial frontal gyrus.

TABLE 5 | Summary of connectivity features.

Paper	Connectivity type	Imaging methods	Connectivity features correlating with trust
Sanders et al. (2019)	Effective	EEG	No specific conclusions due to limited sample size, although they do posit the anterior and posterior cingulate cortex
Goodyear et al. (2016, 2017)	Effective	fMRI	The left anterior precuneus and posterior insular are drivers of the trust network with influences on all other significant activation regions
Pushparaj et al. (2019)	Functional	fMRI	Anterior cingulate cortex and insular cortex are strong seeds of the network, highly connected to other regions including the putamen and nucleus accumbens

decrease in trust (Chen et al., 2011; Hu et al., 2016). As the PFC, responsible for complex cognition and working/short-term memory, was identified by a subset of the reviewed articles, investigations into the neural correlates of trust should control for the co-varying influence of workload on trust perceptions in the accompanying neural signatures. Within interpersonal trust, there is a similar consensus that the cognitive system of trust is primarily comprised of the ventrolateral prefrontal cortex and amygdala and that trust is influenced by deeper brain regions linked to the motivational (risk-reward) and risk cognition systems and to social affect systems (Adolphs, 2003; Yanagisawa et al., 2011). Trust in automation has primarily been discussed similar to risk cognition, where trust is characterized by user uncertainty and vulnerability (Lee and See, 2004), and risk cognition itself has often been measured alongside trust or as subscales of trust (Hopko et al., 2021b). Thus, one can posit that trust relies on ability or willingness to perceive risk in addition to the willingness to be subject one's self to the consequence. There are three identified candidates responsible for the risk-reward evaluation of decision alternatives: the amygdala, ventral striatum, and orbitofrontal cortex (Drnec et al., 2016). The importance of these regions lies on the cognition of an action; the amygdala and lateral orbitofrontal cortex are thought to be responsible for interpreting the negative risk of an action, whereas the ventral striatum and medial orbitofrontal cortex are responsible for the perceiving the rewards (Basten et al., 2010).

GAPS IN NEURAL CORRELATES OF TRUST LITERATURE

Most of the summarized studies in **Table 3** are exploratory or pilot studies that compared neural correlates of human verses technology agents, as the neural correlates of trust have been predominantly studied in interpersonal and reciprocal trust (Parasuraman et al., 2014). These studies reported small sample sizes in highly controlled environments. Many of these studies that compared human with technology agents found statistically significant differences in brain activity between agent type, supporting the claim that people trust technology agents differently than they trust humans (Madhavan and Wiegmann, 2007), measurable in neural activity. There is limited work on neural correlates of trust in automation, where the vast majority of current studies are in cyber aid/detection system technologies. It is still unknown how well the neural correlates of trust will generalize between automation domains.

There is a lot left to unravel about what features in the brain signal trust levels and whether brain activity can capture changes in trust perceptions (i.e., trust building, breach, and/or repair). Systematic investigation into these time-dependent trust markers is warranted to understand, measure, and model effective human-automation trust calibration. Furthermore, mis-calibrated levels of trust do not always influence the user's behavior (Chiou and Lee, 2021). As such, the neural correlates associated with a user's identification of a trust influencer and the decision to act upon the trust perception are needed. The studies reviewed here highlight the potential of using neural signatures, both through activation and connectivity features, in better capturing operator trust in automation. However, only three studies performed any form of connectivity analysis. When data mining the neural correlates of trust, it is important to consider not only which regions are effectively responsible for driving the activations, but also how the different regions of interest are functionally communicating with each other. Connectivity analysis can be used to better understand trust adaptations in different conditions rather than just a snapshot

of activations. Furthermore, the predominant method to capture the neural correlates of trust in existing literature is EEG, which has a disadvantage when measuring region specific dynamics due to poor spatial resolutions. Other neural imaging methods, like fNIRS, have an advantage of offering higher spatial resolution, are less invasive, and are cost effective, and thus might provide additional information, perhaps implemented alongside or independently of EEG.

CONCLUDING REMARKS

This review aimed to provide context onto the relevance of trust-related findings in interpersonal and automation domains in addition to context about the generalizability of trust between various automation domains. Due to the novelty of the neural correlates of trust in technology, there is a dearth of works outside cyber aid technologies. There is still need to understanding trust in other automation domains beyond cyber aid technologies and beyond trust influencers like "reliability" such as investigating how the impacts of "new age" trust influencers [i.e., team autonomy and fluency; (Chiou and Lee, 2021)] affect trust perceptions and corresponding neural correlates. For the studies that consider agent type (namely, human or anthropomorphic agents against technology agents), there were observable differences in the neural correlates of trust. Future research is warred to investigate the similarities or differences between automation levels and interpersonal relation levels as pertaining to trust and its corresponding neural activity. The use of neural activity can provide unique insights other bioinstrumentations may be unable to capture due to its spatial resolution and location-based functionality. Beyond the use of traditional subjective measures, objective correlates of trust can provide high resolution into the temporal aspects of trust and provide a real-time measure for use in trust-sensitive technology. In doing so, automation can be sensitive to physiological indicators of trust and respond accordingly. In short, a neuro ergonomics approach may prove promising to better understand and model human-automation trust.

AUTHOR CONTRIBUTIONS

SH and RM contributed to the conception of the study. SH performed the review and drafted the first version of the manuscript. SH and RM wrote sections of the manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version.

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Plasma Lysophosphatidylcholine and Lysophosphatidylethanolamine Levels Were Associated With the Therapeutic Response to Olanzapine in Female Antipsychotics-naïve First-episode Patients With Schizophrenia

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Background: Accumulating studies have shown that the pathophysiology of schizophrenia may be associated with aberrant lysophospolipid metabolism in the early stage of brain development. Recent evidence demonstrates that antipsychotic medication can regulate the phospholipase activity. However, it remains unclear whether lysophospolipid is associated with the therapeutic response to antipsychotic medication in schizophrenia. This study aimed to investigate the influence of olanzapine monotherapy on lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) and the association between symptom improvement and changes of LPC and LPE levels during treatment in antipsychotic-naïve first-episode (ANFE) patients.

Materials and Methods: The psychotic symptoms were evaluated by the Positive and Negative Syndrome Scale (PANSS). 25 ANFE patients were treated with olanzapine for 1 mo. The levels of LPC and LPE were determined and psychotic symptoms were assessed at baseline and at 1-mo follow-up.

Results: Relative to baseline, the psychotic symptoms were significantly reduced after olanzapine treatment, except for negative symptoms. Moreover, the levels of most LPC and LPE increased after treatment. Interestingly, increased LPC(18:3) and LPC(20:2) levels were positively associated with the reduction rates of PANSS positive subscore. In addition, baseline levels of LPE(20:5), LPE(18:3) and LPE(22:5) were predictors for the reduction of positive symptoms.

Conclusion: Our study reveals that the levels of lysophospolipid are associated with the improvement of positive symptoms, indicating that LPC may be a potential therapeutic target for olanzapine in schizophrenia. Moreover, baseline LPE levels were predictive biomarkers for the therapeutic response to olanzapine in the early stage of treatment in ANFE patients.

Keywords: schizophrenia, lysophospolipid, therapeutic response, olanzapine, association

INTRODUCTION

Schizophrenia is a chronic and severe mental disorder affecting approximately 1% of the population (Barnett 2018). Currently, the first-line therapy for schizophrenia is atypical antipsychotic medication, such as olanzapine, risperidone and ziprasidone (Lieberman 1996). However, the therapeutic response to antipsychotics is heterogeneous between individuals. Current symptom-driven treatment leads to the poor outcome in patients with schizophrenia, especially in the early stage of treatment (Liu-Seifert et al., 2005).

Metabolites are the final products of the biochemical pathways in the human body, and their abnormalities can better reflect the disruption of functional status of the patients (Lindon et al., 2003). Yet, most of small-molecule metabolites are currently difficult to separate and detect (Rojo et al., 2012). Therefore, their roles in the pathophysiology of schizophrenia remain unclear. Recently, liquid chromatography tandem mass spectrometry (LC-MS) based metabolomics provided an opportunity to understand the pathological role of metabolites and develop new predictive biomarkers that can monitor the response to antipsychotics (Kaddurah-Daouk et al., 2008; Paredes et al., 2014; Pickard 2015). Our previous study by metabolomics method showed that olanzapine treatment for 1 month significantly increased the plasma levels of several types of lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) (Liu et al., 2021).

LPC and LPE are the prominent parts of lysophospolipid and play key roles in physiological and pathological processes of nervous system (Steinbrecher et al., 1984). Increasing evidence shows that LPC and LPE are involved in the function of cell membrane, apoptosis, oxidative stress and inflammatory responses. In the body, the peripheral circulating LPE and LPC are produced from hepatic secretion following the hydrolysis of cellular membrane phosphatidylcholine (PC) and phosphatidylethanolamine (PE) catalyzed by both acyltransferases and phospholipase (PLA) (Schmitz and Ruebsaamen 2010). In contrast, the majority of plasma LPC and LPE species may originate from liver secreted lecithincholesterol acyltransferases (LCAT) reaction (Sekas et al., 1985). In mechanism, LPC and LPE serve as a reservoir for arachidonic acid and as a central precursor for lipid-associated signaling molecular. In addition, LPC and LPE are bioactive lipids involved in monocyte recruitment and activation (Park et al., 2015). For example, studies found that LPC and LPE have protective role on ischemic neurons during the oxidative stress (Blondeau et al., 2002; Chen et al., 2019).

Schizophrenia shows complex and unique abnormal characteristics of glycerophospholipids in serum, plasma or brain tissues (Horrobin et al., 1994; Schmitt et al., 2004; He et al., 2012; Ghosh et al., 2017). Non-targeted LC-MS based metabolic profiling studies in schizophrenia spectrum disorders suggest altered glycerophospholipid species and levels in the brain of patients (Fukuzako et al., 1999; Matsumoto et al., 2011). Metabolomics study of prefrontal cortex from schizophrenia patients reported significantly lower levels of PCs in white and grey matter than healthy controls (Schwarz et al., 2008). Further, some studies revealed that after antipsychotics treatment, schizophrenia patients showed increased levels of 22:5, 20:5, and 20:3 within the PC and PE glycerophospholipid classes (Kaddurah-Daouk et al., 2012; Castillo et al., 2016). Particularly, a study comparing the lipid profiles of antipsychotics-free and drug-naïve (ANFE) patients before and after several types of antipsychotics medication for 7 mo showed that 11 glycerophospholipids (nine PCs and two LPCs) were significantly up-regulated after treatment, suggesting that glycerophospholipids may be used for predictive markers to monitor the treatment in the early stage of this disorder (Leppik et al., 2020).

Based on the aforementioned literatures regarding the phospholipids in schizophrenia, we hypothesized that the LPC and LPE levels were associated with the improvement of clinical symptoms after 1-month treatment. Therefore, this study aimed to investigate whether the plasma LPC and LPE levels identified by LC-MS based metabolomics were the predictive markers for the treatment response to olanzapine in the early treatment of ANFE patients with schizophrenia.

METHODS

Subjects

Female ANFE patients with schizophrenia diagnosed as DSM-IV criteria and confirmed by the Structured Clinical Interview for DSM-IV (SCID) were recruited. The inclusion criteria were described in our previous study. In brief, *1*) female Han Chinese; *2*) between 18 and 45 yr old; *3*) antipsychotic free or cumulative antipsychotic treatment <14 days; *4*) without substance abuse, including alcohol and smoking. We acquired a complete physical examination and medical history from all patients to exclude serious physical conditions. A detailed questionnaire including general information, sociodemographic characteristics, and medical and psychological conditions was administered to each subject by a member of the research staff. Additional information was collected from available medical records. The average age and onset age of the ANFE patients were 27.4 ± 7.6 and 26.4 ± 8.9. The mean year of education was 9.1 ± 3.5 and mean BMI was 21.0 ± 3.0 (shown in **Table 1**).

TABLE 1 | Reduction of psychotic symptoms assessed by PANSS after treatment with olanzapine for 4 wk.

	Baseline	Follow-up	Reduction rate	*p* value[a]
Age (years)	27.4 ± 7.6			
Education (years)	9.1 ± 3.5			
BMI (kg/m^2)	21.0 ± 3.6			
Onset age (years)	26.4 ± 8.9			
Clinical symptoms				
P subscore	25.0 ± 6.0	16.4 ± 5.2	0.33	<0.001
N subscore	17.1 ± 4.7	15.6 ± 4.3	0.04	0.23
G subscore	39.7 ± 7.5	32.0 ± 6.0	0.17	<0.001
Total score	81.8 ± 13.9	63.9 ± 13.8	0.21	<0.001

PANSS *the positive and negative syndrome scale.*

[a]*Comparison of psychotic symptoms between baseline and follow-up.*

The study was approved by the Institutional Review Board of Beijing Huilongguan Hospital, and all patients provided the written informed consent.

Study Procedures

It is an open-labeled, single center, longitudinal observational study. ANFE patients were treated with a flexible-dosed (ranged from 10 to 30 mg/day) olanzapine for 1 mo. All patients stayed in hospital and the nurses monitored the adherence to olanzapine treatment throughout the study.

Clinical Symptom Assessment

The Positive and Negative Syndrome Scale (PANSS) was used to evaluate the severity of clinical symptoms by three experienced psychiatrists. Each patient was assessed on PANSS scale at baseline and at 1-mo follow-up. The equation used to calculate the rate of the clinical symptom reduction was $(T_b\text{-}T_a)/T_a\times100\%$, where T_a is the score at baseline and T_b is the score at follow-up.

Plasma Processing and Metabolomics Data Processing

Venous blood samples from participants were collected at two time points after fasting: on admission and 1-mo follow-up. Plasma samples were separated after centrifugation for 15 min at 4°C and stored at −80°C until further processing. The sampling process was described in our previous study (Liu et al., 2021). In brief, 200 μl of plasma was added to a vial, extracted with methanol (600 μl), and centrifuged for 2 min. The supernatant was absorbed and dried in nitrogen at 37°C. Then, the treated supernatant was analyzed using a UPLC-MS metabolomics (Liang et al., 2021; Liu et al., 2021). MS analysis was carried out in positive ion mode. We obtained scans in the mass range of 70–1000 m/z, at three scans per second with a resolution of 70,000. For the MS/MS assay, a normalized collision energy of 35 V, an isolation window of 0.8 m/z, and a mass resolution of 35,000 were used.

Data acquisition from the raw MS output files were achieved using XCALIBUR software (Thermo Fisher Scientific). Progenesis QI (Waters) was conducted to extract the mass spectral features. Principal component analysis (PCA) was conducted using SIMCA-P 13 software (Umetrics). Partial least squares discriminate analysis (PLS-DA) model was performed for the calculation of variable importance in projection (VIP) values. Annotated compounds were identified by searching the accurate mass of the molecular ions and the fragment ions against compound databases.

Statistical Analysis

All the analyses were performed by using SPSS 20.0. The Kolmogorov-Smirnov (K-S) test was performed to test the normality of the relative intensity of the metabolites. Comparisons of plasma metabolites between baseline and follow-up were conducted with paired *t*-test. The analysis of variance (ANOVA) was performed for the group comparison of continuous variables. In addition, Pearson product moment correlation was conducted to analyze the correlations between the rate of reduction of PANSS and the increase in the relative intensity of LPC and LPE. Multiple linear regression analysis was performed to adjust for the confounding factors. In this model, the reduction percentage of PANSS score was the dependent variable and the demographic data and the increase of LPC and LPE were the independent variables. Bonferroni corrections were applied for multiple testing. The significance levels were set at $p < 0.05$.

RESULTS

Psychotic Symptoms at Baseline and After Treatment

Initially, we tested 27 ANFE patients using LC-MS metabolomics approach. Only 25 patients had complete LPC and LPE data, which were included in the following analysis. **Table 1** shows the psychotic symptoms of 25 ANFE patients at baseline and 1-mo follow-up. After 1 mo of olanzapine monotherapy, patients showed a significant improvement in the psychotic symptoms (all $p_{Bonferroni}<0.05$), except for negative symptoms. The mean reduction rate of psychotic symptoms ranged from 0.04 (negative subscore) to 0.33 (positive subscore).

LPC and LPE Levels at Baseline and 1-mo Follow-up

We identified 13 LPC and nine LPE in the differential compound library. The relative intensities of these metabolites were all normally distributed (K-S test: all $p > 0.05$). As shown in **Table 2** and **Figure 1**, the levels of most LPC and LPE were significantly increased after treatment with olanzapine ($p < 0.01$). However, LPE(22:1) and LPC(22:6) levels were significantly decreased (all $p < 0.05$), and LPE(22:6) and LPC(20:4) showed no significant difference after treatment (all $p > 0.05$).

Associations Between Changes in Metabolites and Improvement of Symptoms From Baseline

The increases of LPC(18:3) and LPC(20:2) were positively associated with the reduction rates of PANSS positive subscore (both were: $r = 0.43$, $p = 0.03$) (**Table 3** and **Figure 2**). Moreover, the increase of LPC(18:3) was significantly associated with the increase of LPC(20:2) ($r = 0.65$, $p < 0.001$). Further regression analyses confirmed the relationship between the reduction rate of PANSS positive subscore and the increase of LPC(18:3) (β= 0.68, t = 3.1, $p = 0.006$; $R^2 = 0.41$) or LPC(20:2) (β= 0.56, t = 2.5, $p = 0.02$; $R^2 = 0.33$) after controlling for age, education, baseline BMI, onset age and the increase of BMI.

Association Between Baseline Metabolites and the Improvement of Clinical Symptoms

The reduction rate of PANSS positive subscore was negatively correlated with baseline LPE(20:5) ($r = -0.47$, $p = 0.02$), LPE(18:

TABLE 2 | Changes of metabolites from baseline to 4-wk follow-up (Paired *t* test).

Metabolites	Regulation	Changes from baseline (95% CI)	*p*	Fold changes
LysoPE(20:5)	Up	28008 (10889, 45128)	0.002	0.92 (0.17, 1.35)
LysoPE(18:3)	Up	120565 (36447, 204683)	0.007	0.90 (0.01, 2.35)
LysoPE(16:1)	Up	34625 (15665, 63639)	0.001	1.001 (0.16, 3.09)
LysoPE(20:3)	Up	158801 (110956, 206645)	<0.001	1.36 (0.57,3.87)
LysoPE(22:5)	Up	332257 (199231, 465282)	<0.001	0.54 (0.17, 1.22)
LysoPE(18:2)	Up	1180553 (595466, 1765639)	<0.001	0.72 (0.22, 1.41)
LysoPE(22:1)	Down	−12508 (−18344, −6672)	<0.001	−0.5 (−0.86, −0.22)
LysoPE(22:6)	No change	−1022620 (−2116118, 70878)	0.07	−0.14 (−0.28, 0.10)
LysoPE(22:0)	Up	613281 (300488, 926074)	<0.001	0.55 (0.17, 1.56)
LysoPC(18:0)	Up	59496023 (28783987, 90208060)	0.001	0.17 (−0.08, 0.39)
LysoPC(22:6)	Down	-13073032 (-24702772, -1443292)	0.03	−0.15 (−0.45, 0.17)
LysoPC(20:3)	Up	37830985 (28049234, 47612735)	<0.001	0.98 (0.53, 1.54)
LysoPC(20:4)	No change	−437356 (−908067, 33353)	0.07	−0.14 (−0.30, 0.09)
LysoPC(15:0)	Up	2149849 (729001, 3570697)	0.005	0.30 (0.01, 0.46)
LysoPC(22:4)	Up	597175 (380502, 813847)	<0.001	1.26 (0.34, 1.88)
LysoPC(17:0)	Up	4130458 (1999412, 6261504)	0.001	0.43 (0.08, 10.9)
LysoPC(14:0)	Up	850540 (614758, 1086323)	<0.001	1.16 (0.65, 2.29)
LysoPC(16:1)	Up	1819062 (1271635, 2366489)	<0.001	0.81 (0.33, 1.23)
LysoPC(18:3)	Up	395471 (232238, 558705)	<0.001	0.63 (0.25, 1.40)
LysoPC(20:2)	Up	5180523 (3697696, 6663350)	<0.001	0.59 (0.39, 1.02)
LysoPC(22:2)	Up	32447 (19464, 45431)	<0.001	2.49 (1.45, 4.65)
LysoPC(18:1)	Up	6273223 (3923344, 8623102)	<0.001	0.25 (0.09, 0.45)

PC, phosphatidylcholine; PE, phosphatidylethanolamine; CI, confidence. Change refers to the levels of metabolites at follow-up minus the levels at baseline.

3) (r = −0.53, p = 0.007) and LPE(22:5) (r = −0.42, p = 0.04) (**Table 4** and **Figure 3**). Moreover, LPC(18:3) was negatively correlated with the reduction rate of general psychological subscore (r = −0.41, p = 0.04). Multiple regression analysis identified that baseline LPE(20:5) (β= −0.55, t = 2.7, p = 0.02; R^2 = 0.35), LPE(18:3) (β= −0.56, t = 2.9, p = 0.01; R^2 = 0.39) and LPE(22:5) (β= −0.58, t = 2.7, p = 0.01; R^2 = 0.36) were the contributing factors for the reduction rate of positive subscore.

DISCUSSION

This study found that schizophrenia patients showed increased concentrations of most of LPC and LPE after olanzapine monotherapy for 1 mo. Increased LPC(18:3) and LPC(20:2) levels were correlated with the improvement of positive symptoms. Baseline levels of LPE(20:5), LPE(18:3) and LPE(22:5) also were associated with the reduction rate of positive symptom and may serve as a predictive biomarker for therapeutic response.

Using LC-MS based untargeted metabolomics approach, abnormalities in levels of LPE and LPC have been consistently reported in schizophrenia, indicating that abnormal metabolism of LPE and LPC is a pathophysiological characteristic of this disorder (Law et al., 2019). Lysoglycerophospholipid plays a fundamental role in the neuronal mechanisms underlying the pathophysiological feature of several psychiatric disorders. Moreover, atypical antipsychotic medication has been shown to alter the levels of LPE and LPC in mental disorders (Ask et al., 2016; Cao et al., 2019; Leppik et al., 2020; Canfrán-Duque et al., 2021). In line with our findings, Cao et al. found that after 8-wk treatment with antipsychotics, serum amino acids and LPC levels were increased in first-episode (FEP) patients with schizophrenia (n = 122) (Cao et al., 2019). Leppik et al. profiled 14 LPCs and 76 PCs in serum from 53 FEP patients and found two LPCs and nine PCs levels were significantly increased after antipsychotic treatment for 7 mo (Leppik et al., 2020). Kaddurah-Daouk et al. found that PE levels were reduced in schizophrenia patients, and this change was reversed by olanzapine treatment (Kaddurah-Daouk et al., 2007). Particularly, Qiao et al. reported that decreased LPC (20:3) and LPC (14:0) levels were significantly increased after treatment with olanzapine for 1 mo in female patients with ANFE schizophrenia. However, only 13 metabolites were analyzed in this study (Qiao et al., 2016). The underlying mechanism of increase in the LPC and LPE levels after treatment with olanzapine in patients with schizophrenia is unclear. One possible explanation may be linked with the aberrant activity of phospholipase A_2 (PLA_2) after treatment. It is well known that PLA_2 is a class of enzyme that catalyzes cleavage of fatty acids from the sn-2 position of phospholipids and hydrolyze phospholipids into LPE and LPC (Hishikawa et al., 2014). Several antipsychotics have been reported to influence the activities or levels of PLA_2 in animal models and patients with schizophrenia (Gattaz et al., 1995; Smesny et al., 2005; Smesny et al., 2011; Kim et al., 2012; Shen et al., 2018). Our findings provide further evidence for the regulation of LPE and LPC concentrations by olanzapine treatment in patients with schizophrenia.

Our second finding is that the improvements of clinical symptoms after treatment were associated with the changes of LPC and LPE. The potential mechanism may be related to the physiologic functions of LPC and LPE, such as the formation of

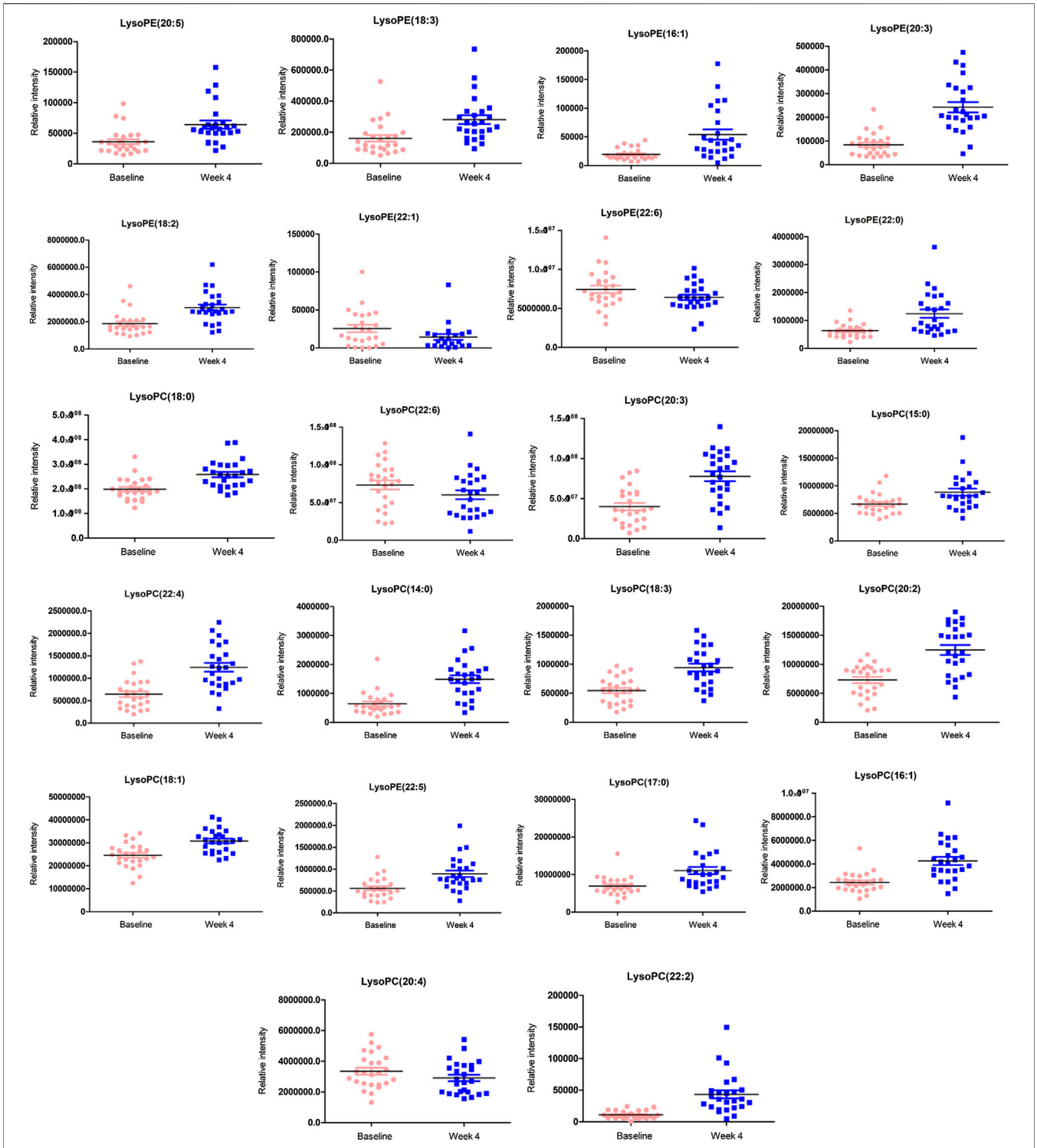

FIGURE 1 | This figure presents the boxplots of the metabolite levels at baseline and at follow-up after treatment with olanzapine for 1 mo. The sample means are indicated by the black bars. The levels of most lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) were significantly increased after treatment with olanzapine (all $p < 0.01$).

cell membranes, vesicle trafficking and bioactive signaling molecules in various cells including neuron (van Meer et al., 2008). As a component of lysophospholipid, LPC and LPE are water-soluble and amphiphilic molecules. They are essential precursors of signaling pathway molecules that activate second messenger to enhance the biological functions. Previous studies

TABLE 3 | The correlation between the increase of metabolite levels and the reduction of clinical symptoms.

Increase of metabolites	P subscore (r/p)	N subscore (r/p)	G subscore (r/p)	Total score (r/p)
LysoPE (20:5)	0.17 (0.41)	−0.14 (0.50)	0.17 (0.41)	0.12 (0.56)
LysoPE (18:3)	0.25 (0.23)	−0.11 (0.61)	0.12 (0.56)	0.12 (0.58)
LysoPE (16:1)	−0.18 (0.39)	0.06 (0.79)	0.09 (0.67)	−0.01 (0.97)
LysoPE (20:3)	0.07 (0.73)	−0.10 (0.63)	0.26 (0.21)	0.16 (0.43)
LysoPE (22:5)	0.03 (0.88)	−0.13 (0.55)	0.15 (0.46)	0.07 (0.73)
LysoPE (18:2)	0.32 (0.12)	−0.09 (0.66)	0.23 (0.26)	0.22 (0.29)
LysoPE (22:1)	0.14 (0.51)	−0.10 (0.62)	0.11 (0.59)	0.12 (0.56)
LysoPE (22:6)	−0.04 (0.85)	−0.37 (0.07)	−0.14 (0.51)	−0.18 (0.38)
LysoPE (22:0)	−0.05 (0.82)	−0.11 (0.59)	0.01 (095)	−0.03 (0.88)
LysoPC(18:0)	0.06 (0.79)	0.05 (0.83)	−0.03 (0.91)	0.04 (0.84)
LysoPC(22:6)	0.04 (0.87)	−0.11 (0.60)	0.06 (0.77)	0.04 (0.84)
LysoPC(20:3)	0.25 (0.22)	−0.02 (0.91)	0.003 (0.99)	0.08 (0.71)
LysoPC(20:4)	−0.14 (0.51)	−0.17 (0.43)	−0.15 (0.48)	−0.17 (0.41)
LysoPC(15:0)	−0.14 (0.50)	−0.23 (0.26)	−0.12 (0.56)	−0.20 (0.35)
LysoPC(22:4)	0.17 (0.41)	−0.10 (0.65)	0.02 (0.94)	0.05 (0.81)
LysoPC(17:0)	−0.23 (0.26)	−0.16 (0.45)	−0.02 (0.94)	−0.13 (0.52)
LysoPC(14:0)	−0.10 (0.63)	−0.09 (0.67)	0.09 (0.69)	−0.002 (0.99)
LysoPC(16:1)	−0.13 (0.53)	0.06 (0.78)	0.16 (0.46)	0.08 (0.72)
LysoPC(18:3)	0.43 (0.03)[a]	−0.04 (0.87)	0.18 (0.40)	0.26 (0.21)
LysoPC(20:2)	0.43 (0.03)[a]	−0.04 (0.86)	0.02 (0.94)	0.14 (0.51)
LysoPC(22:2)	−0.14 (0.50)	−0.13 (0.52)	−0.08 (0.70)	−0.14 (0.50)
LysoPC(18:1)	0.23 (0.26)	−0.33 (0.11)	−0.16 (0.46)	−0.10 (0.63)

[a]$p < 0.05$.

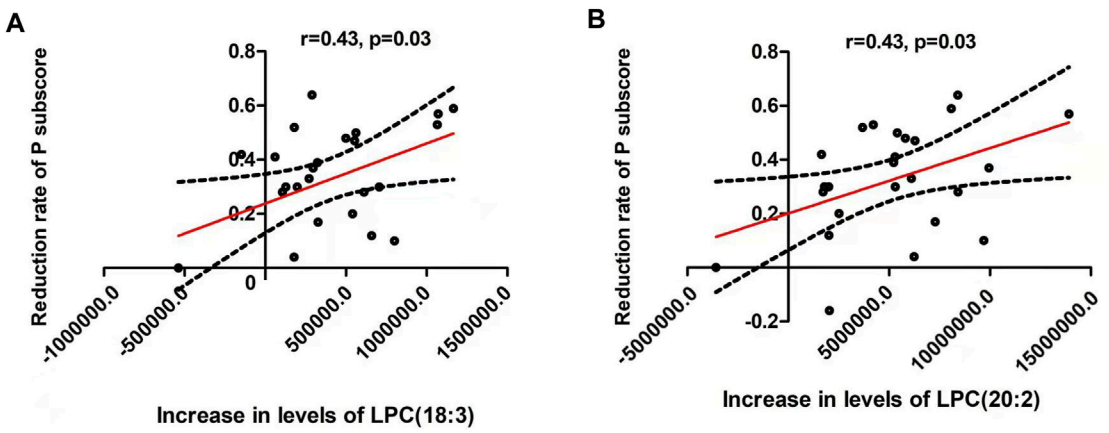

FIGURE 2 | There were significant positive associations between the reduction of positive symptoms and the increase of lysophosphatidylcholine (LPC) (18:3) **(A)** and LPC(20:2) **(B)** (both were: $r = 0.43$, $p = 0.03$). The red line represents the trend line, and the dashed black line is the 95% confidence interval.

have shown that lysophospholipid can stabilize and enhance G protein-coupled receptor G2A signaling pathway for calcium flux by preventing its reuptake or altering its surface expression and localization on the cell surface (Wang et al., 2005; Frasch et al., 2007). There is robust evidence that inflammation and redox imbalance play a crucial role in the pathophysiology of schizophrenia (Zhang et al., 2017; Zhang et al., 2018; Xiu et al., 2019; Xiu et al., 2020). LPC and LPE have also been found to be associated with the regulation of redox system and inflammatory response systems. For example, Cui et al. found that plasma PC (36:4) was correlated with concentration of tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) in septic rat models (Cui et al., 2020). Carneiro et al. found that LPC triggered Toll-like receptors 2 (TLR2)- and TLR4-mediated signaling pathways but counteracted LPS-induced NO synthesis by inhibiting nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) translocation and mitogen activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK) phosphorylation (Carneiro et al., 2013). In particular, a recent randomized, double-blind and placebo-controlled trial showed that a decomposition product of pig liver, which is a rich source of LPC and LPE, has been identified as a nutrient to improve cognitive function in the healthy individuals over the age of 40 (Matsuda et al., 2020). Moreover, administration with LPC and LPE on the lipopolysaccharides (LPS)- stimulated SIM-A9 microglia cells significantly reduced the expression of IL-6 and the production of reactive oxygen species (ROS) (Tsukahara et al.,

TABLE 4 | The correlation between the baseline metabolite levels and the reduction of clinical symptoms.

Baseline metabolite levels	P subscore (r/p)	N subscore (r/p)	G subscore (r/p)	Total score (r/p)
LysoPE (20:5)	**−0.47 (0.02)**[a]	0.11 (0.61)	−0.02 (0.93)	−0.12 (0.58)
LysoPE (18:3)	**−0.53 (0.007)**[b]	0.11 (0.61)	−0.05 (0.81)	−0.14 (0.50)
LysoPE (16:1)	−0.14 (0.49)	016 (0.43)	0.004 (0.99)	0.002 (0.99)
LysoPE (20:3)	−0.22 (0.30)	0.09 (0.66)	−0.09 (0.67)	−0.09 (0.66)
LysoPE (22:5)	**−0.42 (0.04)**[a]	0.12 (0.58)	−0.09 (0.68)	−0.16 (0.43)
LysoPE (18:2)	−0.57 (0.003)	0.08 (0.70)	−0.10 (0.65)	−0.21 (0.33)
LysoPE (22:1)	−0.04 (0.85)	0.09 (0.66)	−0.13 (0.53)	−0.09 (0.66)
LysoPE (22:6)	0.03 (0.90)	0.33 (0.11)	0.17 (0.42)	0.18 (0.38)
LysoPE (22:0)	0.17 (0.41)	0.19 (0.36)	0.14 (0.52)	0.20 (0.34)
LysoPC(18:0)	0.26 (0.20)	0.13 (0.54)	0.12 (0.56)	0.21 (0.32)
LysoPC(22:6)	0.01 (0.97)	0.07 (0.73)	−0.31 (0.13)	−0.20 (0.33)
LysoPC(20:3)	−0.13 (0.53)	−0.01 (0.98)	−0.33 (0.11)	−0.27 (0.19)
LysoPC(20:4)	0.07 (0.75)	0.03 (0.88)	−0.14 (0.49)	−0.09 (0.66)
LysoPC(15:0)	−0.02 (0.91)	0.28 (0.17)	−0.05 (0.81)	0.20 (0.93)
LysoPC(22:4)	−0.27 (0.19)	0.09 (0.68)	−0.38 (0.06)	−0.32 (0.12)
LysoPC(17:0)	0.20 (0.33)	0.24 (0.26)	0.09 (0.68)	0.19 (0.37)
LysoPC(14:0)	−0.05 (0.83)	0.09 (0.66)	−0.07 (0.75)	−0.02 (0.92)
LysoPC(16:1)	−0.06 (0.77)	0.17 (0.41)	−0.14 (0.51)	−0.07 (0.75)
LysoPC(18:3)	−0.38 (0.06)	0.02 (0.94)	**−0.41 (0.04)***	−0.38 (0.06)
LysoPC(20:2)	0.02 (0.94)	0.12 (0.56)	−0.31 (0.13)	−0.18 (0.39)
LysoPC(22:2)	−0.07 (0.74)	0.14 (0.51)	−0.16 (0.45)	−0.09 (0.68)
LysoPC(18:1)	−0.29 (0.16)	0.27 (0.19)	−0.27 (0.20)	−0.22 (0.29)

[a]p < *0.05*.
[b]p < *0.01*.

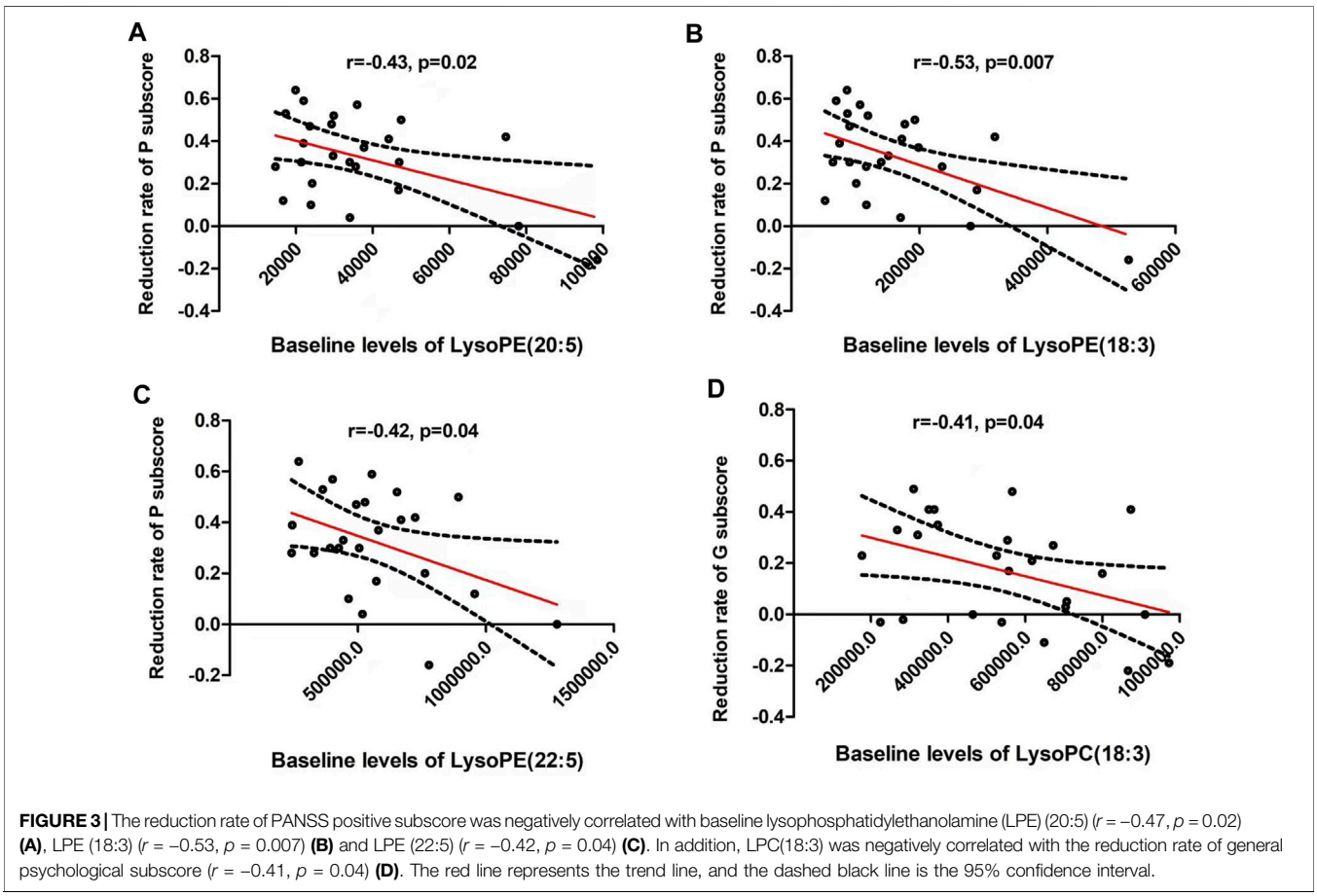

FIGURE 3 | The reduction rate of PANSS positive subscore was negatively correlated with baseline lysophosphatidylethanolamine (LPE) (20:5) ($r = -0.47$, $p = 0.02$) **(A)**, LPE (18:3) ($r = -0.53$, $p = 0.007$) **(B)** and LPE (22:5) ($r = -0.42$, $p = 0.04$) **(C)**. In addition, LPC(18:3) was negatively correlated with the reduction rate of general psychological subscore ($r = -0.41$, $p = 0.04$) **(D)**. The red line represents the trend line, and the dashed black line is the 95% confidence interval.

2021). However, it remains unclear whether the increased levels of LPC and LPE are the causes or results of the improvement of clinical symptoms. The exact mechanism needs further study.

Specifically, we found that the different classes of lysophospholipids were involved in the improvement of clinical symptoms in this study. Baseline LPE levels including (20:5), (18:3) and (22:5) and the increase in LPC levels including (18:3) and (20:2) were associated with the clinical symptom improvements. In line with our findings, previous studies also supported that lysophospholipid with different group has different functional role. In the studies of prehypertension, increased LPC(16:0) was found to cause oxidative stress, thereby increases inflammation and arterial stiffness (Kim et al., 2014). LPE (18:0) can increase Ca^{2+} concentration in nerve cells through the G2A receptor pathway, resulting in calcium overload (Lee et al., 2015). While, in myocardial infarction induced by a high isoprenaline dose in rat, the enhancement of oxidative stress was observed to be related to the decrease of LPCs [(18:0) and (20:3)] levels (Liu et al., 2013). Increased PC(38:4) levels were found in good responders treated with olanzapine and risperidone. All these above-mentioned studies support our findings that different classes of LPC and LPE were associated with the outcome of treatment in schizophrenia.

This study has a few limitations. First, the sample size is relatively small, which reduces the statistical power of the present study. Second, further study recruiting healthy controls is warranted to verify the clinical value of these predictive biomarkers for the therapeutic efficacy of olanzapine. Third, considering that some patients have been treated with antipsychotics for a short time (less than 14 days) in our study, we cannot completely rule out the possible impact of previous antipsychotics on the results. Fourth, the metabolites were analyzed in plasma rather than in CNS. Future study is warranted to compare the LPC and LPE levels between peripheral (plasma) and CNS (cerebrospinal fluid). Fifth, in a previous study, we published the same cohort of patients and same metabolomic methods (Liu et al., 2021). Although part of methods is similar, the two studies have focused on different clinical problems. Liu et al. focused on the severe side effect of weight gain after treatment with olanzapine. This study focused on the therapeutic response to olanzapine.

Overall, this study provides new evidence that the changes in LPC(18:3) and LPC(20:2) levels were associated with the improvement of positive symptoms, suggesting that LPC may be a potential therapeutic target for olanzapine. Moreover, the baseline levels of LPE(20:5), LPE(18:3) and LPE(22:5) might be highly useful as novel plasma biomarkers for the prediction of therapeutic response in the early stage of schizophrenia. However, this study is limited by the lack of the healthy controls and a small ample size. Replication is warranted in further longitudinal studies with a large sample size to introduce LPC and LPE biomarkers into clinical practice.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The study was approved by the Institutional Review Board of Beijing Huilongguan Hospital, and all patients provided the written informed consent. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MX, XL, and JL were responsible for study design, statistical analysis and manuscript preparation. HL, JW, and XL were responsible for recruiting the patients, performing the clinical rating and collecting the clinical data. XL and JW were evolving the ideas and editing the manuscript. HL and MX were involved in writing the protocol, and cowrote the paper. All authors have contributed to and have approved the final manuscript.

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Resurgent Sodium Current in Neurons of the Cerebral Cortex

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In the late '90, Dr. Indira Raman, at the time a postdoctoral fellow with Dr. Bruce Bean, at Harvard University, identified a new type of sodium current, flowing through the channels that reopens when the membrane is repolarized. This current, called "resurgent Sodium current," was originally identified in cerebellar Purkinje neurons and has now been confirmed in around 20 different neuronal types. Since moving to Northwestern University in 1999 to establish her own research group, Dr. Raman has dedicated great efforts in identifying the mechanisms supporting the resurgent Sodium current and how its biophysical properties shape the firing of the different cell types. Her work has impacted greatly the field of cellular neurophysiology, from basic research to translation neuroscience. In fact, alterations in the resurgent sodium currents have been observed in several neuropathologies, from Huntington's disease to epilepsy. In this Perspective we will focus on the current knowledge on the expression and function of the resurgent Sodium current in neurons of the cerebral cortex and hippocampus. We will also briefly highlight the role of Dr. Raman's as teacher and mentor, not only for her pupils, but for the whole scientific community.

Keywords: resurgent sodium current, cortical neurons, hippocampus, sodium channels, neurophysiology

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INTRODUCTION

Neuronal voltage-gated Sodium channels (VGSCs) are responsible for the large and transient inward current (I_{NaT}) underlying the upstroke of the action potential (AP) (Hodgkin and Huxley, 1952a). After opening, VGSCs quickly inactivate and require strong membrane hyperpolarization to become available (Hodgkin and Huxley, 1952b). Many neurons express subthreshold non-inactivating Sodium currents: a persistent sodium current (I_{NaP}) flowing through non-inactivated VGSCs, and resurgent Sodium current (I_{NaR}) that activates upon membrane repolarization (Stafstrom et al., 1982; Crill, 1996; Raman and Bean, 1997). Subthreshold sodium currents can powerfully shape spike after-potentials and repetitive firing (Raman and Bean, 1997; Khaliq et al., 2003; Yamada-Hanff and Bean, 2013). I_{NaR} has first been described in isolated cerebellar Purkinje neurons, where it contributes to the generation of complex spikes and repetitive spontaneous firing (Raman and Bean, 1997; Khaliq et al., 2003). I_{NaR} has been identified in many different cell-types throughout the brain, with conserved biophysical properties (Lewis and Raman, 2014). Thanks to the elegant work of Dr. Raman and collaborators, I_{NaR}, together with I_{NaT}, is the best characterized component of the Sodium current. Indeed, Dr. Raman's original description highlighted the role of I_{NaR} in bursting in Purkinje neurons and developed a model for its generation hypothesizing the existence of a blocking particle that enters the pore at open states and is released upon repolarization (Raman and Bean, 1997, 2001). Later, Dr. Raman led her laboratory on a quest to decipher the structural and molecular mechanisms of I_{NaR}, discovering the interaction of alpha and beta subunits

of VGSCs underlying open channel block and the identity of the blocking particle (Grieco et al., 2005; Aman and Raman, 2007, 2010; Aman et al., 2009; Bant and Raman, 2010; Lewis and Raman, 2011, 2013). Following its description in Purkinje cells, the discovery of I_{NaR} in other cell-types has propelled research on the molecular underpinnings and physiological role of I_{NaR} in the brainstem, basal ganglia and peripheral sensory neurons (Do and Bean, 2003; Cummins et al., 2005; Enomoto et al., 2006; Barbosa et al., 2015), as well as its involvement in disorders such as pain, long QT syndrome and epilepsy (Jarecki et al., 2010; Hargus et al., 2011). In this review we want to focus on I_{NaR} in neurons of the cerebral cortex, where the work of Dr. Raman inspired our own first steps in the field of neurophysiology. The authors started their journey in neuroscience by studying the expression of I_{NaR} in cortical neurons and were deeply inspired by the solidity and elegance of the experimental work of Dr. Raman. One of us (GQ) had the chance to attend Dr. Raman's "Great experiments in Cellular Neurophysiology" course at Northwestern University, experiencing her outstanding teaching.

EXPRESSION OF I_{NaR} IN CORTICAL AND HIPPOCAMPAL NEURONS

Soon after Raman and Bean (1997) first described it in cerebellar Purkinje cells, the $_{INaR}$ has been identified in several cell types of the cerebellum, brainstem, basal ganglia, and dorsal root ganglia (Lewis and Raman, 2014). Raman and Bean (1997) also described the absence of I_{NaR} in acutely isolated pyramidal neurons of the CA3 region. In the cerebral cortex, I_{NaR} was first reported in layer II of the rat perirhinal cortex (Castelli et al., 2007a). This first report was followed shortly by an examination of the expression of I_{NaR} across the hippocampus and parahippocampal region (Castelli et al., 2007b). In the perirhinal and entorhinal cortices 75–100% of excitatory neurons express I_{NaR} depending on layer localization. In these regions the resurgent conductance amounts to 1.5–3% of the conductance of the transient component. As opposed to other types of neurons, the channels responsible for the I_{NaR} in cortical neurons are enriched in the axon initial segment. Indeed, application of TTx to the axon initial segment of perirhinal neurons abolished I_{NaR} to a larger extent than when applied to the soma/proximal apical dendrite (Castelli et al., 2007a). Moreover, patch clamp recordings from acutely isolated cortical neurons rarely show I_{NaR} (Castelli et al., 2007a). In the parahippocampal region, I_{NaR} is expressed most prominently by layer II excitatory neurons of the medial entorhinal cortex (MEC) (Castelli et al., 2007b). In MEC layer II, I_{NaR} was found in all recorded neurons, with an amplitude representing 3.6% that of I_{NaT}, the second largest among neurons of the cerebral cortex (Castelli et al., 2007b; Nigro et al., 2012). MEC layer II neurons express all three components of the Sodium current: I_{NaT}, I_{NaP}, and I_{NaR}. The developmental expression of the I_{NaR} in MEC layer II follows a trajectory that is independent from that of the other components, increasing steadily in amplitude from postnatal day (P) 5 to P10 (Nigro et al., 2012). In parallel, the percent of neurons expressing I_{NaR} also increases in the same developmental window (Nigro et al., 2012).

In the hippocampus I_{NaR} is expressed in subpopulations of excitatory neurons in the dentate gyrus (60%), ventral CA1 (40%), and the majority of subicular neurons (Castelli et al., 2007b; Barker et al., 2017). Interestingly, I_{NaR} does not seem to be expressed by pyramidal neurons of the dorsal hippocampus or in CA3 pyramidal neurons recorded from brain slices (Castelli et al., 2007b). Future studies correlating I_{NaR} expression to transcriptomic cell types might shed light on the molecular identity of neurons expressing I_{NaR} and the molecular mechanisms underlying its expression in the cortex (see below).

CONTRIBUTION OF I_{NaR} TO FIRING PROPERTIES OF CORTICAL NEURONS

Several neuron types expressing I_{NaR} show spontaneous firing with relatively high firing rates and bursting, e.g., Purkinje cells, subthalamic neurons, neurons of the cerebellar nuclei (Lewis and Raman, 2014). I_{NaR} endows these cell-types with the ability to produce repetitive spiking spontaneously (i.e., in absence of synaptic activity) at high frequencies by preventing fast inactivation through open channel block (Raman and Bean, 2001; Khaliq et al., 2003). At depolarized membrane potentials, the open channel block competes with fast inactivation, and during repolarization, the transition from open channel block to open states allows Sodium ions to flow and initiate a new cycle of spiking (Raman and Bean, 2001). Excitatory neurons of the cerebral cortex do not produce spontaneous repetitive firing, nor they reach high firing frequency, but fire trains of action potentials with different degrees of adaptation. I_{NaR} has been shown to contribute to repetitive firing in layer II pyramidal neurons of the rat perirhinal cortex (Castelli et al., 2007a). These neurons produce repetitive firing up to 30 Hz upon depolarizing current injection, a firing frequency much lower than other I_{NaR} expressing neurons outside the cerebral cortex. However, even at those firing frequencies I_{NaR} contributes to most of the Sodium current during the interspike interval promoting depolarization and repetitive firing (Castelli et al., 2007a).

By injecting AP waveforms (recorded in current clamp) in voltage clamp experiments, Raman and Bean (1997) demonstrated that I_{NaR} provides a major contribution to the generation of complex spikes in Purkinje neurons. Inspired by these original experiments, Alessi et al. (2016) tested the contribution of different ionic conductances to the generation of the depolarizing afterpotential (DAP) in MEC layer II stellate cells. The authors described two mechanisms generating DAPs in these cells acting at different membrane voltages. At hyperpolarized holding potentials T-type Calcium channels provide most of the depolarization following the fast afterhyperpolarization (fAHP). However, at holding voltages closer to the resting potential, subthreshold Sodium currents (I_{NaP} and I_{NaR}) are the major contributors to the DAP (Alessi et al., 2016). During spatial navigation, MEC layer II stellate cells show a spatially modulated firing pattern characterized by regularly spaced firing fields arranged in a hexagonal matrix, as described for grid cells (Fyhn et al., 2004; Hafting et al., 2005; Domnisoru et al., 2013; Schmidt-Hieber and Hausser, 2013;

Rowland et al., 2018). MEC stellate cells with grid firing patterns also show a higher probability of generating bursts of APs during navigation (Latuske et al., 2015; Bant et al., 2020). Interestingly, bursting probability and amplitude of I_{NaR} show a gradient along the dorso-ventral axis of the MEC that correlates with the gradient in spacing and field size of grid cells along the same axis (Bant et al., 2020). The authors observed that using bursts increased the performance of decoding the animal's position during navigation as compared to isolated spikes. The higher information content of burst points to a cellular mechanism to maximize signal-to-noise ratio in dorsal MEC grid cells (Bant et al., 2020). We would like to highlight that these interesting results were obtained by Dr. Jason Bant, a previous student of Dr. Raman, teaming up with Dr. Lisa Giocomo, who pioneered the study of the topographic organization of biophysical properties in MEC.

MOLECULAR MECHANISM OF I_{NaR} IN CORTICAL NEURONS

Patch clamp experiments from Purkinje neurons obtained from Nav1.6 (*Scn8a*) null mice showed that this subunit contributes to most of the I_{NaR} in these neurons (Raman et al., 1997). However, the alpha subunit on its own cannot generate I_{NaR}. Indeed Nav1.6 is also expressed in CA3 neurons that do not express I_{NaR} (Raman and Bean, 1997). The mechanism generating I_{NaR} involves a blocking particle that interacts with the alpha subunit (Raman and Bean, 2001). In a series of elegant experiments, Grieco et al. (2005) demonstrated that the blocking particle consists of the beta subunit Navβ4 (Grieco et al., 2005). Knockdown expression of the Navβ4 in cerebellar granule cells and peripheral sensory neurons strongly reduced I_{NaR}, further highlighting the role of this beta subunit in generating I_{NaR} (Bant and Raman, 2010; Barbosa et al., 2015). In cortical neurons, Nav1.6 is expressed at high levels in the axon initial segment correlating with the subcellular expression of I_{NaR} in these neurons (Castelli et al., 2007a; Royeck et al., 2008; Buffington and Rasband, 2013). On the other hand, most of the cortical cells in which I_{NaR} has been observed express Navβ4 at very low levels (Buffington and Rasband, 2013) or completely lack *scn4b* expression (Yu et al., 2003; Lewis and Raman, 2014; **Table 1**). *In situ* hybridization (ISH) data from the Allen Brain Institute suggest a low level of expression in a subpopulation of layer II stellate cells in the dorsal MEC, which correlates with the dorso-ventral gradient of I_{NaR} in these neurons (Lein et al., 2007; Bant et al., 2020). In the hippocampus, *scn4b* is expressed in the dorsal CA1, where I_{NaR} is not expressed (Castelli et al., 2007b). The absence of I_{NaR} in CA1 pyramidal neurons might be explained by a slicing artifact by which the axon initial segment of CA1 neurons was lost. However, the recordings in CA1 were performed from coronal slices that preserve cellular integrity (Dougherty et al., 2012). Additionally, *scn4b* expression might not be sufficient for I_{NaR} expression in all cell types. A recent single cell transcriptomic analysis of the whole cortex and hippocampus by the Allen Brain Institute (Yao et al., 2021) showed that expression of *scn4b* is restricted to a handful of layer V neurons, none of whom are known to express I_{NaR}. The discrepancy between ISH and single cell transcriptomic in MEC might be due to the sparseness of *scn4b* expressing neurons in this region (**Figure 1**). Additionally, this discrepancy may arise because of sample processing differences between ISH and single cell transcriptomics. In ISH, the tissue remains intact and preserves cytosolic compartments, like distal neuronal processes, that are otherwise destroyed in tissue homogenization for single cell RNA processing. Alternatively, single cell RNA sequencing intrinsically yields low rates of capture (Zheng et al., 2017) and read efficiency which must be accounted for computationally (Galfrè et al., 2021). Tissue homogenization is similar between bulk and single cell RNA sequencing and differences in technique should yield similar results. Bulk transcriptomic analysis comparing the dorsal and ventral MEC from adult mice in Ramsden et al. (2015), however, demonstrates that *scn4b* is significantly upregulated in the dorsal MEC at P60 (**Figure 1B**). Moreover, it is expressed at a level which drives distance-based clustering separation between the dorsal and ventral MEC (Ramsden et al., 2015). This discrepancy in the single cell dataset may not be related to weakness in gene detection or homogenization methods. Instead, gene expression differences in the dorsal-ventral axis in the adult single cell dataset may not have been considered and thus cells in ventral layer II may mask *scnb4* expression in dorsal layer II. Interestingly, a subgroup of pyramidal tract (PT) projecting layer V neurons showed a significant expression of *scn4b*, suggesting the possible expression of I_{NaR} in this type of cells. The expression of *scn4b* in layer V cortical neurons shows a gradient along the rostrocaudal axis of the telencephalon. Strong *scn4b* expression in layer V is evident in motor areas, sensory cortices, anterior cingulate and retrosplenial cortex. *Scn4b* seems to be absent in association areas, including the insula, parahippocampal area and medial prefrontal areas (**Figures 1C–G**). Future electrophysiological experiments might corroborate the presence of I_{NaR} in these cortical neurons. The molecular nature of the blocking particle in cortical neurons also remains enigmatic, particularly in the perirhinal cortex, ventral CA1 and dentate gyrus. Current approaches to characterize the transcriptomic and electrophysiological profile of neurons will shed light on the molecular underpinnings of I_{NaR} in cortical neurons (Cadwell et al., 2016).

I_{NaR} IN EPILEPSY

The contribution of I_{NaR} to repetitive spiking, DAP and burst generation suggests that it might play a role in disorders where neuronal excitability is altered, such as epilepsy. This component of the Sodium current is expressed in cortical areas that are strongly affected in temporal lobe epilepsy (TLE), such as MEC, perirhinal cortex, hippocampus and subiculum. I_{NaR} expressing MEC layer II neurons are spared in temporal lobe epilepsy, but they show an increased excitability (Bear et al., 1996). The increased excitability is in part due to a reorganization of the synaptic network (Kuman et al., 2007), however intrinsic mechanisms are also at play. Indeed, in the absence of synaptic inputs, layer II MEC neurons show a higher firing rate in response to current injection in a rat model of TLE (Hargus et al., 2011). These changes in intrinsic excitability correlate

TABLE 1 | Cell-types where I_{NaR} expression and/or *scn4b* has been investigated.

Cell type	I_{NaR} expression	*scn4b* expression by ISH	References
CA3 pyramidal neuron	No	No	Raman and Bean, 1997; Yu et al., 2003 Allen Institute
Dorsal CA1 pyramidal neuron	No	Low	Yu et al., 2003 Allen Institute Castelli et al., 2007b
Ventral CA1 pyramidal neuron	35% of tested neurons	No	Yu et al., 2003 Allen Institute Castelli et al., 2007b
Dentate granule cells	60% of tested neurons	No	Yu et al., 2003 Allen Institute Castelli et al., 2007b
Subiculular pyramidal neurons	Most tested neurons	Yes	Allen Institute Barker et al., 2017
MEC LII stellate cells	Yes	Low in dorsal MEC (not detectable with single cell transcriptomics)	Allen Institute Castelli et al., 2007b
MEC LII pyramidal cells	Yes	Possibly in dorsal MEC	Allen Institute Castelli et al., 2007b
MEC LIII pyramidal cells	80% of tested neurons	No	Allen Institute Castelli et al., 2007b
MEC LV pyramidal neurons	70% of tested neurons	No	Allen Institute Castelli et al., 2007b
Perirhinal LII pyramidal neurons	90% of tested neurons	No	Allen Institute Castelli et al., 2007b
Perirhinal LV pyramidal neurons	80% of tested neurons	No	Allen Institute Castelli et al., 2007b
Neocortical LV pyramidal neurons	Unknown	Yes (detectable also with single cell transcriptomics)	Yu et al., 2003 Allen Institute

with an increased amplitude of all components of the Sodium current (Hargus et al., 2011). The molecular underpinnings of the increased excitability reside in an increased expression of the Nav1.6 subunit. Indeed, pharmacological blockade of Nav1.6 channels with 4,9-anidro-tetrodotoxin rescued the excitability of TLE MEC layer II neurons to levels like control neurons (Hargus et al., 2013). Moreover, similar alterations in intrinsic excitability and Sodium currents are recapitulated in mice carrying a mutated Nav1.6 isoform (Ottolini et al., 2017; Pan and Cummins, 2020). An increased excitability and augmented I_{NaR} and I_{NaP} have recently been described in human excitatory cortical neurons differentiated from pluripotent stem-cells obtained from patients affected by early infantile epileptic encephalopathy type 13 (*Scn8a*-related epilepsy) (Tidball et al., 2020).

DISCUSSION

In the current perspective we aimed at reviewing the state-of-the-art of the research on the I_{NaR} in neurons of the cerebral cortex. The expression of I_{NaR} has been reported in nine cortical cell-types, and we propose its expression in a population of layer V PT neurons based on their expression of Navβ4. The contribution of I_{NaR} to the firing behavior of cortical neurons has been well demonstrated in the perirhinal and entorhinal cortices. In these areas, I_{NaR} supports repetitive firing, DAPs and bursting. Bursting in LII MEC neurons has been proposed to maximize signal-to-noise ratio in grid cells and might represent a cellular mechanism for a reliable transmission of spatial information to the hippocampus (Bant et al., 2020).

The molecular mechanisms underlying I_{NaR} in cortical neurons are yet to be described. The molecular identity of the blocking particle in many cortical cell types remains unknown and future studies describing the correlation of trascriptomics and electrophysiology will allow to uncover potential candidates. These studies will also provide molecular targets for pharmacological treatments of epileptic encephalopaties involving I_{NaR}.

With this perspective, we wished to emphasize the pivotal influence of Dr. Raman's work on the mechanism of open-channel block, molecular identity of the blocking particle, and physiological role of I_{NaR} in cerebellar Purkinje neurons. Her findings sparked the quest for I_{NaR} in the cerebral cortex and provided the foundations for our current understanding of the role of I_{NaR} in the firing properties of cortical neurons.

In addition to her scientific contribution, Dr. Raman has also always been interested in forging new generations of scientists. Anyone who had the privilege of attending a class or a lecture given by Dr. Raman knows it will not be boring. Her scientific knowledge will engage you and her ability to introduce interesting anecdotes on the "behind the scenes" will enchant you. Her outstanding teaching abilities have been recognized by multiple awards received at Northwestern University, but her drive to good mentoring and good science has not stopped in Chicago. Dr. Raman has contributed to a series of feature articles titled "Living science" published in eLIFE from 2015 to 2019 (Raman, 2015a,b, 2016, 2017, 2019). In addition, her piece on good mentoring (Raman, 2014) faithfully describes the challenges faced by both the trainee and the advisor.

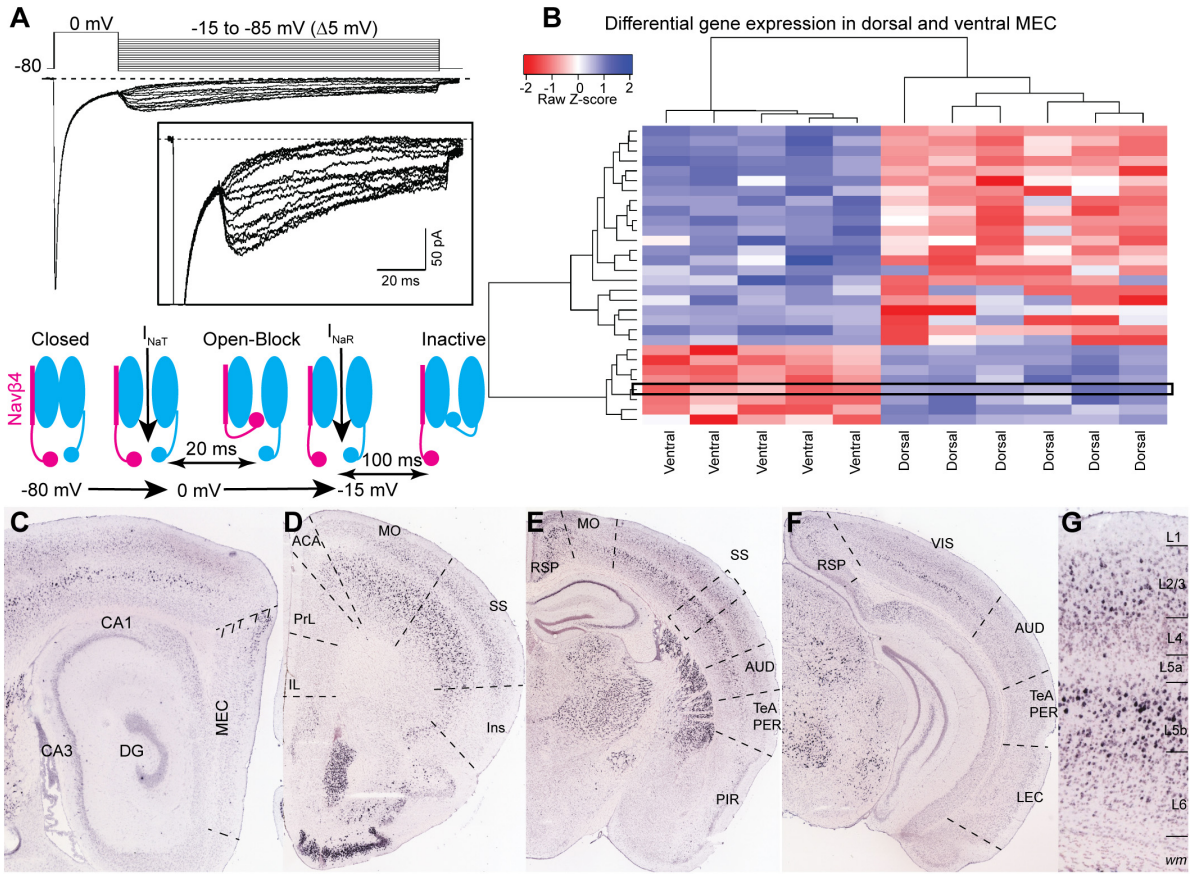

FIGURE 1 | (A) Representative voltage-clamp recording of sodium currents in a layer II neuron in MEC. Sodium currents were evoked by the voltage steps shown in the upper panel. A 20 ms step to 0 mV evoked INaT and was followed by a family of repolarizing steps from −15 to −85 mV to elicit INaR. Insert shows an expanded view of the INaR. The schematic in the lower panel shows the sequence of events generating INaR in sodium channels associated with a Navβ4 subunit. **(B)** Differentially expressed gene analysis of data from Ramsden et al. (2015). Gene expression driven separation between ventral and dorsal medial entorhinal cortex from P60 mice. *Scn4b* is highlighted by a black rectangle and appears as one of 8 genes significantly enriched in the dorsal adult MEC. Heatmap displays 34 genes significantly up or downregulated in either ventral or dorsal MEC samples (p-val. < 0.05, $\log_2$FC threshold $\pm$ 1.5, Benjamini-Hochberg corrected; hierarchical clustering performed by pairwise-Spearman correlation matrix). **(C–G)** ISH experiments from the Allen Institute showing the expression of *scn4b* across the cerebral cortex. **(C)** *Scn4b* is expressed in the dorsal MEC. **(D–F)** Expression of *scn4b* in the isocortex decreases from rostral **(C)** to caudal **(F)** regions. **(G)** Magnification of the somatosensory (SS) area highlighted by a dotted box in **(C)**. CA, Cornu Ammonis; DG, dentate gyrus; MEC, medial entorhinal cortex; IL, infralimbic; PrL, prelimbic; ACA, anterior cingulate area; MO, motor area; SS, somatosensory area; Ins, insula; AUD, auditory area; TeA, temporal association area; PER, perirhinal cortex; PIR, piriform cortex; RSP, retrosplenial area; VIS, visual area; LEC, lateral entorhinal cortex. Image credit: For **(A)**: adapted from Nigro et al. (2012) with permission from Elsevier. For **(C–G)** Allen Institute. 2004 Allen Institute for Brain Science. Allen Mouse Brain Atlas. Available from: https://mouse.brain-map.org/.

Dr. Raman contribution to science and her effort to make the scientific world a better place for young trainees and women has left a mark on the authors as well as, we are sure, on all the people that had trained with her and have known her.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://mouse.brain-map.org/search/show?page_num=0&page_size=44&no_paging=false&exact_match=true&search_term=Scn4b&search_type=gene. The bulk sequencing dataset can be found here: https://www.ebi.ac.uk/ena/browser/view/PRJNA267227?show=reads.

AUTHOR CONTRIBUTIONS

GQ and MN conceived the study and analyzed the data. KD analyzed the data. All authors wrote the manuscript.

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Better Screening Value of Sylvian Fissure Ratio on Cognitive Decline Among Female Compared to Male: An Observational Study in Elderly Patients With Cerebral Small Vessel Disease in Soochow

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Background: Cognitive decline (CD) occurs frequently in elderly patients with cerebral small vessel disease (CSVD). In China, elderly patients are more likely to enter healthcare in community hospitals where no magnetic resonance imaging (MRI) is available. This study aimed to explore the screening value of Sylvian fissure ratio (SFR) on CD and compare its gender difference from community-transferred patients.

Methods: We performed a single-center, observational study (collected between April 1, 2016, and March 1, 2019) to evaluate the association between Montreal Cognitive Assessment (MoCA) and SFR in 203 eligible community-transferred patients. Baseline characteristics of patients were collected during hospitalization. Multiple linear regression analyses were used to estimate the effect of variables on MoCA, and interactions between select variables were analyzed in different models. Receiver operating characteristic (ROC) curve analysis was used to evaluate the discriminative effect of SFR to severe CD.

Results: We identified that a meaningful SFR cutoff of 0.05 had important screening value (likelihood ratio test, p = 0.067) on CD. The ratio had a lower screen value in males when compared to females (adjusted β, −5.54; 95% CI, −8.78 to −2.30 vs. adjusted β, −1.01; 95% CI, −2.84 to 0.82). The gender difference was further verified by ROC curve analysis, in which this discriminative effect was more potent in females (from 0.878 to 0.948) compared to males (from 0.838 to 0.837).

Conclusion: An SFR of 0.05 may be more useful to distinguish CD in female patients with CSVD than male patients in whom the syndrome is suspected clinically.

Keywords: cognitive decline, sylvian fissure ratio, cerebral small vessel disease (CSVD), MoCA, females

INTRODUCTION

Cerebral small vessel disease (CSVD) is a kind of cerebrovascular diseases which frequently co-occurs with the development of cognitive decline (CD). The burden of co-existing CSVD and CD is found in the elderly (Liu et al., 2019). However, the specific pathophysiological mechanisms of CD that accompany CSVD remains unknown (Liu et al., 2018). Additionally, the clinical manifestation of CD is nonspecific, there is a lack of objective diagnostic criteria, and there is notable heterogeneity of CD in patients with CSVD. Therefore, distinguishing vascular cognitive disorders (VCD) is a persisting challenge. To our knowledge, CSVD is an important subtype of vascular cognitive impairment (VCI) and is the most common cause of vascular dementia (Dichgans and Leys, 2017). Neuroimaging techniques provide tools for early diagnosis and may play an important role for cognitive endpoints in clinic (Zanon Zotin et al., 2021). The presentations of abnormalities on neuroimaging increased diagnostic accuracy (Tullberg et al., 2004; Sachdev P. et al., 2014). Therefore, not only latest international guidelines but recent Chinese guidelines which are relevant to VCD refer to its importance in identifying CD (Sachdev P. et al., 2014; Skrobot et al., 2018; Peng, 2019; Zhang et al., 2020). Consequently, identifying VCD may be highly dependent on neuroimaging techniques, which are not universally available in all Chinese healthcare settings (Chen et al., 2019).

In China, the use of magnetic resonance imaging (MRI) in regional central hospitals has become increasingly common in recent years, which has increased CSVD detection rates at the population-level (Wu et al., 2019). However, uneven distribution of neuroimaging resources exists in China over the non-central areas (i.e., non-polyclinic hospitals where shortage in professional neurologists and imaging equipment). At present, in China, a significant number of community/non-polyclinic doctors do not receive training on cognitive assessment tools (Zhuang et al., 2021). Moreover, in addition to the insufficient trained doctors, the poor compliance and inner resistance for diagnosis of dementia among the patients or their families may also underlie the low detection rate of mild cognitive impairment (MCI). However, a higher prevalence of MCI in the rural than in the urban population has been reported in China (Wang et al., 2011; Jia et al., 2014). Therefore, it is common that with low scores on cognitive screening tools such as MoCA, the patients still remain undetected by the healthcare system in China. In those non-central areas, non-contrast computerized tomography (CT) is the most common imaging technique available in their hospitals. Of patients with ischemic stroke, approximately 90% of patients receive brain CT, whereas only about 50% of patients receive MRI in China (Huang et al., 2010). Using linear measurements based either on CT or MRI could provide useful prognostic information eliminating the need for specialized software (Wardlaw et al., 2013). Among these approaches, the use of a parameter Sylvian fissure ratio (SFR) which represents cerebral atrophy is recommended. SFR is defined as the average maximum width of Sylvian fissures divided by the transpineal inner table diameter (Gomori et al., 1984; van Zagten et al., 1999). There is potential for SFR to predict outcomes in the 90-day functional profile of patients with moderate-volume basal ganglia hemorrhage (Sato et al., 2016; Kwon et al., 2018).

Until now, the screening value of SFR on CD in CSVD patients has not been fully examined. This study aimed to investigate its association with CD in patients with CSVD and compare the gender difference in the screening value of SFR.

MATERIALS AND METHODS

Study Populations

Between April 1, 2016, and March 1, 2019, an inpatient-based observational cohort study was conducted in the Stroke Center, at the First Affiliated Hospital of Soochow University. A total of 203 eligible community-transferred patients (mean age 82.24 $\pm$ 4.91 years) were enrolled in this study. The inclusion criteria were (1) age equal to or greater than 65 years; (2) suspected presence of CSVD with cognitive impairment assessed by community doctors; (3) receipt of comprehensive clinical evaluation that included a detailed medical history, clinical diagnoses, laboratory tests, and screen testing with a standardized test battery; and (4) receipt of an MRI scan of the brain. The following patients were excluded: (1) cognitive impairment diagnosed with clear etiology, such as poisoning, infection, degeneration disease (Parkinson's disease, multiple system atrophy, corticobasal degeneration, and dementia with Lewy bodies, etc.), immune demyelination (multiple sclerosis, Balo's concentric sclerosis, etc.); (2) patients with intracranial tumors, large cerebral infraction, hydrocephalus; and (3) patients with visual or hearing impairment or language barrier which may affect their participation in the test or psychiatric symptoms with definite etiology. The ethics committees of The First Affiliated Hospital of Soochow University approved this study, which has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Cognitive Assessment and Image Analysis

Montreal Cognitive Assessment (MoCA) was used to evaluate the cognitive functioning of the study population (Davis et al., 2015). The raw MoCA score was adjusted for educational attainment (1 extra point for 10–12 years of formal education; 2 points added for 4–9 years of formal education). Severe CD was determined by MoCA scores of $\leq$20 and were calculated using spline regression (see **Table 1**). All scales were evaluated by well-trained physicians independently blinded to the patient's clinical details. Imaging of the brain was performed with a 3.0 T MRI scanner which included axial T1-weighted, axial T2-weighted, susceptibility-weighted, and fluid attenuated inversion recovery (FLAIR). White matter lesions were evaluated in four grades from the T2-weighted and FLAIR MR images by the supervision of two neuroradiologists. The differences between two neuroradiologists were solved by a senior consensus, according to the modified Fazekas visual scale (Fazekas et al., 1987). Briefly, Grade 0 was defined as No lesions; Grade 1: Mild lesions defined as pencil-like or cap-like thin lesions; Grade 2: Moderate lesions defined as smooth haloes; and Grade 3: Severe lesions defined as large

TABLE 1 | Threshold effect of SFR on MoCA using two-piecewise linear regression.

	β (95% CI) *p* value	LR test *p* value	Predicted MoCA at threshold (95% CI)
Total		0.067	20.04 (18.75, 21.32)
SFR ≤ 0.05	−109.37 (−185.97, −32.77) 0.0056		
SFR > 0.05	−28.86 (−48.88, −8.83) 0.0052		
Male		0.272	20.23 (18.70, 21.76)
SFR ≤ 0.05	−92.18 (−186.39, 2.03) 0.0571		
SFR > 0.05	−33.55 (−56.86, −10.23) 0.0055		
Female		0.047	19.25 (16.85, 21.64)
SFR ≤ 0.05	−162.18 (−290.93, −33.43) 0.0169		
SFR > 0.05	−10.04 (−49.50, 29.41) 0.6200		

fused lesions (Caprio et al., 2013). SFR was defined as the average of the maximal Sylvian widths taken from the cut showing the widest Sylvian fissure divided by the transpineal coronal inner table diameter (van Zagten et al., 1999). We adopted the criteria for the diagnosis of CSVD as STRIVE Recommendation mentioned (Wardlaw et al., 2013). The diagnosis of CSVD contains at least one of the following neuroimaging markers: (1) Lacunar infarction includes recent small subcortical infarct (RSSI) and lacune of presumed vascular origin. RSSI refers to recent infarction in the territory supplied by a perforating arteriole, which is less than 20 mm in the axial plane in its maximum diameter. The occurrence of lesion is consistent with imaging features or clinical syndrome in the previous few weeks. Lacune of presumed vascular origin was defined as a CSF-filled cavity (3–15 mm in diameter, with CSF similar signal on all sequences) surrounded by a high signal rim on T2-FLAIR. (2) White matter hyperintensity of presumed vascular origin is defined as punctate, patchy, or confluent hyperintense signal on T2-weighted and FLAIR sequences, and isointensity or hypointensity signal on T1-weighted sequence. The hyperintense signal in subcortical gray matter and brainstem is not included. (3) Cerebral microbleed is defined as small hypointensity signal with diameter ranging from 2 to 10 mm on susceptibility weighted imaging but no corresponding signal on FLAIR, T1-weighted, or T2-weighted sequences. (4) Perivascular space is defined as round (axial) or linear (parallel to vessels) fluid-filled space with diameter of less than 3 mm. The signal is similar to CSF that is hypointense on T1-weighted and hyperintense on T2-weighted sequence without hyperintense rim on T2-weighted or FLAIR sequence. We omit the definition of brain atrophy in our manuscript due to lack of specificity of atrophy and selection bias to our study. The assessment of SFR was defined as follows: based on the previous researches (Gomori et al., 1984; van Zagten et al., 1999), SFR was defined as the average of the maximal Sylvian widths taken from the cut showing the widest Sylvian fissure divided by the transpineal coronal inner table diameter.

Data Collection

From the clinical dataset between April 1, 2016, and March 1, 2019, we obtained the following data elements for each patient: age; sex; Fazekas scale; and past and current diagnoses, such as hypertension, diabetes, coronary heart disease, stroke; patient-reported laboratory tests, such as creatinine, uric acid, α-hydroxybutyrate dehydrogenase (α-HBDH), high-sensitivity C-reactive protein, total cholesterol, total triglyceride, high-density lipoprotein cholesterol (HDLC), low-density lipoprotein cholesterol, apolipoprotein A (APOA), and apolipoprotein B.

Statistical Analysis

Continuous variables were expressed as mean ± standard deviation (SD) when data accorded with normal distribution. Otherwise, data were expressed in terms of quartile. Data were compared using an unpaired, two-tailed *t*-test or Mann–Whitney *U* test. Categorical variables were compared using χ^2 test or Fisher's exact test. Furthermore, to help account for the nonrandomized allocation of SFR, we performed propensity-score methods to reduce the effects of potential confounding. A two-piecewise linear regression model was used to examine the threshold effect of SFR on MoCA through spline smoothing. We also applied a likelihood model to compare the one-linear regression model with a two-piecewise regression model. The βs and 95% CIs of MoCA in response to dichotomous SFR categorical variables across sex stratification were estimated, and their interactions were analyzed. Multiple linear regression analyses were also used to estimate the effect of variables on MoCA, with the lower dichotomous as the reference. The βs and 95% CIs were estimated by three kinds of adjusted models. The final model retained selected factors to develop an integrative predictive model to discriminate patients with severe CD (MoCA ≤ 20). The 95% CI with the area under the curve (AUC) was estimated using the bootstrapping method (500 iterations). The image of scatter plots with the best linear fit, together with the shadows up and down showing the 95% CI, was processed using MATLAB R2017b (version 9.3.0.713579; The Mathworks, Inc., Natick, MA, United States). All analyses were performed using the statistical package R version 3.6.3[1].

RESULTS

Association Between Montreal Cognitive Assessment Score and Sylvian Fissure Ratio

A total of 203 patients who received a screening test (MoCA) were enrolled in the study. The detailed information on the demographic characteristics of the sample before analysis was shown in **Supplementary Table 1**. The illustrative brain MRI images with a continuous range of SFR measurements are provided in **Figure 1**. Scatter plots of raw values are shown in **Figure 2A**, and the regression was presented with the best linear fit plotted in a continuous line, with shadows up and down

[1]http://www.r-project.org/

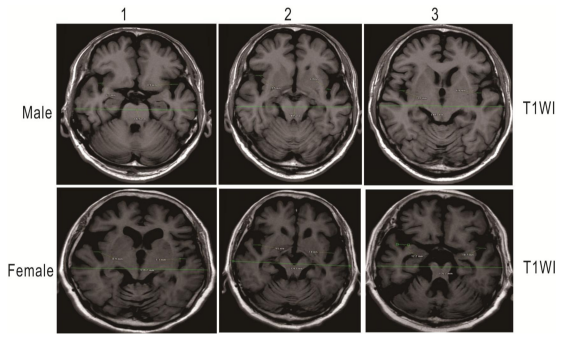

FIGURE 1 | As a measure of external cerebral atrophy, SFR, the average of the maximum width of the two Sylvian fissures on the section showing them at their widest, divided by the transpineal coronal inner table diameter. Illustrative measurement of brain MRI images showing green lines in female and male patients, respectively. Measurement values are automatically generated by imaging software. Upper: A 73-year-old man with SFR 0.051: MoCA score: 20 on T1WI. Lower: A 76-year-old woman with SFR 0.068: MoCA score: 14 on T1WI. T1WI, T1 weighted image.

showing the 95% CI. When analyzed as continuous variables, the linear regression model showed that SFR was inversely associated with the MoCA score (**Figure 2A**). Smoothing spline plots suggested that an SFR score of 0.05 may represent a threshold to reliably distinguish the two-piecewise effect of SFR on MoCA (likelihood ratio test, p = 0.067) which corresponded to the predictive MoCA score of 20.04 (95% CI 18.75–21.32; **Table 1**). The two-piecewise effect of SFR was statistically significant in females, compared to males (**Figure 2B** and **Table 1**, likelihood ratio test, p = 0.047 vs. 0.272). The figures (half-violin plots with raw values) of MoCA in different cohorts that were divided into dichotomous SFR are shown in **Figure 3**.

Characteristics of the Study Population

The cohort was divided into dichotomous SFR categorical variables according to the threshold, that is, those with SFR of greater than 0.05 and less than or equal to 0.05 comprised the study groups, respectively. Of these 203 unmatched patients, 132 (65%) had an SFR greater than 0.05, while 71 had no more than 0.05. The unmatched cohort was propensity score-matched (1:1) for age and gender. The distribution of the cohort's baseline characteristics according to SFR categorical variables is shown in **Table 2** and **Supplementary Table 2**. Patients with SFR greater than 0.05 had older age, lower MoCA, lower APOA, and higher Fazekas scale than patients whose SFR was $\leq$0.05 ($p < 0.05$). In propensity score-matched analytic patients, statistical differences were found among age, MoCA, HDLC, and Fazekas scale ($p < 0.05$).

Associations of Montreal Cognitive Assessment and Sylvian Fissure Ratio Categorical Variables by Subgroup Analysis

The regression coefficients (95% CI) for the association between MoCA and SFR categorical variables in the stratified cohort were explored. As shown in **Table 3**, interaction effects were also analyzed among the stratum of sex. There was a statistically significant modification effect of sex on the associations of MoCA with SFR categorical variables (p = 0.0114 and p = 0.0129, respectively) in the two adjusted models.

Linear Regression Analyses and Development of a Model Assessing Cognition

Three regression models were analyzed for crude adjustment for risk factors of CSVD including Fazekas; age; hypertension; diabetes; coronary heart disease; and stroke; the corresponding βs (95% CI) are depicted in **Tables 4**, **5**. Having higher Fazekas scale scores, experiencing stroke in both male (**Table 4**) and female patients (**Table 5**), and having SFR > 0.05 were statistically and clinically significant. The factors of Fazekas scale, occurrence of stroke, SFR threshold (0.05), and age were

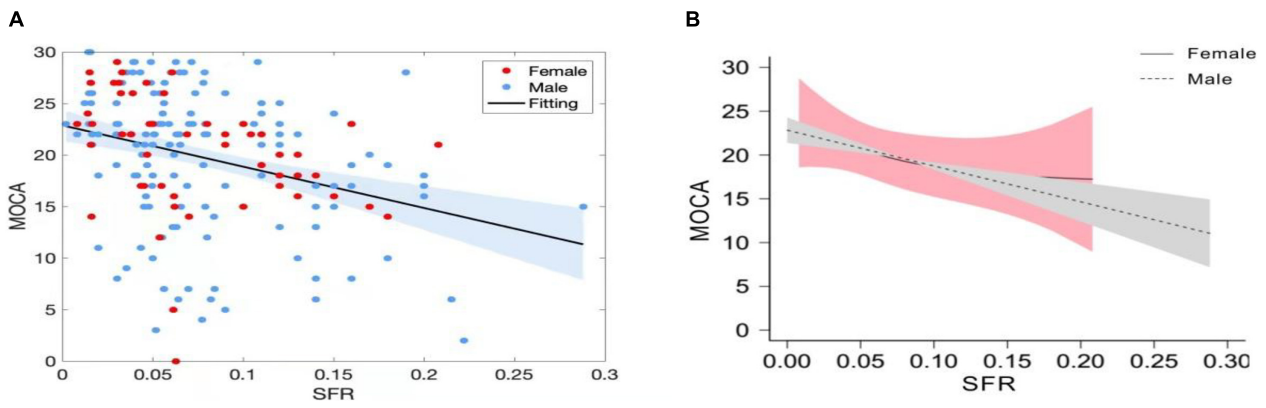

FIGURE 2 | (A) Sylvian fissure ratio (SFR) was inversely associated with MoCA score. Scatter plots with the best linear fit were presented with the shadows up and down showing the 95% CI. Red dots represent female patients and blue dots represent male patients. **(B)** Smoothing spline plots of MoCA by SFR in stratified patients. Dashed line with gray shadows showing the 95% CI represents male patients, and solid line with pink shadows showing the 95% CI represents female patients.

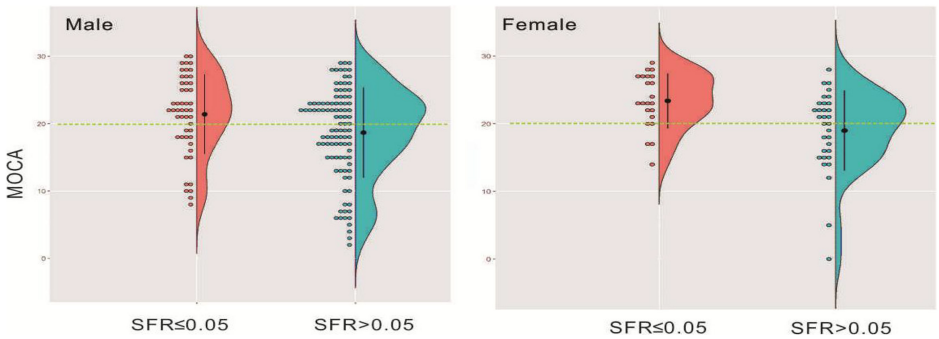

FIGURE 3 | Distribution of MoCA in gender-stratified patients who were divided into dichotomous SFR illustrated in the half-violin plots with raw values.

retained in the final analysis to develop an integrative predictive model to discriminate patients with severe CD (MoCA $\leq$ 20). Two Receiver operating characteristic models adjusted for the resulting factors (model 1, SFR rule in, and model 2, SFR rule out), with bootstrap validation, and were applied and compared (**Figures 4A,B**). The predictive performance was improved as evidenced by a higher AUC in female patients (0.878 vs. 0.948), whereas an improvement was not observed in male patients (0.838 vs. 0.837).

TABLE 2 | Characteristics of patients divided into dichotomous SFR categorical variables before matching.

Characteristic	Mean (SD) or median (Q1–Q3)/N (%)		*p*-Value
	SFR $\leq$ 0.05 (*n* = 71)	SFR > 0.05 (*n* = 132)	
Age	80.75 ± 4.28	83.04 ± 5.06	0.001
MoCA	22.00 ± 5.43	18.49 ± 6.42	<0.001
SFR	0.03 ± 0.01	0.10 ± 0.05	<0.001
Creatinine	88.42 ± 36.93	84.65 ± 34.95	0.473
Uric acid	347.27 ± 94.33	350.52 ± 116.81	0.841
α-HBDH	151.09 ± 38.55	149.61 ± 33.70	0.778
HSCRP	3.43 ± 4.17	4.39 ± 11.95	0.514
TC	4.25 ± 0.80	4.16 ± 0.89	0.471
TG	1.25 ± 0.61	1.44 ± 0.83	0.094
HDLC	1.24 ± 0.32	1.17 ± 0.32	0.137
LDLC	2.50 ± 0.64	2.44 ± 0.74	0.563
APOA	1.39 (1.21–1.63)	1.29 (1.12–1.49)	0.005
APOB	0.90 ± 0.22	0.89 ± 0.24	0.688
Fazekas scale, No. (%)			0.002
0	29 (40.8)	26 (19.7)	
1	22 (31)	36 (27.3)	
2	15 (21.1)	45 (34.1)	
3	5 (7)	25 (18.9)	
Sex-No. (%)			0.300
Male	49 (69)	100 (75.8)	
Female	22 (31)	32 (24.2)	
Hypertension, No. (%)			0.301
No	8 (11.3)	22 (16.7)	
Yes	63 (88.7)	110 (83.3)	
Diabetes, No. (%)			0.087
No	47 (66.2)	71 (53.8)	
Yes	24 (33.8)	61 (46.2)	
Coronary heart disease, No. (%)			0.956
No	53 (74.6)	99 (75)	
Yes	18 (25.4)	33 (25)	
Stroke, No. (%)			0.269
No	60 (84.5)	103 (78)	
Yes	11 (15.5)	29 (22)	

MoCA denotes, Montreal Cognitive Assessment; SFR, Sylvian fissure ratio; α-HBDH, α-hydroxybutyrate dehydrogenase; HSCRP, high-sensitivity C-reactive protein; TC, total cholesterol; TG, total triglyceride; HDLC, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholesterol; APOA, apolipoprotein A; and APOB, apolipoprotein B.

DISCUSSION

Our analysis identified a clinically significant value of 0.05 for the SFR in that this threshold was specifically useful as a screen predictor of CD in female patients with CSVD (Adjusted β, −5.54; 95% CI, −8.78 to −2.30).

Cerebral small vessel disease patients with manifesting latent and chronic CD is very common. The type and degree of CD is related to the neuroimaging features, the number of lesions, and the location of lesions (Cannistraro et al., 2019). Thus, the management of its diagnostic approaches needs to

TABLE 3 | Modification effect of gender on the association between MoCA and SFR categorical variables.

	β (95% CI) *p* value		
	Non-adjusted	Adjust I	Adjust II
Total			
0.03 ± 0.01	Ref.	Ref.	Ref.
0.10 ± 0.05	−3.49 (−5.25, −1.72) 0.0001	−1.89 (−3.45, −0.32) 0.0190	−1.70 (−3.27, −0.14) 0.0344
Male			
0.03 ± 0.01	Ref.	Ref.	Ref.
0.10 ± 0.05	−2.72 (−4.92, −0.52) 0.0168	−0.92 (−2.75, 0.90) 0.3218	−1.01 (−2.84, 0.82) 0.2808
Female			
0.03 ± 0.01	Ref.	Ref.	Ref.
0.10 ± 0.04	−5.43 (−8.13, −2.72) 0.0003	−5.80 (−8.72, −2.88) 0.0003	−5.54 (−8.78, −2.30) 0.0020
P for interaction	0.1713	0.0114	0.0129

Adjusted model 1 was adjusted for risk factors of CSVD (Fazekas; age; hypertension; diabetes; coronary heart disease; and stroke). Adjusted model 2 was adjusted for all covariables. SFR denotes Sylvian fissure ratio.

TABLE 4 | Effect of variables on MoCA in three linear regression models among male patients.

Variable	β (95%CI) *p*-Value		
	Non-adjusted	**Adjust I**	**Adjust II**
Age	−0.24 (−0.46, −0.03) 0.0295	−0.11 (−0.29, 0.06) 0.1932	−0.10 (−0.29, 0.09) 0.2880
Fazekas			
1	−2.72 (−5.00, −0.45) 0.0203	−2.67 (−4.91, −0.43) 0.0211	−2.76 (−5.04, −0.49) 0.0188
2	−9.00 (−11.29, −6.72) <0.0001	−8.21 (−10.54, −5.88) <0.0001	−8.49 (−10.85, −6.13) <0.0001
3	−10.07 (−12.67, −7.46) <0.0001	−9.19 (−11.87, −6.51) <0.0001	−9.34 (−12.08, −6.60) <0.0001
HBP	−3.76 (−6.61, −0.90) 0.0110	−1.60 (−3.93, 0.73) 0.1792	−1.22 (−3.60, 1.15) 0.3151
Diabetes	0.19 (−1.97, 2.35) 0.8638	1.48 (−0.21, 3.17) 0.0891	1.54 (−0.25, 3.34) 0.0943
CHD	−0.51 (−2.93, 1.91) 0.6803	−0.79 (−2.67, 1.09) 0.4131	−0.80 (−2.89, 1.28) 0.4526
Stroke	−4.98 (−7.42, −2.54) 0.0001	−2.95 (−4.98, −0.92) 0.0051	−2.98 (−5.10, −0.87) 0.0064
SFR >0.05	−2.72 (−4.92, −0.52) 0.0168	−0.92 (−2.75, 0.90) 0.3218	−1.01 (−2.84, 0.82) 0.2808
Creatinine > = 111	0.89 (−1.84, 3.63) 0.5224	1.88 (−0.42, 4.17) 0.1112	2.48 (−0.79, 5.75) 0.1392
Uric acid > = 428	−0.81 (−3.30, 1.67) 0.5231	−0.02 (−2.03, 1.98) 0.9820	0.82 (−2.14, 3.79) 0.5875
α−HBDH > = 182	0.02 (−3.15, 3.18) 0.9914	0.26 (−2.23, 2.76) 0.8368	−0.89 (−4.71, 2.93) 0.6501
HSCRP > = 3	0.08 (−2.19, 2.35) 0.9465	0.72 (−1.03, 2.47) 0.4221	0.52 (−1.48, 2.51) 0.6131
TC > = 5.2	−1.70 (−5.57, 2.17) 0.3901	−1.32 (−4.35, 1.72) 0.3966	−2.40 (−6.35, 1.56) 0.2370
TG > = 1.7	0.60 (−1.94, 3.14) 0.6443	0.42 (−1.57, 2.42) 0.6773	−0.76 (−4.21, 2.69) 0.6681
HDLC > = 1	0.85 (−1.38, 3.08) 0.4570	−0.35 (−2.18, 1.49) 0.7124	−0.63 (−3.07, 1.81) 0.6153
LDLC > = 3.4	−0.12 (−4.15, 3.92) 0.9544	−0.34 (−3.50, 2.81) 0.8321	−1.32 (−5.37, 2.73) 0.5251
APOA > = 1.6	3.31 (0.54, 6.08) 0.0205	2.16 (−0.05, 4.38) 0.0579	1.34 (−1.26, 3.94) 0.3135
APOB > = 1.25	2.71 (−2.64, 8.06) 0.3219	4.62 (0.51, 8.72) 0.0290	2.51 (−3.17, 8.19) 0.3889

Adjusted model 1 was adjusted for risk factors of CSVD (Fazekas; age; hypertension; diabetes; coronary heart disease; and stroke). Adjusted model 2 was adjusted for all covariables.

HBP, hypertension; CHD, coronary heart disease; SFR, Sylvian fissure ratio; α-HBDH, α-hydroxybutyrate dehydrogenase; HSCRP, high-sensitivity C-reactive protein; TC, total cholesterol; TG, total triglyceride; HDLC, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholesterol; APOA, apolipoprotein A; and APOB, apolipoprotein B.

be further improved in order to improve outcomes among these patients (Teng et al., 2017; Smith and Beaudin, 2018). Traditionally, CD is primarily assessed by screening tools, such as Mini-Mental State Examination (MMSE), MoCA, Addenbrooke's Cognitive Examination-Revised, etc. Compared to other screen tools, MoCA has many advantages: (1) It has higher sensitivity for detecting MCI and is superior for the detection of post-stroke cognitive impairment (PSCI; Dong et al., 2010; Pendlebury et al., 2012). (2) Although specificity of the MoCA is slightly lower than the MMSE, it still performs good (87%; Nasreddine et al., 2005; Shi et al., 2018). (3) Since 2006, MoCA has been recommended as a clinical screening instrument for VCI by the NINDS-CSN working group (Hachinski et al., 2006). It is valid and reliable in the patients with VCI and CSVD and has good psychometric properties across a wide range of VCI-related conditions and is free for clinical and research use (Wong et al., 2009, 2013). Different from the MMSE, MoCA covers most of the domains considered typically affected in cerebrovascular diseases (executive functions, attention, and concentration) and has been extensively applied in studies (particularly in Asia; Salvadori et al., 2020). (4) According to the latest guidelines (Jia et al., 2014; Skrobot et al., 2018), MoCA is better than MMSE in identifying mild VCI and can be applied to the early screening and overall cognitive assessment of suspected VCI (Koski, 2013). Nevertheless, the threshold of MoCA adopted in PSCI has not reached a consensus (Davis et al., 2015). Neuroimaging techniques provide tools for early diagnosis and may play an important markers in clinical practice (Zanon Zotin et al., 2021). The presentations of neuroimaging is critical to increase diagnostic inaccuracy in the subtypes of CSVD (Tullberg et al., 2004; Sachdev P. et al., 2014). Therefore, not only latest international guidelines but recent Chinese guidelines which are relevant to VCD refer to its importance in identifying CD (Sachdev P. et al., 2014; Skrobot et al., 2018; Peng, 2019; Zhang et al., 2020). Therefore, objectiveness, homogeneity, and patient compliance

TABLE 5 | Effect of variables on MoCA in three linear regression models among female patients.

Variable	β (95% CI) *p*-Value		
	Non-adjusted	**Adjust I**	**Adjust II**
Age	−0.30 (−0.59, −0.01) 0.0482	−0.22 (−0.51, 0.08) 0.1604	−0.01 (−0.40, 0.37) 0.9397
Fazekas			
1	0.44 (−3.34, 4.21) 0.8218	1.41 (−2.30, 5.11) 0.4603	1.54 (−2.60, 5.68) 0.4699
2	−3.48 (−7.06, 0.10) 0.0623	−1.72 (−5.36, 1.92) 0.3599	−2.53 (−7.40, 2.33) 0.3145
3	−3.92 (−9.82, 1.97) 0.1983	−0.34 (−6.72, 6.04) 0.9179	−3.10 (−10.07, 3.86) 0.3887
HBP	−2.78 (−7.23, 1.66) 0.2251	−1.33 (−5.78, 3.11) 0.5592	−0.26 (−4.96, 4.45) 0.9159
Diabetes	−0.80 (−3.83, 2.22) 0.6040	0.63 (−2.34, 3.61) 0.6781	−1.71 (−4.94, 1.52) 0.3057
CHD	−1.82 (−5.32, 1.69) 0.3150	−0.55 (−4.10, 2.99) 0.7614	−1.85 (−5.61, 1.91) 0.3421
Stroke	−6.34 (−10.24, −2.44) 0.0024	−5.35 (−9.95, −0.74) 0.0276	−4.29 (−9.16, 0.58) 0.0930
SFR >0.05	−5.43 (−8.13, −2.72) 0.0003	−5.80 (−8.72, −2.88) 0.0003	−5.54 (−8.78, −2.30) 0.0020
Creatinine > = 111	4.43 (−2.07, 10.93) 0.1874	5.05 (−1.38, 11.47) 0.1309	−3.14 (−14.19, 7.90) 0.5804
Uric acid > = 428	−1.29 (−5.17, 2.60) 0.5190	−0.67 (−4.68, 3.35) 0.7463	−3.32 (−10.13, 3.49) 0.3460
α−HBDH > = 182	−0.88 (−4.32, 2.57) 0.6210	−0.25 (−3.76, 3.25) 0.8881	1.25 (−4.59, 7.09) 0.6777
HSCRP > = 3	−1.05 (−4.68, 2.58) 0.5743	−1.57 (−5.17, 2.02) 0.3959	−2.39 (−9.89, 5.11) 0.5368
TC > = 5.2	−0.41 (−4.17, 3.34) 0.8298	−1.08 (−4.91, 2.75) 0.5829	0.44 (−5.59, 6.47) 0.8876
TG > = 1.7	0.47 (−2.98, 3.93) 0.7886	−0.37 (−3.85, 3.12) 0.8373	−4.02 (−11.05, 3.00) 0.2691
HDLC > = 1	0.67 (−3.22, 4.57) 0.7363	0.11 (−3.79, 4.01) 0.9547	0.29 (−5.17, 5.75) 0.9178
LDLC > = 3.4	−0.83 (−5.33, 3.68) 0.7204	−1.77 (−6.21, 2.66) 0.4377	−4.93 (−13.16, 3.30) 0.2487
APOA > = 1.6	−0.57 (−3.95, 2.80) 0.7403	−0.34 (−3.97, 3.29) 0.8554	−2.42 (−8.33, 3.49) 0.4284
APOB > = 1.25	0.58 (−4.23, 5.40) 0.8133	−0.44 (−5.22, 4.34) 0.8574	−3.95 (−10.91, 3.00) 0.2728

Adjusted model 1 was adjusted for risk factors of CSVD (Fazekas; age; hypertension; diabetes; coronary heart disease; stroke). Adjusted model 2 was adjusted for all covariables.

HBP, hypertension; CHD, coronary heart disease; SFR, Sylvian fissure ratio; α-HBDH, α-hydroxybutyrate dehydrogenase; HSCRP, high-sensitivity C-reactive protein; TC, total cholesterol; TG, total triglyceride; HDLC, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholesterol; APOA, apolipoprotein A; and APOB, apolipoprotein B.

may be optimized by combing neuropsychiatric inventory and neuroimaging.

Thus far, it is also difficult to conduct widespread cognitive screening assessment in China, especially in non-polyclinic hospitals, primary care settings, and community clinics where there is a shortage of neuropsychiatric professionals. However, a higher prevalence of MCI in the rural than in the urban population has been reported in China (Wang et al., 2011; Jia et al., 2014). Moreover, in addition to the insufficient trained doctors, the poor compliance and inner resistance for diagnosis of dementia in the patients or their families may also underlie the low prevalence of MCI (US Preventive Services Task Force et al., 2020). At present, in China, neuroimaging, especially CT, is widely used in many non-polyclinic hospitals (Wang et al., 2017, 2019). Many elderly people will go to non-polyclinic hospitals for treatment because of dizziness, headache, limb weakness, and other symptoms due to its convenience when compared to regional center hospitals. At this time, it is more acceptable for patients and their families when they are suggested to receive a neuroimaging exam to preclude diseases such as cerebral infarction or hemorrhage (He et al., 2019; Wang et al., 2020). Linear measurement plays an important role in the screening of CD (Bermel et al., 2002; Del Brutto et al., 2018), especially in identifying cerebral atrophy, which has been implicated in the pathophysiology of CD. Previous studies have found that the volume of nucleus accumbens, amygdala, caudate nucleus, thalamus, and brain stem is closely related to cognition in stroke patients (Hilal et al., 2015). One study showed that of 393 patients with transient ischemic attack or suspected stroke, 169 patients had a medial temporal lobe atrophy finding that medial temporal lobe atrophy was significantly related to memory, naming, perception, executive ability, speed, and attention (Kebets et al., 2015). According to the STRIVE recommendation, the neuroimaging markers of cerebral atrophy are nonspecific (Wardlaw et al., 2013). In the indices of brain atrophy, the Sylvian fissure is an important structure of both

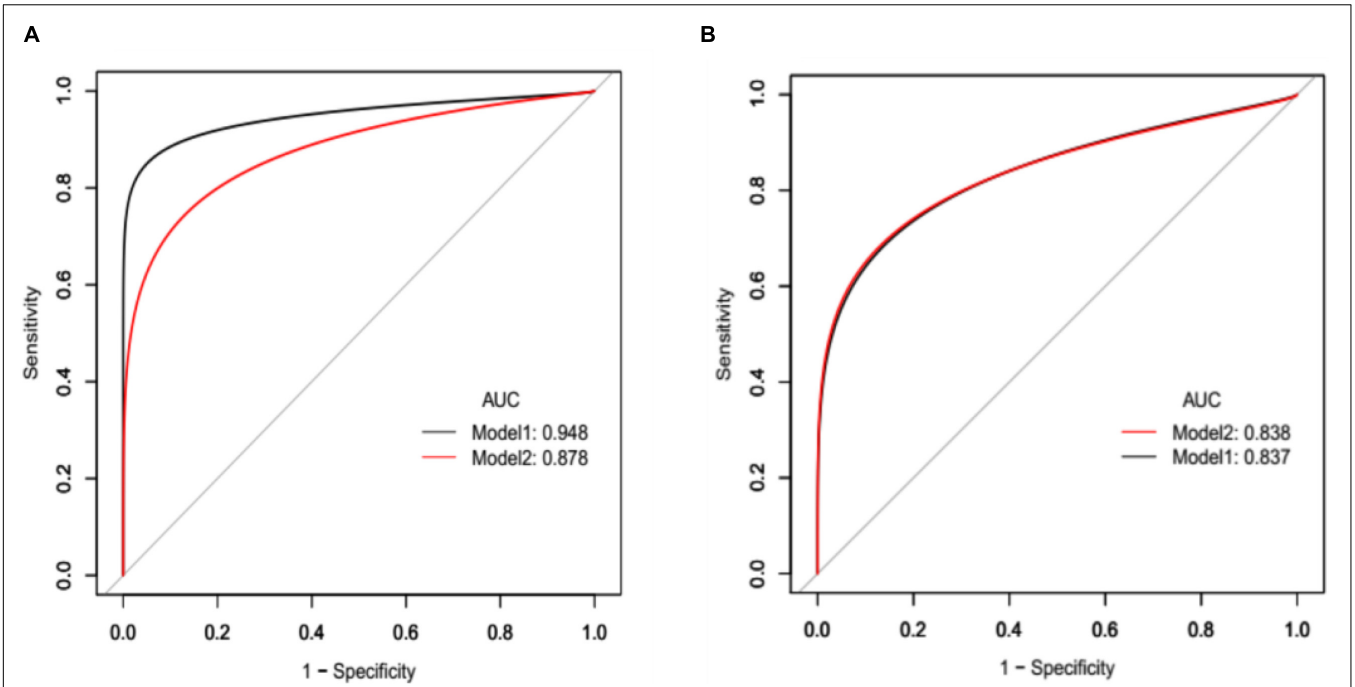

FIGURE 4 | (A) Receiver operating characteristic (ROC) curve analyses of integrative variables (Fazekas scale, having stroke, SFR categorical variables, and age) to discriminate female patients with severe cognitive impairment. Area under the ROC curve (AUC) for model 1, SFR rule in was 0.9477 (95% CI, 0.8115–0.9730), and for model 2, SFR rule out was 0.8775 (95% CI 0.7490–0.9466). **(B)** ROC analyses of integrative variables (Fazekas scale, having stroke, SFR categorical variables, and age) to discriminate male patients with severe cognitive impairment. AUC for model 1, SFR rule in was 0.8367 (95% CI, 0.7662–0.8941), and for model 2, SFR rule out was 0.8380 (95% CI 0.7585–0.8899).

pathophysiological and microsurgical significance (Balak, 2014). The SFR has been shown to be associated not only with clinical outcomes in patients with CSVD (Sato et al., 2016) or with basal ganglia hemorrhage (Kwon et al., 2018) but also with survival in elderly individuals after age 85 (Olesen et al., 2011). Concerning the overall significance, similar to stroke center referrals, physicians in non-polyclinic hospitals primarily screen suspected patients of CSVD with CD through neuroimaging, and send them to regional central hospitals where there is an abundance of neuropsychiatric professionals for further confirmation. The practical application of using the SFR may increase the diagnostic accuracy and screening rate under the current Chinese healthcare system. In addition, it will be of great benefit to find out comparatively objective and simple parameters with limited harms of screening (e.g., labeling people with dementia) in the elderly (US Preventive Services Task Force et al., 2020).

Our primary finding is, to our knowledge, the first to identify a clinically significant screening value of 0.05 for SFR as a threshold associated with CD in patients with CSVD, specifically in female patients. We speculated that due to the higher weight of visuospatial and execution in MoCA, the scores of female patients may be lower than male patients in the same stage and that this may have clinical significance (Munro et al., 2012; Laws et al., 2016). Females have been found to experience more rapid cerebral decline compared to male patients with respect to the development of MCI in Alzheimer's disease (AD; Perneczky et al., 2007; Bramen et al., 2011; Giedd et al., 2012; Koolschijn and Crone, 2013). The proposed difference of downward trend is consistent with our finding. Additionally, estrogen has been shown to have a protective effect on the nervous system, and estrogen can regulate glucose transport, aerobic glycolysis and mitochondrial function, thus generating ATP in multiple brain regions (such as medial temporal lobe, cingulate gyrus, and frontal cortex) involved in cognitive function, providing energy for brain tissue (Munro et al., 2012; Mosconi et al., 2018). During menopause, estrogen level decrease, increasing the risk of AD in female. In our study, all female patients were over 65 years old and were in menopause. Other factors that may lead to sex differences are that the risk of AD in females with the apoE4 gene is three to four times higher than that in non-carriers; this risk in men with or without apoE4 gene is relatively low (Bertram and Tanzi, 2008). There may be confounding due to the fact that life expectancy among females is higher than males in China, and that AD is predominately present in those older than 65 years (Clarfield and Dwolatzky, 2013). To improve the generalizability of the conclusions drawn, we also performed additional analysis using public data from ADNI. It was found that the optimal threshold increased based on the cohort analysis of the ADNI database when compared to our cohort (data not shown). Meanwhile, with the same increase of SFR, females have been found to experience more rapid CD compared to male patients in MMSE scores. However, the gender difference

was not as big as the cohort with MOCA screen testing. We speculate that this may be related to the different sensitivity between MMSE and MOCA.

Our study has several limitations. The data were validated with a single-center observational study, and the optimal threshold for SFR may differ when other confounders are controlled in randomized controlled trials. Therefore, inferences derived from our results may lack generalizability and maybe only applicable to specific populations similar to our study population. SFR thresholds require further study in both female and male populations in studies with larger sample sizes using randomized methods. According to the *Diagnostic and Statistical Manual of Mental Disorders* (*DSM-V*; Sachdev P.S. et al., 2014), diagnostic criteria for mild/major neurocognitive disorder encompass evidence of six key cognitive domains (complex attention, executive function, learning and memory, language, perceptual–motor, or social cognition), together with capacity for independence in everyday activities. In our study, we only use MoCA as a screening tool. However, MoCA is not a diagnostic criterion of CD. The association of SFR and tests for different domains in *DSM-V* should be performed to indicate the predictive power of SFR. In future studies, specific tests should be administered per cognitive domain according to *DSM-V*, and further detailed evaluations of cognitive impairment with each domain together with IADL, NPI, etc., should be further investigated. Other limitations of this study include missing data for some important variables, such as lack of regular follow-up after discharge, lack of further evaluation of CD and SFR after discharge, lack of patient genotype records, etc. Finally, the single-center design may limit the generalizability of this conclusion.

In conclusion, among patients undergoing CSVD, higher SFR may be highly associated with a greater risk of CD. A ratio of 0.05 may represent a cutoff point defining higher risk for female patients in whom the disorder is clinically suspected. SFR may be a useful, low-resource, and objective measurement to screen CSVD patients with CD, especially for female elderly in non-polyclinic hospitals such as community hospitals and rural clinics where there is a shortage of professional neurologists and imaging equipment.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committees of The First Affiliated Hospital of Soochow University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YQ and QX: study design. DA: data acquisition. XG and TL: statistical analysis. SD and QX: data supervisor. YQ and YL: the manuscript preparation. HZ and YW: language editing. YW: the manuscript review. AJ: the manuscript review and revision (second round). YQ and YW: funding support. All authors contributed to the article and approved the submitted version. QF: director of the Stroke Center has organized all authors to finish this project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.729782/full#supplementary-material

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OPINION
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The Female Impact in the World of Neurodegeneration

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INTRODUCTION

Scientific research fuels advances in medicine, but the contributions of individual scientists, especially women, are often underappreciated. The field of neuroscience is vast, with many important gaps in knowledge remaining; thus, it is imperative for women to be able to contribute. A pivotal 2018 study revealed that women were still shockingly underrepresented in academic neurology, representing only 30.8% of academic neurologists (McDermott et al., 2018). Of additional concern, this gender gap grows with increasing academic rank, with only 13.8% of neurology professors being women (McDermott et al., 2018). Although women have faced much adversity in the field of neuroscience, there have been pioneers who have paved a path for other women. Moreover, these women made important contributions that enhanced scientific understanding of neurodegeneration and neurodegenerative diseases. The purpose of this paper is to highlight notable, but underappreciated, female neuroscientists and their important contributions to modern understanding of neurodegeneration.

Overview of Neurodegeneration

The term neurodegeneration is a mixture of two words—"neuro", which refers to nerve cells and then "degeneration", which refers to progressive damage. The term in its entirety, "neurodegeneration", can be used to describe various conditions that result in the loss of nerve structure and function. This deterioration of neural structures results in a loss of cognitive abilities such as memory and decision making (Murman, 2015). Loss of neural function is a key hallmark in many neurodegenerative diseases, including Parkinson's disease (PD), Huntington's disease (HD), Multiple Sclerosis (MS), Alzheimer's disease (AD), and many rare disorders (Przedborski et al., 2003).

An important area of research is exploring the mechanisms that underlie neurodegeneration and plasticity; this includes investigation of proteins implicated in AD (e.g., tau; amyloid-beta) and Parkinson's (e.g., alpha-synuclein). Tau is a protein found in the brain that serves to stabilize microtubules (Gao et al., 2018); tau hyperphosphorylation results in its aggregation into insoluble tangles, a characteristic biomarker of AD. Another characteristic feature of AD is the aggregation of amyloid beta protein into extracellular deposits known as amyloid plaques (Bloom, 2014). Alpha-synuclein is a neuronal protein that normally regulates trafficking of synaptic vesicles for neurotransmitter release. If mutated, the once soluble protein aggregates into insoluble fibrils forming Lewy bodies, a hallmark feature of PD (Stefanis, 2012). Notably, there are currently no known therapies that can prevent or reverse neurodegeneration. Rather, for each particular disease, medications may be available that reduce symptom burden and improve quality of life. Ongoing research efforts focus on the similarities between neurodegenerative these diseases in the hope of 1 day finding a cure. In this manuscript, notable contributions

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FIGURE 1 | The above timeline outlines a few key events that contributed to knowledge of neurodegeneration. Some of the events shown are the first descriptions of neurodegenerative diseases, others are key discoveries regarding the mechanisms of neurodegeneration, one is a clinical trial.

of women to neurodegeneration research are highlighted. These contributions are vast and include discoveries about neuronal pathways, proteins, vascular effects on neurodegeneration, nerve growth factor, and novel imaging techniques. In total, 12 female neuroscientists are celebrated below, namely: Rita Levi-Montalcini, Valina L. Dawson, Eva-Maria Mandelkow, Tara Spires-Jones, Maria Grazia Spillantini, Miia K. Kivipelto, Vivian Tabar, Marian Diamond, Elizabeth Roboz-Einstein, Cécile Vogt-Mugnier, Patricia Goldman-Rakic, and Anita Harding. A timeline of select key advancements in neurodegeneration research is provided below (see **Figure 1**).

Rita Levi-Montalcini

Rita Levi-Montalcini was born on April 22, 1909 in Turin, Italy, and went on to make pivotal contributions to our understanding of neuroscience (Aloe, 2011). In 1947, Levi-Montalcini went to Washington University, in St. Louis, Missouri, to study nerve tissue growth in chick embryos in Viktor Hamburger's laboratory (Zeliadt, 2013). Hamburger and Levi-Montalcini discovered what they called nerve growth factor (NGF) (Aloe, 2011). Their discovery of NGF earned them the Nobel Prize in Medicine in 1986. The historic discovery of NGF has allowed scientists to study neural growth, and enhanced understanding of neurodegenerative diseases such as AD (Scott and Crutcher, 1994), PD (Mogi et al., 1999) and MS (Laudiero et al., 1992).

Valina L. Dawson

Valina L. Dawson, professor of Neurology at Johns Hopkins Medical School, researches cell signaling pathways that lead to neuronal death (Zhang et al., 2017). She also studies EIF4G1 and LRRK2, two mutations associated with familial Parkinson's disease (Martin et al., 2014). Dawson uses animal models (Lee et al., 2012) as well as post-mortem brains (Martin et al., 2010) to explore these signaling pathways. Using an animal model, Dawson characterized a pathway of Parkinson's disease called Parthanatos (Fatokun et al., 2014). Dawson's genetic work led to insights into the complicated origins and causes of neurodegenerative diseases such as PD. Dawson has received over 60 awards and honors in her career, including Thomson Reuters: The World's Most Influential Minds, 2014 and the Javits Neuroscience Investigator Award, 2014.

Eva-Maria Mandelkow

Eva-Maria Mandelkow is a neuroscientist who began studying tau (a protein that stabilizes axonal microtubules) in the context of AD in 1989. Mandelkow's research demonstrated that tau is one of the best indicators of the presence of AD (Mandelkow and Mandelkow, 1998), paving the way for more extensive study of this protein. Mandelkow's research involves studying the spread of tau pathology (Mudher et al., 2017) and tau's role in brain development (Sapir et al., 2012).

Tara Spires-Jones

Tara Spires-Jones is a professor at the University of Edinburgh. Like Dawson, Jones studies mechanisms of AD and other

neurodegenerative diseases. Jones' found that amyloid beta and tau proteins contribute to neurodegeneration and that lowering the levels of these proteins can "prevent and reverse phenotypes in model systems" (Spires-Jones and Hyman, 2014). Jones has also developed high-resolution imaging techniques to view post-mortem brains; her technique has provided evidence for the accumulation of amyloid and tau proteins in brains with neurodegenerative diseases (Meyer-Luehmann et al., 2008; Henstridge et al., 2015).

Maria Grazia Spillantini

Maria Grazia Spillantini is a molecular neurologist who specializes in neurodegenerative disease mechanisms and treatment. Like Dawson and Jones, Spilantini also studies the mechanisms underlying neurodegenerative diseases. Spillantini specifically studies tau and Lewy bodies, which are abnormal proteins which aggregate within the brains in people with PD and other disorders associated with Lewy bodies (Spillantini et al., 1997a,b). Spillantini is famous for discovering the protein alpha-synuclein which is a very important component in Lewy bodies (Spillantini et al., 1997a). Further work revealed that a specific mutation in the gene encoding alpha-synuclein is associated with certain types of PD (Bennett, 2005; Nussbaum, 2017).

Miia K. Kivipelto

Miia K. Kivipelto is a Finnish neuroscientist and professor, whose research has shown that there is a relationship between dementia and vascular risk factors, such as high blood pressure and elevated blood lipids. From 2009 to 2011, Kivipelto led a clinical trial called the Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability (FINGER) (Kivipelto et al., 2013). This study found that a healthy diet, exercise, and cognitive training may reduce loss of cognitive function in people who are at a high risk of dementia. Kivipelto is currently running the worldwide FINGER initiative, which aims to replicate the results in a global sample.

Vivian Tabar

Vivian Tabar is an American neurosurgeon and researcher. Tabar has been recently approved to conduct a clinical trial which tests the ability of stem cells to repair damaged brain cells in people with PD (NCT04802733) (Phase 1). Currently, the main treatment for PD is the drug L-DOPA which was first used about 60 years ago, is non-curative, and eventually stops working. Tabar's clinical trial brings promise and hope to people suffering from PD, and represents a regenerative treatment that could be later trialed for other neurodegenerative diseases.

Marian Diamond

Mariana Diamond was a professor at the University of California Berkeley and is considered one of the founders of modern neuroscience. She famously studied Albert Einstein's brain, and found that it contained more glial cells than the average male brain from the control group (Diamond et al., 1985). Beyond this critical work, Diamond is most lauded for her pioneering research in anatomical neuroscience. Her work highlights that neuroplasticity at the cellular level maintains neuronal connections (Dorszewska et al., 2020). Prior to her seminal research, many thought that the brain and the way it functions was determined by genetics and could not be changed (Kentner et al., 2019). Although her work was initially doubted by other scientists, it eventually gained respect and brought neuroplasticity into the limelight. Other experiments on rats demonstrated the benefits of enriched environments at any age, including a thicker cortex and enhanced learning capacity (Diamond et al., 1964).

Elizabeth Roboz-Einstein

Elizabeth Roboz-Einstein was born in Hungary in 1904 and relocated to the United States in 1940 in response to nazi forces invading Hungary. She married Hans Einstein, the first son of Albert Einstein. In an effort to better teach her students, she studied neurochemistry. This led to her interest in neuroscience, particularly myelin, an insulating fat around nerves. She isolated the myelin basic protein (MBP) using an MS animal model (Einstein et al., 1972). This discovery allowed Roboz-Einstein and other neuroscientists to determine which specific regions of MBP were antigenic. Ultimately her work led to an improved model and progressed research on immunotherapies for MS and other demyelinating diseases (Meinl and Hohlfeld, 2002).

Cécile Vogt-Mugnier

Vogt received her medical doctorate from Paris in 1900 and is widely renowned for her work as a neurologist. She is most famous for her discoveries regarding the neuroanatomy of the thalamus. She and her husband both played a vital role in understanding the functional anatomy of the brain and subsequent mapping of the human brain (Kreutzberg et al., 1992). She was nominated for a Nobel Prize, elected to the German Academy of Science, and was awarded the National Prize of East Germany (Kreutzberg et al., 1992).

Patricia Goldman-Rakic

Patricia Goldman-Rakic was The Eugene Higgins Professor of Neuroscience at Yale School of Medicine. Patricia Goldman-Rakic conducted seminal research on the frontal lobe and memory (Goldman-Rakic, 1990). This work greatly enhanced our understanding of both memory and behavior in the context of neurodegenerative diseases including PD and AD (Rajkowska et al., 1998). Because of Patricia Goldman-Rakic scientists were better able to understand the basis of higher cognitive function and, ultimately, of neurodegenerative diseases such as PD and AD.

Anita Harding

Anita Harding graduated in 1975 from the Royal Free Hospital School of Medicine and became a leading clinical scientist of her time. She is considered a pioneer of neurogenetics because she anticipated the entry of molecular genetics into neurology. Some of her major accomplishments included the first identification of the mitochondrial DNA mutation in human disease and identifying trinucleotide repeat sequences in

degenerative neurological diseases such as Huntington's (Poulton and Huson, 1996).

CONCLUSION

This paper celebrates the notable research contributions of 12 women, whose work enhanced scientific understanding of neurodegenerative diseases and changed the direction of neuroscience. Overall, the contributions of these and other female neuroscientists are vast and include the creation of novel imaging techniques as well as enhanced understanding of proteins, genetics, and environmental factors that contribute to brain health and neurodegenerative disorders. The work summarized in this manuscript and other contributions of women neuroscientists helped to advance neurodegenerative research, though their important contributions may not be widely known or taught in schools. This manuscript serves as a primer meant to celebrate select seminal contributions of women to neurodegeneration research; interested readers are encouraged to explore more work by these and other female neuroscientists.

AUTHOR CONTRIBUTIONS

CG and PG conducted literature reviews to gather research from different sources and wrote sections of the draft. CG compiled drafts to create the final manuscript and designed the figure. All authors worked to revise the draft with NO contributing significantly to outlining the paper as well as the revision process.

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BRIEF RESEARCH REPORT
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Imbalances in Copper or Zinc Concentrations Trigger Further Trace Metal Dyshomeostasis in Amyloid-Beta Producing *Caenorhabditis elegans*

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Alzheimer's Disease (AD), a progressive neurodegenerative disease characterized by the buildup of amyloid-beta (Aβ) plaques, is believed to be a disease of trace metal dyshomeostasis. Amyloid-beta is known to bind with high affinity to trace metals copper and zinc. This binding is believed to cause a conformational change in Aβ, transforming Aβ into a configuration more amenable to forming aggregations. Currently, the impact of Aβ-trace metal binding on trace metal homeostasis and the role of trace metals copper and zinc as deleterious or beneficial in AD remain elusive. Given that Alzheimer's Disease is the sixth leading cause of adult death in the U.S., elucidating the molecular interactions that characterize Alzheimer's Disease pathogenesis will allow for better treatment options. To that end, the model organism *C. elegans* is used in this study. *C. elegans*, a transparent nematode whose connectome has been fully established, is an amenable model to study AD phenomena using a multi-layered, interconnected approach. Aβ-producing and non-Aβ-producing *C. elegans* were individually supplemented with copper and zinc. On day 6 and day 9 after synchronization, the percent of worms paralyzed, concentration of copper, and concentration of zinc were measured in both groups of worms. This study demonstrates that dyshomeostasis of trace metals copper or zinc triggers further trace metal dyshomeostasis in Aβ-producing worms, while dyshomeostasis of copper or zinc triggers a return to equilibrium in non-Aβ-producing worms. This supports the characterization of Alzheimer's Disease as a disease of trace metal dyshomeostasis.

Keywords: Alzheimer's, amyloid-beta, copper, zinc, trace metal, dyshomeostasis, *Caenorhabditis elegans*, imbalances

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INTRODUCTION

Alzheimer's Disease (AD) is the 6th leading cause of death in the U.S., with one in ten people age 65 or older having AD (Alzheimer's Association, 2021). As a progressive neurodegenerative disease, AD is characterized by extra-neuronal amyloid-beta plaques and intraneuronal tau neurofibrillary tangles which affect memory and cognition. Amyloid-beta plaques are aggregates of the amyloid-beta peptide (Aβ), a cleavage product of the amyloid precursor protein (APP). Buildup of Aβ causes neural death and neuroinflammation.

Neurodegenerative diseases such as Alzheimer's, Parkinson's, and Wilson's Diseases have been associated with metal dyshomeostasis, which often accompanies aging (Luo et al., 2011; Squitti, 2012; Singh et al., 2013). Metal dyshomeostasis occurs when metal levels increase or decrease beyond normal bounds. As important components of vitamins and enzymes, trace metals play a crucial role in neural and biochemical processes. When in homeostasis, these trace metals facilitate proper brain functioning and growth by protecting against reactive oxygen species (ROS), regulating gene expression, and activating enzymes. The dyshomeostasis of trace metals results in cellular damage and oxidative injury, induced by the formation of ROS (Grochowski et al., 2019).

Both trace metals copper and zinc play key roles in proper brain functioning. Copper is an essential trace element that plays a key role in energy production, free radicals scavenging, and neurotransmission (Singh et al., 2013). Zinc is another essential trace element that plays a key role in neurotransmission and redox regulation (Grochowski et al., 2019). Amyloid beta plaques have high affinity to trace metals copper and zinc and have thus been found to contain high concentrations of these trace metals (Bush et al., 1994; Atwood et al., 1998; Lovell et al., 1998; Sayre et al., 2000; Suh et al., 2000; Cherny et al., 2001; Dong et al., 2003; Miller et al., 2006; Mital et al., 2015; Ejaz et al., 2020). For instance, a 339% increase in Zn and a 466% increase in Cu were found in amyloid beta plaques of AD patients in comparison to healthy subjects (Leskovjan et al., 2009). The levels of copper and zinc in AD, however, remains controversial (Huang et al., 2000; Strausak et al., 2001; Cerpa et al., 2005; Kessler et al., 2005; Watt et al., 2010; Bagheri et al., 2018; Rana and Sharma, 2019). Some studies indicate copper deficiency in AD, suggesting a need for supplementation (Borchardt et al., 1999; Exley, 2006; Jiao and Yang, 2007; Kessler et al., 2008; Vural et al., 2010; Kaden et al., 2011; Exley et al., 2012; Xu et al., 2017), while others indicate copper excess in AD, suggesting a need for chelating agents (Cherny et al., 2001; Sparks et al., 2006; Hua et al., 2011; Luo et al., 2011; Ceccom et al., 2012; Eskici and Axelsen, 2012; Brewer, 2014; Squitti et al., 2014; Yu et al., 2015; Patel and Aschner, 2021). Similarly, some studies indicate zinc deficiency in AD (Kapaki et al., 1989; Molina et al., 1998; Brewer et al., 2010; Rivers-Auty et al., 2021), while others indicate zinc excess (Lovell et al., 1998; Religa et al., 2006; Bonda et al., 2011; Greenough et al., 2013; James et al., 2017). These conflicting findings could be partially due to differences in the brain regions in which copper and zinc were measured. With over 5 million Americans currently living with AD and nearly 14 million projected to be living with AD by 2050, better understanding the molecular mechanisms characterizing the involvement of copper and zinc dyshomeostasis in AD will allow for better treatment options and outcomes (Alzheimer's Association, 2021).

Caenorhabditis elegans, a non-parasitic nematode whose connectome has been fully established, is an advantageous model for studying the molecular mechanisms in Alzheimer's Disease (Caito et al., 2012). The nematode's simple nervous system and transparency allow for the study of the effects of AD on neuronal pathways and function. Roughly 38% of worm genes have a human ortholog, such as APP and tau, making *C. elegans* an excellent *in vivo* model for the study of AD (Shaye and Greenwald, 2011). Since the toxic Aβ42-peptide is expressed in muscle cells in *C. elegans* strain CL2006, Aβ aggregations result in the paralysis of *C. elegans*, thus allowing the extent of Aβ aggregation in response to different treatments to be viewed macroscopically (Saharia et al., 2016).

Given that molecular mechanisms characterizing the interaction between copper, zinc, and amyloid-beta remain elusive, the present study aims to elucidate whether the dyshomeostasis of one trace metal induces the dyshomeostasis of other trace metals and of amyloid-beta in Alzheimer's Disease. It is hypothesized that increases in amyloid-beta aggregations are part of a failed protective homeostatic mechanism to bind excess trace metals copper and zinc. The present study newly shows that dyshomeostasis of trace metals copper or zinc triggers further trace metal dyshomeostasis in Aβ-producing worms while dyshomeostasis of copper or zinc triggers a return to equilibrium in non-Aβ-producing worms.

MATERIALS AND METHODS

Nematode Strains and Maintenance

Caenorhabditis elegans strains were received from the Caenorhabditis Genetics Center (CGC). The transgenic *C. elegans* strain CL2006, which expresses human $A\beta_{1-42}$ in body-wall muscle cells, is characterized by progressive, adult-onset paralysis and a roller phenotype (Link, 1995). The *C. elegans* strain N2 represents the wild type. During two independent trials, worm strains were synchronized according to the following procedure: Adult hermaphrodite worms were transferred to fresh plates and allowed to lay eggs for 2–4 h. After removal of the adult parental worms, the synchronized progeny were allowed to reach adulthood, then later scored for paralysis (Fonte et al., 2002). The worms were propagated at 20°C on Nematode Growth Media (NGM) plates seeded with the bacterial strain OP50 and supplemented with either copper or zinc (Brenner, 1974).

Supplementation With Copper and Zinc

$CuCl_2$ was used to supplement the worms with copper. $CuCl_2$ stock solution was diluted into a live E. coli OP50 suspension, reaching a final concentration of 150 μM, and was placed on the surface of the NGM plates. Once the worms reached adulthood (day 3), a group of synchronized CL2006 worms and a group of synchronized N2 worms were placed on the copper-supplemented plates. $ZnSO_4$ was used to supplement the worms with zinc. $ZnSO_4$ stock solution was diluted into a live E. coli OP50 suspension, reaching a final concentration of 500 μM, and was placed on the surface of the NGM plates. Once the worms reached adulthood, a group of synchronized CL2006 worms and a group of synchronized N2 worms were placed on these zinc-supplemented plates.

Paralysis Assay

On days 6 and 9[1] after synchronization, 20 worms from the copper-supplemented and zinc-supplemented CL2006 and N2 groups were tested for paralysis. Paralysis indicates the extent of Aβ-aggregation development. The worms were tested for paralysis by tapping their noses with a platinum wire pick. Worms that moved their noses but failed to move their bodies were scored as "paralyzed" (Luo et al., 2011).

Lysis Procedure

On days 6 and 9, thirty worms from each of the four groups: (1) copper-supplemented CL2006, (2) zinc-supplemented CL2006, (3) copper-supplemented N2, (4) zinc-supplemented N2, were lysed in preparation for copper and zinc colorimetric assays. The following procedure is especially useful for dauer larvae lysis. Worms were spun in a centrifuge at 4,000 rpm for 1 min to a pellet. The supernatant was removed, and the pellet was washed in 1.5 mL of ice cold L15 buffer. The worms were centrifuged, and the supernatant was removed once again. Twenty-five microliters of the pellet was pipetted onto a glass slide. A 50 mm glass coverslip was added on top, and pressure was applied to the coverslip using a pipette. When viewed under a microscope, head disruption head could be visualized as pressure was applied with the pipette tip. Pressure continued to be applied until most of the worms were exploded. The contents on the coverslip and slide were washed off with 1 ml of cold L15 into a test tube. Finally, this L15-cell solution was pipetted vigorously 25 times to ensure the *C. elegans* were completely lysed.

Copper and Zinc Colorimetric Assays

To quantify the amount of copper in the *C. elegans* on days 6 and 9, a copper colorimetric assay (Elabscience) was applied to the lysed *C. elegans* solution. Similarly, to quantify the amount of zinc in the *C. elegans* on days 6 and 9, a zinc colorimetric assay (Elabscience) was applied to the lysed *C. elegans* solution. Once the standard wells were created for both assays, the percent transmittance of the standards and test groups was measured using a colorimeter. The percent transmittance was converted to ion content (μmol/L) as specified by the Elabscience assays.

Statistical Analysis

All values were expressed as mean ± SEM. Statistical analysis involving two groups was conducted using a *t*-test. Statistical analysis involving more than two groups was conducted using a one-way analysis of variance (ANOVA) followed by a *post-hoc* analysis using Tukey test. The differences were considered to be significant at $p < 0.05$.

RESULTS

To elucidate the differences in trace metal homeostasis maintenance in amyloid-beta producing *C. elegans* compared to non-amyloid-beta producing *C. elegans*, both strains of *C. elegans* were supplemented with copper and zinc individually.

Zinc Concentration Changes in Response to Copper Supplementation

When Aβ-producing *C. elegans* were supplemented with copper, the zinc concentration increased significantly ($p = 0.013$) from day 6 (13.5 ± 0.6 μmol/L) to day 9 (20.1 ± 0.9 μmol/L). Likewise, when wild-type worms were supplemented with copper, the zinc concentration increased significantly ($p = 0.041$) from day 6 (16.0 ± 0.9 μmol/L) to day 9 (19.3 ± 0.6 μmol/L, **Figure 1**). Additionally, the percent change in zinc content from day 6 to day 9 in Aβ-producing C. elegans (49% increase) was more than double the percent change in wild-type *C. elegans* (21% increase). This indicates that a high copper concentration results in a larger change in the zinc concentration in Aβ-producing *C. elegans* compared to non-Aβ-producing *C. elegans*.

Copper Concentration Changes in Response to Zinc Supplementation

When Aβ-producing *C. elegans* were supplemented with zinc, the copper concentration increased significantly ($p = 0.022$) from day 6 (27.8 ± 4.9 μmol/L) to day 9 (58.6 ± 3.7 μmol/L). In contrast, when wild-type *C. elegans* were supplemented with zinc, the copper concentration decreased significantly ($p = 0.012$) from day 6 (60.8 ± 2.4 μmol/L) to day 9 (24.7 ± 5.4 μmol/L, **Figure 2**). In fact, the copper content on day 6 in mutant *C. elegans* was roughly equivalent to the copper content on day 9 in wild-type *C. elegans* ($p = 0.9$). Similarly, the copper content on day 9 in mutant *C. elegans* was roughly equivalent to the copper content on day 6 in wild-type *C. elegans* ($p = 0.9$). This indicates that a high zinc concentration through supplementation results in an increase in the copper content of Aβ-producing *C. elegans* that is roughly equal in magnitude to the decrease in copper content in non-Aβ-producing *C. elegans*.

Effect of Copper and Zinc Dyshomeostasis on Aβ Aggregations

To characterize the effect of imbalances in such trace metals on the extent of Aβ aggregations, the percent of worms paralyzed was measured in Aβ-producing *C. elegans* supplemented with copper or zinc. The percent of worms paralyzed significantly increased in both copper-supplemented mutant worms ($p = 0.0142$) from day 6 (37 ± 4%) to day 9 (92 ± 8%) and zinc-supplemented mutant worms ($p = 0.0187$) from day 6 (67 ± 0%) to day 9 (87 ± 4%), as shown in **Figure 3**. The change in percent paralyzed from day 6 to day 9 was larger for the copper-supplemented group (145% increase) compared to the zinc-supplemented group (31% increase). Also, the percent paralyzed was significantly higher on day 6 for the zinc-supplemented group compared to the copper-supplemented group ($p = 0.0098$), while there was no significant difference in percent of the worms paralyzed between the two groups on day 9 ($p = 0.3483$). Overall, high concentrations of both copper and zinc are positively correlated with increases in the percent of worms paralyzed.

[1]Day 6 was chosen to ensure that the effects of supplementation, which occurred when worms reached adulthood on day 3, will be significant. Day 9 was chosen as the last date of data collection to ensure that dead worms were not mistaken for paralyzed worms.

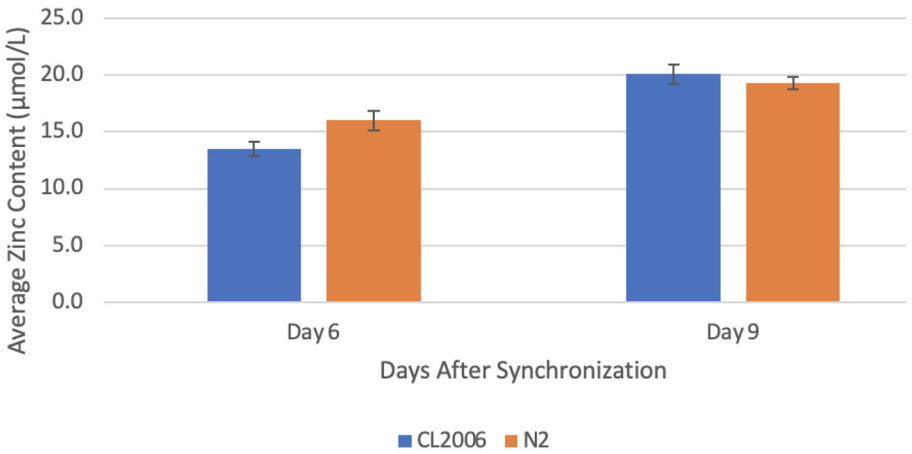

FIGURE 1 | Average zinc content in *C. elegans* supplemented with copper. When amyloid-beta producing *C. elegans* (CL2006) were supplemented with copper, a statistically significant ($p = 0.013$) increase in the average zinc content occurred from day 6 (13.5 ± 0.6 µmol/L) to day 9 (20.1 ± 0.9 µmol/L). Similarly, when non-amyloid-beta producing *C. elegans* (N2) were supplemented with copper, a statistically significant ($p = 0.041$) increase in the average zinc content occurred from day 6 (16.0 ± 0.9 µmol/L) to day 9 (19.3 ± 0.6 µmol/L). Values are mean ± SEM and are representative of 2 experiments where 30 *C. elegans* were analyzed at each time point.

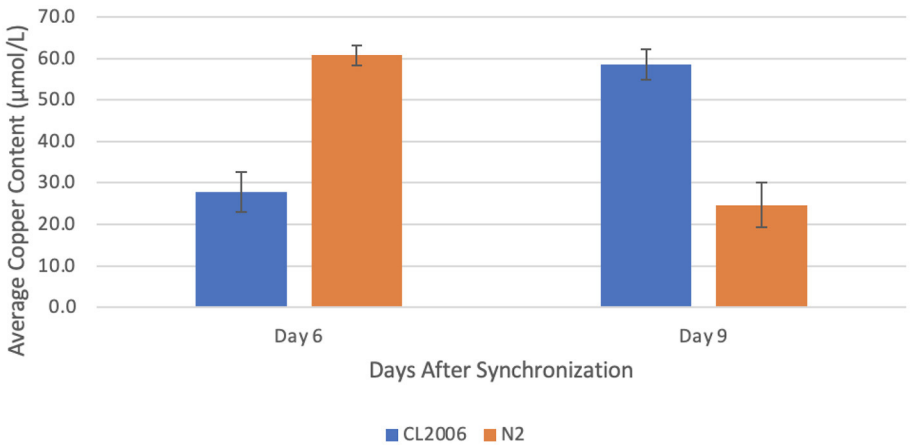

FIGURE 2 | Average copper content in *C. elegans* supplemented with zinc. When amyloid-beta producing *C. elegans* (CL2006) were supplemented with zinc, a statistically significant ($p = 0.022$) increase in the average copper content occurred from day 6 (27.8 ± 4.9 µmol/L) to day 9 (58.6 ± 3.7 µmol/L). In contrast, when non-amyloid-beta producing *C. elegans* (N2) were supplemented with zinc, a statistically significant ($p = 0.012$) decrease in the average copper content occurred from day 6 (60.8 ± 2.4 µmol/L) to day 9 (24.7 ± 5.4 µmol/L). Values are mean ± SEM and are representative of 2 experiments where 30 *C. elegans* were analyzed at each time point.

DISCUSSION

Aging has been found to trigger copper and zinc dyshomeostasis (Myhre et al., 2013; McCord and Aizenman, 2014; Nuttall and Oteiza, 2014; Malavolta et al., 2015). While trace metals copper and zinc are crucial for normal functioning, excess copper, and zinc are highly damaging to proteins. Excess copper and zinc are known to bind with high affinity to Aβ, resulting in visible precipitation into an aggregated form (Bush et al., 1994; Huang et al., 1997, 2000; Atwood et al., 2000; Kumar et al., 2016; Bagheri et al., 2018; Barykin et al., 2018). Therefore, it is of particular interest to determine how changes in the homeostasis of a given trace metal influence the homeostasis of other trace metals and the aggregation state of Aβ.

The present study has found that in Aβ-producing *C. elegans,* imbalances in trace metals copper or zinc trigger further trace metal dyshomeostasis. When supplemented with copper, zinc levels increase significantly and when supplemented with zinc, copper levels increase significantly. Thus, an imbalance in

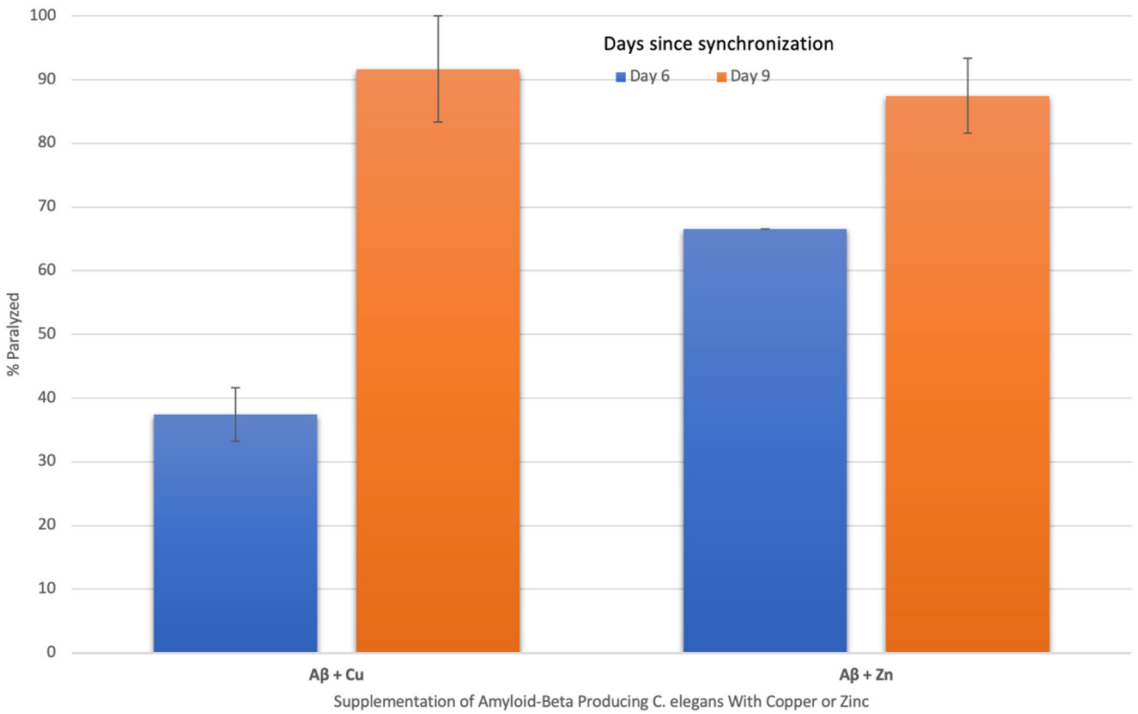

FIGURE 3 | The effect of supplementing amyloid-beta producing *C. elegans* with copper and zinc on percent paralyzed over time. When amyloid-beta producing *C. elegans* were supplemented with copper, a statistically significant ($p = 0.014$) increase in % paralyzed occurred from day 6 (37 ± 4) to day 9 (92 ± 8). When supplemented with zinc, a statistically significant ($p = 0.019$) increase in % paralyzed also occurred from day 6 (67 ± 0) to day 9 (87 ± 4). The change in percent paralyzed is larger for the copper-supplemented group compared to the zinc-supplemented group. Values are mean ± SEM and are representative of 2 experiments where 20 *C. elegans* were analyzed at each time point.

either trace metal causes a cascading effect resulting in further imbalances. This triggering of further trace metal dyshomeostasis might explain why the percent of worms paralyzed, which correlates to Aβ-aggregation levels, significantly increases in both copper and zinc supplemented mutant worms from day 6 to day 9.

However, in wild-type worms, dyshomeostasis of copper or zinc ultimately triggers a return to equilibrium. When copper levels increase through supplementation, zinc levels correspondingly increase from day 6 to day 9. In contrast, when zinc levels increase through supplementation, copper levels decrease from day 6 to day 9. Therefore, it is possible that through a negative feedback mechanism loop, an increase in copper triggers an increase in zinc which ultimately causes a decrease in copper and a return to equilibrium.

Increases in the concentration of zinc or copper, through supplementation, both result in increases in the percent of worms paralyzed, reflecting higher Aβ-aggregation levels, from day 6 to day 9. Given that trace metal levels naturally increase to a degree during the aging process, it is possible that in certain populations more prone to developing amyloidogenic diseases, trace metal levels dramatically increase during aging. Since Aβ avidly binds to trace metals copper and zinc, it is possible that when trace metal levels increase during the aging process, Aβ levels increase in an effort to bind excess copper and zinc (Squitti et al., 2021). The binding of trace metals such as copper and zinc to Aβ is known to trigger an Aβ conformational shape change (Barykin et al., 2018; Kim et al., 2018; De Benedictis et al., 2019), thus transforming Aβ into a configuration more amenable to forming aggregations.

While both copper and zinc dyshomeostasis result in an increase in the percent of worms paralyzed over time, copper might have a stronger effect on the percent of worms paralyzed, reflecting Aβ aggregations, compared to zinc. The zinc supplementation concentration (500 μM $ZnSO_4$) was over three times higher than the copper supplementation concentration (150 μM $CuCl_2$); however, the percent of worms paralyzed on day 6 in the zinc supplemented group was only about 1.8 times higher than the copper supplemented group. The zinc supplementation (Kumar et al., 2016) and copper supplementation (Minniti et al., 2009) were chosen based on previous publications that found considerable changes in amyloid-beta aggregations, but did not measure whether dyshomeostasis of one trace metal triggers dyshomeostasis of other trace metals. Additionally, despite the higher zinc supplementation, there was no significant difference in the percent of worms paralyzed by day 9 when comparing the zinc supplemented group and the copper supplemented group. While

this could simply be due to natural age-related Aβ aggregation development caused by the inserted Aβ gene as the mutant *C. elegans* approach the end of their lifespan, more trials would be needed to better understand if there is a significant difference between the effect of copper vs. zinc on Aβ aggregations. It would be particularly useful to measure the percent of worms paralyzed when supplementing with the same concentration of $CuCl_2$ and $ZnSO_4$ in the future. Future work also includes measuring the levels of copper and zinc in Aβ-producing and non-Aβ-producing *C. elegans* without any supplementation to determine the copper and zinc homeostatic ranges.

Overall, the novelty of this study is the experimental demonstration that dyshomeostasis of trace metals copper or zinc triggers further trace metal dyshomeostasis in Aβ-producing worms, while dyshomeostasis of copper or zinc triggers a return to equilibrium in non-Aβ-producing worms. Future directions include determining how increases in amyloid-beta aggregations might be part of a failed protective homeostatic mechanism to bind excess trace metals copper and zinc. Additional future directions will include elucidating the mediating factors that facilitate Aβ-trace metal binding and testing the effects of trace metal chelators on Aβ levels.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Spatially Covarying Patterns of Gray Matter Volume and Concentration Highlight Distinct Regions in Schizophrenia

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Introduction: Individuals with schizophrenia have consistent gray matter reduction throughout the cortex when compared to healthy individuals. However, the reduction patterns vary based on the quantity (concentration or volume) utilized by study. The objective of this study was to identify commonalities between gray matter concentration and gray matter volume effects in schizophrenia.

Methods: We performed both univariate and multivariate analyses of case/control effects on 145 gray matter images from 66 participants with schizophrenia and 79 healthy controls, and processed to compare the concentration and volume estimates.

Results: Diagnosis effects in the univariate analysis showed similar areas of volume and concentration reductions in the insula, occipitotemporal gyrus, temporopolar area, and fusiform gyrus. In the multivariate analysis, healthy controls had greater gray matter volume and concentration additionally in the superior temporal gyrus, prefrontal cortex, cerebellum, calcarine, and thalamus. In the univariate analyses there was moderate overlap between gray matter concentration and volume across the entire cortex (r = 0.56, p = 0.02). The multivariate analyses revealed only low overlap across most brain patterns, with the largest correlation (r = 0.37) found in the cerebellum and vermis.

Conclusions: Individuals with schizophrenia showed reduced gray matter volume and concentration in previously identified areas of the prefrontal cortex, cerebellum, and thalamus. However, there were only moderate correlations across the cortex when examining the different gray matter quantities. Although these two quantities are related, concentration and volume do not show identical results, and therefore, should not be used interchangeably in the literature.

Keywords: schizophrenia, multivariate analysis, Jacobian scale modulation, gray matter concentration (GMC), gray matter volume (GMV)

INTRODUCTION

Schizophrenia is a severe mental illness characterized by cognitive, behavioral, and emotional dysfunction (American Psychiatric Association, 2013). Numerous structural MRI studies report cortical reductions in individuals with schizophrenia compared to healthy controls in the frontal and temporal lobes, thalamus, and cerebellum (McCarley et al., 1999; Wright et al., 2000; Shenton et al., 2001; Honea et al., 2005; Glahn et al., 2008; Segall et al., 2009; Turner et al., 2012; Gupta et al., 2015). The current understanding is that the reductions are largely clustered in certain regions and not uniform throughout the brain (McCarley et al., 1999; Shenton et al., 2001; Kubicki et al., 2002), though more subtle effects can be found with larger samples (van Erp et al., 2013, 2018). With sufficient sample size (N $\sim$ 10,000) the entire cortex shows reduced thickness in schizophrenia (van Erp et al., 2018). However, in individual studies the results can largely depend on pre-processing protocols, statistical analyses, and sample sizes (Senjem et al., 2005; Silver et al., 2011; Chen et al., 2014; Radua et al., 2014). Study-specific decisions in pre-processing protocols can result in different smoothing kernel sizes, segmentation parameters, and the addition of Jacobian-scaled modulation. All of these decisions can ultimately change the resulting estimates of diagnostic group differences (Eckert et al., 2006; Radua et al., 2014).

Voxel Based Morphometry

Voxel based morphometry (VBM) involves segmentation of structural MRI images and spatial normalization of these images to the same stereotactic space, usually for voxel-wise analysis of gray matter values (Ashburner and Friston, 2000). There are two estimates of gray matter used in VBM studies of gray matter differences in schizophrenia, that we consider for the purposes of this paper: concentration and volume. Concentration can be thought of as the proportion of gray matter to other tissue types within a given voxel, whereas volume is the absolute amount of gray matter in each voxel, as originally described in Ashburner and Friston, 2000. The quantification of gray matter, either concentration or volume, is determined by the addition of Jacobian-scaled modulation (as described below) during the pre-processing step of segmentation. This step of modulation allows for the preservation of gray matter volume by multiplying the images by the relative voxel volumes or the Jacobian determinants of the deformation field, explained in more detail in the previous literature (Ashburner and Friston, 2000; Radua et al., 2014).

Pre-processing Without Modulation

Following spatial normalization, the global differences in brain shape are removed (Eckert et al., 2006; Bora et al., 2011), and the resulting images are interpreted as gray matter concentration (Ashburner and Friston, 2000). These values represent the probability that the voxels in question ascribe as gray matter (Good et al., 2001). Gray matter concentration is not the comparison of relative concentration of gray or white matter structures in the spatially normalized images, but rather the proportion of gray matter assigned to those tissue types; it can also be useful for identifying cortical regions with poor registration (Mechelli et al., 2005).

Pre-processing With Jacobian-Scaled Modulation

Pre-processing involves spatial normalization that contracts or expands brain regions to match a pre-identified template (Ashburner and Friston, 2000). Jacobian-scaled modulation accounts for the amount of contraction needed to move voxels in the native space image to corresponding voxels in the template by multiply of the spatially normalized gray matter by its relative volume before and after spatial normalization: $I_b = I_a \times (V_a/V_b)$ (V_a = volume before normalization, V_b = volume of the template (Jacobian determinants of deformation field), I_a = spatially normalized intensity, and I_b = intensity in signal after modulation) (Mechelli et al., 2005). In other words, the Jacobian determinants are the measures of warping applied to each image to match the template. The modulation step preserves local volumes of gray matter that were detected prior to normalization. For example, if a brain region is enlarged to match the standard template during normalization, then the partial volumes should be proportionally reduced to maintain the original amount of volume (Radua et al., 2014).

The use of Jacobian-scaled modulation is consistently cited in the literature examining degenerative diseases or atrophy, as it can ensure that inter-subject alignment preserves the intergroup differences (Eckert et al., 2006). However, there are some drawbacks to consider when including Jacobian-scaled modulation. Modulation can decrease the sensitivity to detect mesoscopic abnormalities due to the cluster-based statistics, as well as introduce multiplicative noise (Radua et al., 2014). Modulation also results in increased variance across groups when compared to non-modulation (Eckert et al., 2006; Meda et al., 2008; Segall et al., 2009; Radua et al., 2014; Torres et al., 2016). On the other hand, Jacobian-scaled modulation may help to analyze macroscopic regional differences in the absolute volume of gray matter as these differences might be removed during the non-linear registration but are re-introduced with the modulation (Good et al., 2001; Keller et al., 2004; Ranlund et al., 2018). The relationship between regional differences is important for understanding not only diagnostic differences but also potential medication effects or developmental trajectories (Gupta et al., 2015).

Source Based Morphometry

Source-based morphometry (SBM) is a multivariate extension of VBM (Xu et al., 2009). SBM identifies patterns which covary among the participants whereas the VBM approach identifies gray matter differences through a voxelwise comparison. In SBM, each image is decomposed into a linear combination of components through an independent component analysis (ICA) and then normalized by multiplying the weight of each of those components in each participant's image (Xu et al., 2009). Simply, it is a linear model with the sum of component maps and participant loadings making up the input segmentation maps. In that sense there is a direct link between the SBM information and

the input voxel information (either concentration or volume). This technique results in components or patterns of gray matter variation for all subjects included in the analysis, which can be analyzed for differences among the groups in question. The contribution of component for each participant, or the individual loading coefficient, indicates that that pattern of gray matter variation is more strongly weighted for that individual (Xu et al., 2009). SBM can be applied to both concentration and volumetric gray matter images. For the purposes of the current study, we utilized SBM analyses in addition to the univariate VBM analyses.

Study Overview

There are inconsistencies in the current schizophrenia literature on the use of Jacobian modulation in the pre-processing pipeline of MRI images. In addition, a number of studies do not cite either step in the methods section or use concentration and volume interchangeably, thus creating an unclear picture of what the results are depicting (Gur et al., 1999; Kubicki et al., 2002; Brent et al., 2016). Given the steps of both pre-processing pipelines, there is an assumption that the resulting images do not differ significantly and therefore, both classifications of gray matter (concentration or volume) should yield similar regional conclusions. The main difference between the Jacobian-scaled modulation and non-modulation is that modulated images represent the regional differences in the absolute amount, or volume, of gray matter and the unmodulated images represent the regional differences in the concentration of gray matter as it reported per unit volume in native space (Radua et al., 2014).

For the purposes of this study, the modulated images will be referred to as gray matter volume and unmodulated images will be referred to as gray matter concentration. We sought to examine the differences between gray matter concentration and volume within a schizophrenia dataset, and present how modifying the signal intensity of a voxel (to account for the expansion/contraction during the process of standardizing into Montreal Neurological Institute (MNI) coordinate space) can result in differences in gray matter findings.

MATERIALS AND METHODS

Participants

Imaging and behavioral data were from the Center for Biomedical Research Excellence (COBRE) dataset, accessed *via* COINS and Schizconnect [RRID:SCR_010482,[1,2]; (Scott et al., 2011)]. Details of the participant recruitment can be found in Aine et al. (2017). The variables included in analyses were diagnosis of schizophrenia, determined by the Structural Clinical Interview for DSM-4; smoking status, determined from self-report responses on the Fagerstrom Test for Nicotine Dependence (FTND) (Heatherton et al., 1991); the mean translational motion corrections estimated from the resting state scan from the same dataset; intracranial volume (ICV) determined from Freesurfer segmentations (Fischl, 2012); olanzapine equivalents for current medication (Aine et al., 2017); gender; and age. Three participants were removed prior to analyses because their images did not meet quality assurance standards (correlation $r < 0.90$ when compared to the MNI template). Seven participants were removed for missing responses on smoking status ($N = 2$) or for missing resting state images ($N = 5$) because of head motion correction. Motion during functional scans is stable within individuals, negatively correlated to quality of structural scans, and can produce a potential confound on structural MRI measurements (Savalia et al., 2017) and therefore, resting state images were used for head motion correction. Following data curation, sixty-six adults with schizophrenia (54 males; mean age 38.08 ± 14.21 years) and 79 healthy control adults (59 males; mean age 37.66 ± 11.79 years) were included in the present study. See **Table 1** for more participant demographic information.

MRI Acquisition and Preprocessing

High resolution T_1-weighted images were acquired on a Siemens 3T TIM Trio scanner with a five-echo multi-echo MPRAGE (MEMPR) sequence [TE = 1.64, 3.5, 5.36, 7.22, 9.08 ms, TR = 2.53 s, inversion time = 1.2 s, 7° flip angle, number of excitations (NEX) = 1, slice thickness = 1 mm, FOV = 256 mm, resolution = 256 × 256].

We utilized the optimized VBM pre-processing procedure and SPM12 for image pre-processing and statistical analysis (Good et al., 2001). Images were normalized to MNI space with DARTEL and segmented in SPM12, a high-dimensional normalization pipeline. The non-brain tissues were stripped and gray matter, white matter, and cerebral spinal fluid (CSF) were segmented and normalized. We completed the preprocessing pipeline in two parallel steps to create both the gray matter concentration and gray matter volume images. Following segmentation, gray matter volume images were then smoothed to an 8 mm × 8 mm × 8 mm Gaussian kernel. Both VBM and SBM analyses were performed with the gray matter concentration and volume images.

Voxel Based Morphometry Analysis

We performed a univariate VBM analysis on the dataset using SPM12[3]. In two separate general linear models (GLMs) for smoothed gray matter concentration and volume images, we added the covariates of age, gender, smoking status, intracranial volume (ICV), and average head motion. A second linear regression model examined the significant voxelwise

[1]http://coins.trendscenter.org

[2]http://schizconnect.org

[3]https://www.fil.ion.ucl.ac.uk/spm/software/spm12/

TABLE 1 | Participant demographic information.

	SZ (*N* = 65)	HC (*N* = 79)	Total (*N* = 145)	*p*
Males (%)	53 (81.81%)	62 (78.48%)	116 (80%)	0.30
Smokers (%)	23 (34.85%)	14 (17.72%)	37 (25.52%)	0.02*
Age ± SD	38.08 ± 14.21	37.66 ± 11.79	37.85 ± 12.90	0.85
Head motion ± SD	0.40 ± 0.28	0.46 ± 0.37	0.43 ± 0.33	0.33
OLZ ± SD	15.47 ± 11.11	–	–	–

*SD, Standard Deviation; HC, healthy controls; SZ, individuals with schizophrenia; OLZ, total olanzapine equivalent dose; *p < 0.05.*

results for olanzapine equivalents effects in individuals with schizophrenia. Statistical results for group comparisons (healthy controls > schizophrenia) were thresholded at a $q < 0.001$ with family discovery rate (FDR) correction.

Following VBM preprocessing, we utilized a voxelwise correlation on all voxels that contained ≥ 20% gray matter and intersected between the two sets of images (concentration and volume), to identify the correlations between gray matter concentration and volume.

Source-Based Morphometry Analysis

The preprocessed images went through component estimation using the minimum description length algorithm (Rissanen, 1978). For the concentration images, there were 14 distinct gray matter components and for the volume images, there were 12 distinct gray matter components computed by the infomax algorithm (Bell and Sejnowski, 1995). We used the SBM module of the GIFT Toolbox[4] to perform independent component analysis (ICA) decompositions (Xu et al., 2009) on the dataset. ICASSO (Himberg et al., 2004) with 20 ICA runs was used to ensure the stability of components.

A multivariate analysis of covariance (MANCOVA) model using R v3.6.2 (R Core Team, 2016) was completed with SBM components' loading coefficients as dependent variables, diagnosis as a factor, gender and smoking status as dummy-coded covariates, and age, intracranial volume (ICV), and average head motion as covariates in separate models. A secondary linear regression model examined the significant case/control spatial maps for olanzapine equivalents effects in individuals with schizophrenia. We utilized a threshold of $q < 0.05$ with false discovery rate (FDR) correction.

Following the SBM analysis, we performed Pearson's correlations on the ICA concentration spatial patterns (14 total) against the volume spatial patterns (12 total). We also performed a Jaccard Index to determine similarity between the two thresholded ($z \geq |2|$) concentration and volume images using the spatial patterns. Briefly, Jaccard Index is a measurement of similarity between samples that is defined as the size of the intersection divided by the size of the union of those samples (Real and Vargas, 1996).

RESULTS

Voxel Based Morphometry Results

Effect of Diagnosis

We evaluated relative voxelwise concentration and volume differences between diagnostic groups using a FDR correction. In **Figure 1**, the images show an overall pattern of gray matter reduction in individuals with schizophrenia. For the concentration results, the maximal differences were in the left insula ($p < 0.001$), the orbitofrontal cortex/Brodmann Area 11 ($p < 0.001$), and the right primary visual cortex ($p < 0.001$). For the volume images, the maximal differences were located in the temporopolar area ($p < 0.001$) and the left fusiform gyrus ($p = 0.043$). There was no significant effect of olanzapine equivalents in either the concentration or volume images. See **Figure 1** and **Table 2** for more details on group differences.

[4]http://trendscenter.org/software/gift/

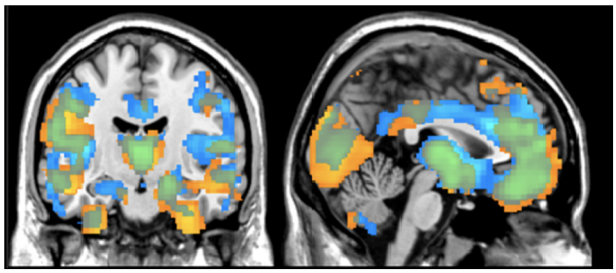

FIGURE 1 | Regional Differences in Gray Matter Results from VBM analysis showing differences between healthy controls and individuals with schizophrenia in terms of gray matter concentration (blue) and gray matter volume (yellow). Results are thresholded at $z > |2|$ ($p < 0.05$, FDR corrected).

TABLE 2 | Peak levels and MNI coordinates for significant voxel-based morphometry results.

Gray matter	Peak T	Peak *x, y, z*	Brain location	# Of voxels	*p**
Concentration	7.35	−36, 18, −2	L Insula	6,193	1.53e-05
	7.19	−4, 44, −20	OFC/BA11	12,098	1.62e-05
	4.97	10, −62, 10	R Calcarine	848	0.0011
Volume	6.85	34, 22, −30	Temporopolar area/ R BA38	3,088	1.32e-04
	5.23	−46, −60, −4	Left fusiform gyrus	389	0.0434

*L, left; R, right; OFC, Orbitofrontal cortex; BA, Brodmann Area; *FDR corrected $p < 0.05$.*

Effect of Quantity

The average voxelwise correlation per individual between the gray matter concentration and volume images was $r = 0.56$, largely seen in voxels containing mostly gray matter. We separately compared the concentration and volume images by diagnosis to examine the pattern differences between diagnostic groups. Images from the healthy controls had an average correlation of $r = 0.51$, $p < 0.05$ (max $r = 0.99$) and images from the schizophrenia group had an average correlation of $r = 0.60$, $p = 0.03$ (max $r = 0.97$). To visualize the distribution of correlation, different levels of correlations were mapped onto corresponding voxels. **Figure 2** shows those voxelwise correlations between concentration and volume in healthy controls (A) and individuals with schizophrenia (B). Negative correlations in both maps made up less than 4% of the total number of voxels and therefore, were not included in the visual mapping. As the correlation threshold was increased from $r = 0.2$ to $r = 0.9$, overlap between the two sets of images dropped to the outer edges of the cortex.

Source-Based Morphometry Results

Effect of Diagnosis

In the concentration images, three spatial patterns showed a significant effect of diagnosis (see **Table 3** and **Figure 3A** for brain locations). The component with the largest effect (HC > SZ) was

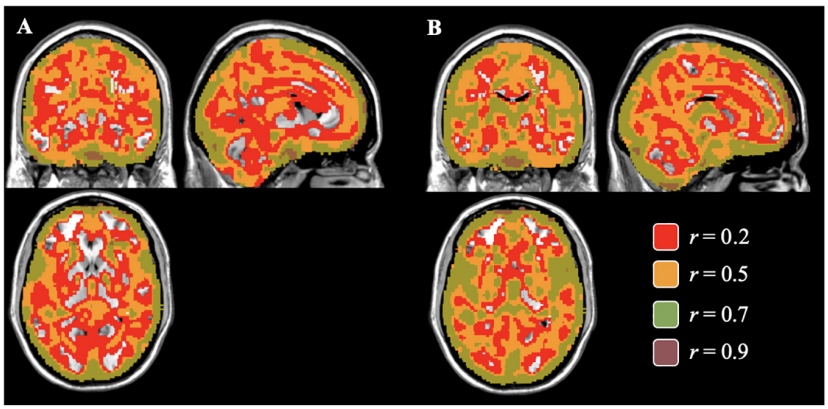

FIGURE 2 | Voxelwise Correlations between Concentration and Volume for Controls **(A)** and Individuals with Schizophrenia **(B)** Colored correlations set at different thresholds between gray matter concentration and volume images of healthy controls **(A)** and individuals with schizophrenia **(B)** in the brain. The areas of overlap between the concentration and volume results were found mostly in areas of gray matter and gradually decreasing in the subcortical regions as the correlation threshold increases.

located in the superior temporal pole (component 2; $F = 49.06$, $p < 0.0001$). None of the three spatial patterns showed a significant effect of olanzapine equivalents.

In the volume images, six spatial patterns showed a significant effect of diagnosis (see **Figure 3B**). The components with the largest effects (all HC > SZ) were located in the right prefrontal cortex ($F = 15.71$, $p = 0.0001$), the thalamus ($F = 11.71$, $p < 0.001$), and bilaterally at the superior temporal gyrus ($F = 6.24$, $p < 0.05$). None of the six spatial patterns showed a significant effect of olanzapine equivalents. Refer to **Table 4** and **Figure 3B** for more peak coordinates for the six spatial patterns of diagnostic differences.

Effect of Quantity

The resulting 168 non-thresholded Pearson's correlations between the two sets of images ranged from low to moderate with a median correlation of $r = 0.01$ (range from $r = -0.37$ to 0.37). The strongest correlations were with component 6 from the concentration images and component 3 and component 6 from the volume images ($r = 0.37$ and -0.37, respectively). Of note, none of these three (3) components were significant for diagnostic differences, with the majority of the brain region identified being in the cerebellum and vermis (see **Figure 4** for more details on correlation results).

TABLE 3 | Brain labels and peak scores for concentration ICA components showing diagnostic differences.

Component	Diagnostic effect	Peak *x, y, z*	Brain location	Peak | Z|
2	HC > SZ	−43, 16, −17	L superior temporal pole	5.21
	HC > SZ	43, 16, −17	R superior temporal pole	4.51
4	HC > SZ	23, −53, 15	Retrosplenial cortex	6.64
14	SZ > HC	1, −16, 56	SMA	4.52

ICA, independent component analysis; L, left; R, right; SMA, supplementary motor area; HC, healthy controls; SZ, individuals with schizophrenia.

Jaccard Index

After thresholding the spatial patterns at $z \geq |2|$, the concentration and volume components showed a median of 2.73% (SD = 4.04%; range: 0.22 to 27.89%) similarity across voxels that passed threshold as a union (see **Figure 5** for more details). These results indicate little overlap between the multivariate results. For example, the largest gray matter difference between diagnostic groups (HC > SZ) was found primarily in the gray matter concentration of the superior temporal pole [component 2; $F(1,123) = 49.39$, $p = 9.42E\text{-}11$]. However, the Jaccard Index shows minimum similarity (range: 0.74–10.67%) with voxels in this region. The largest overlap (27.89%) between the concentration and volume spatial patterns was primarily in the cerebellum (see **Figure 6** for more details).

DISCUSSION

To date, this is the first study to compare the univariate and multivariate results from gray matter concentration and volume images using a dataset of individuals with schizophrenia. After accounting for age, gender, smoking status, ICV, olanzapine equivalents, and average head motion, the gray matter differences between healthy controls and individuals with schizophrenia were largely seen in the frontal lobe, temporal gyrus, and cerebellum; which is consistent with current schizophrenia literature including previous analyses completed with this dataset (McCarley et al., 1999; Wright et al., 2000; Shenton et al., 2001; Honea et al., 2005; Glahn et al., 2008; Segall et al., 2009; Turner et al., 2012; Gupta et al., 2015). The univariate results showed largely overlapping case/control differences for concentration and volume throughout the cortex (**Figure 1**). The lack of complete overlap between the images may highlight poor gray and white matter differentiation that may even expand down into the subcortical regions. This finding underlines the fact that volume VBM reflects warping, which is not captured in concentration VBM. However, the voxel by voxel correlations

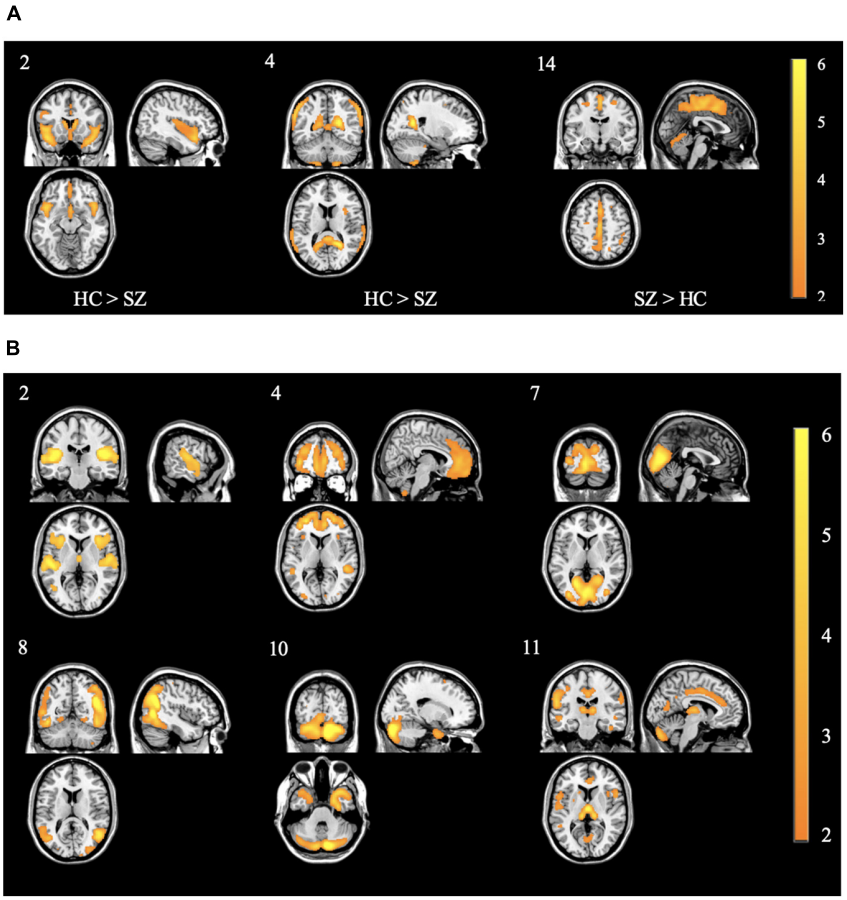

FIGURE 3 | (A) Spatial patterns of the concentration components showing control > case effect from the primary SBM decomposition. Component 2 showed the strongest control > case effect Of Note. All images are thresholded at $|z| > 2$. The color bar indicates the color mapping for the component weights. Component numbers are listed in the top left corner of every image. HC: healthy controls; SZ: individuals with schizophrenia. **(B)** Spatial patterns of the volume components showing control > case effect from the primary SBM decomposition. Of note, component 2 showed similar patterns to concentration component 2 in panel **(A)** Of Note. All images are thresholded at $|z| > 2$. The color bar indicates the color mapping for the component weights. Component numbers are listed in the top left corner of every image. All components showed control > case effects.

between the quantities had a moderate to high correlation of $r = 0.56$ (range: $r = 0.00$ to 0.99; negative $r < 4\%$) indicating sufficient overlap.

Our gray matter concentration VBM and SBM results were largely replications of previous COBRE analysis with small variations in the subcortical regions, brain stem, and insula (Gupta et al., 2015). This lack of exact replication may be explained by the addition of the covariates of ICV, head motion, and smoking status that were not previously explored with this dataset (Gupta et al., 2015). Although there was not exact replication between our concentration spatial patterns and the previous COBRE analyses, the regions identified have been previously recognized as part of larger networks associated with schizophrenia (Wright et al., 2000; Honea et al., 2005; Glahn et al., 2008; Vita et al., 2012). However, the gray matter volume images, primarily from the SBM results, showed marked differences from the previous COBRE analyses and schizophrenia literature.

The multivariate analysis identified very different patterns with diagnostic effects across gray matter concentration and volume. The concentration images identified mostly areas in the superior temporal pole whereas the volume images were mostly in the prefrontal cortex, thalamus, and anterior cingulum. There was moderate overlap in the temporal gyrus and cerebellum (see **Figure 6**). Overall, the greatest difference between diagnostic groups (HC > SZ) was located in the bilateral superior temporal pole (Component 2 in **Table 3**). Our analysis showed only one spatial pattern (Component 14 in **Table 3**) where individuals with schizophrenia had increased gray matter concentration around the motor cortex/sensorimotor area (SMA). This may be explained by noise/movement or alternatively, by an overall reduction of gray matter concentration in the sensorimotor area as individuals age (Douaud et al., 2009). Neither the voxelwise results nor the spatial maps showed an effect of olanzapine equivalents, however, the effect of antipsychotic medication on the cortex is still unclear and in need of further investigation (Roiz-Santiañez et al., 2015).

The addition of Jacobian-scaled modulation can create different gray matter boundaries and therefore, the results may

TABLE 4 | Brain labels and peak scores for volume ICA components showing diagnostic differences.

Component	Diagnostic effect	Peak *x, y, z*	Brain location	Peak | Z|
2	HC > SZ	47, −22, 13	R superior temporal gyrus	7.16
	HC > SZ	−42, −28, 8	L superior temporal gyrus	8.17
4	HC > SZ	26, 56, −2	R prefrontal cortex	5.68
	HC > SZ	−26, 56, −3	L prefrontal cortex	6.00
7	HC > SZ	−18, −66, 8	L lingual gyrus	7.83
8	HC > SZ	43, −59, 28	Angular gyrus	9.48
10	HC > SZ	13, −84, −29	Cerebellum/Crus II	9.71
11	HC > SZ	3, −13, 5	R thalamus	7.39
	HC > SZ	3, 28, 23	R anterior cingulum	4.63
	HC > SZ	−57, −18, 23	Somatosensory cortex	4.66

ICA, independent component analysis; L, left; R, right; HC, healthy controls; SZ, individuals with schizophrenia.

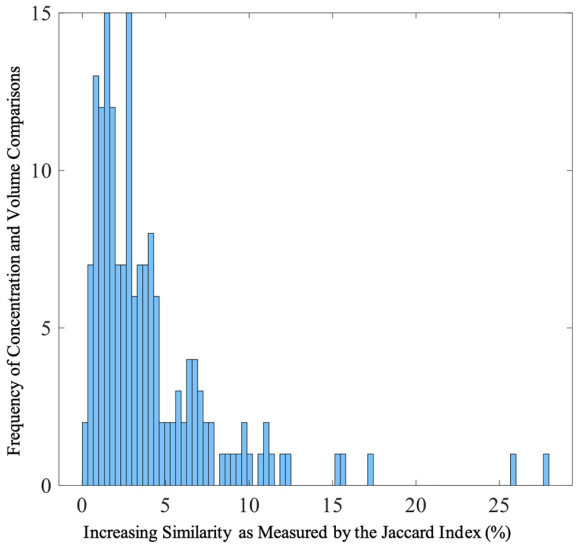

FIGURE 5 | Distribution of Jaccard Indices between Gray Matter Concentration and Volume Spatial Patterns Frequencies of Jaccard Indices showing similarities between gray matter concentration and volume spatial patterns. The range of Jaccard indices was 0.22–27.89% (SD = 4.04%) with a median of 2.73%. The Jaccard indices were calculated from thresholded ($z > |2|$) voxels that passed threshold.

vary depending on how the gray matter is quantified (Good et al., 2001; Radua et al., 2014; Ranlund et al., 2018). Another reason for the differences between gray matter concentration and volume being more pronounced in the multivariate analyses is that, by definition, these analyses are showing patterns of structural changes within the cortex. Therefore, multivariate results indicate divergent neuroanatomical differences. Neural packing varies in density throughout the cortex and can affect these multivariate patterns (Soares and Mann, 1997; Stockmeier and Rajkowska, 2004).

In regards to whole brain automated analysis, another comparison may need to be made between voxel-based morphometry (VBM) and the surface-based analysis of the commonly used FreeSurfer methods[5]. Freesurfer's segmentation and cortical percolation allows semi-automated regional measures, which have been extensively used in schizophrenia

[5]http://surfer.nmr.mgh.harvard.edu/

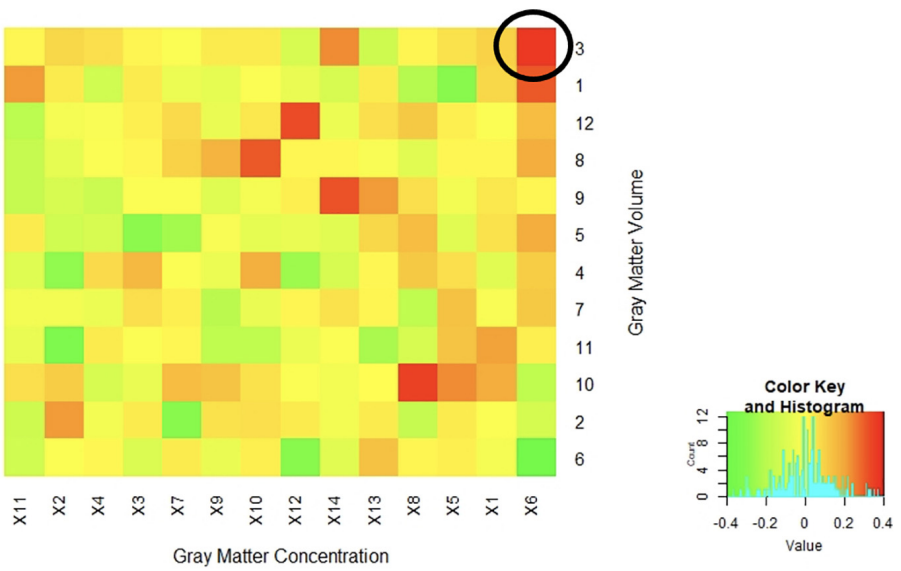

FIGURE 4 | Correlations between Concentration and Volume ICA Components Correlations between the z-weights in the spatial patterns of the gray matter concentration (x axis) and volume (y axis) ICA components from SBM analysis were low to moderate. The highest positive correlation was between spatial patterns in the cerebellum and vermis ($r = 0.37$) circled in the above figure. The majority of the correlations fell between $-0.20 < r < 0.20$ indicating a generally low overlap between the identified regional patterns.

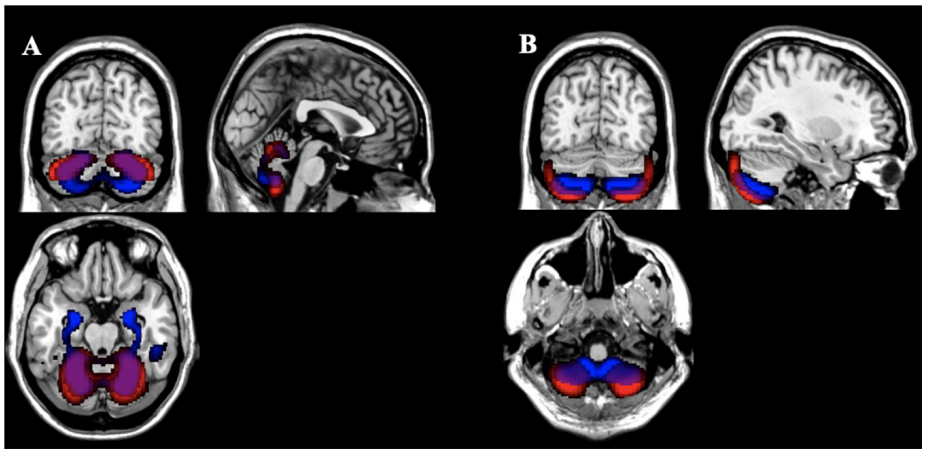

FIGURE 6 | Comparison of Gray Matter Concentration and Volume Spatial Patterns Gray matter concentration (red) and volume (blue) spatial patterns thresholded at $z > |2|$ with the largest Jaccard Index between them [**(A)** 27.89%; **(B)** 25.73%] showing overlap primarily in the cerebellum for those voxels that passed threshold as a union.

imaging studies (van Erp et al., 2013, 2018). While the atlases included with Freesurfer are useful in producing robust, reproducible effects, the regional averages of thickness or volume do not allow for more specific localization of effects; for example, not all the areas we found in the voxel-wise analysis follow sulcal and gyral boundaries like the caudal anterior cingulate region (see **Figure 1** for further details). In the vertex-wise analyses, FreeSurfer is more similar to the voxel-wise analyses in its ability to localize effects, but in contrast to VBM, FreeSurfer provides estimates of localized volume, thickness, and surface area rather than volume and concentration (Fischl et al., 2002; Hutton et al., 2009; Fischl, 2012). There are noted discrepancies between modulated VBM or vertex-wise FS approaches that may be related to surface area, gyrification, and curvature (Palaniyappan and Liddle, 2012; Kong et al., 2015). In addition, the estimate of concentration produces similar results to cortical thickness, but is not reflected in volumetric analyses (Narr et al., 2005).

Future Directions

Given the results, future studies may seek to examine the lack of overlap in the concentration and volume of gray matter. As previously mentioned, there may be a difference in the neural packing (or potential thinning of gray matter) seen commonly in mood disorders that causes lower gray matter concentration but does not proportionally affect the boundaries of gray matter (Soares and Mann, 1997; Stockmeier and Rajkowska, 2004). Although such differences may not be able to be examined directly in VBM or SBM analyses, post-mortem studies may be able to examine if the differences observed are a result of neural discrepancies. Previous post-mortem studies have identified decreases in the volume and total number of neurons in key areas such as the caudate and putamen in individuals with schizophrenia (Kreczmanski et al., 2007). Neuron density may not be specific to diagnosis and should be examined individually as well as with a variety of populations. Of note, in this study the differences in concentration and volume were not limited to diagnostic differences, indicating that other results (i.e., age differences, gender differences) may also be impacted by how the gray matter is quantified.

With the rise of 7T imaging, a more robust picture regarding the localization of gray matter effects to specific cortical layers (e.g., the gray matter effects in superior temporal gyrus (STG) may not arise from the same mechanism as the effects in the hippocampus, in schizophrenia) may be possible as other studies have shown finer scale organization and more specified delineation of cortical boundaries using 7T imaging (Geyer et al., 2011).

Limitations

Limitations of this study include examination of differences between gray matter concentration and volume images only in a schizophrenia population. These findings may not generalize to other population groups that do not have as widespread cortical effects as individuals with schizophrenia, or individuals with more severe atrophy. The addition of Jacobian-scaled modulation may not be sensitive enough to detect mesoscopic abnormalities or cortical thinning so these findings may not be generalizable to other imaging studies (Radua et al., 2014). However, these results are still robust enough to warrant replication and should be examined in different populations.

Conclusion

These results highlight the differences between gray matter concentration and gray matter volume and the importance of distinction between these two quantities in the schizophrenia literature. Furthermore, these results show that there is a relationship between concentration and volume of gray matter but that these two quantities are not interchangeable, especially when examined in multivariate analyses. Therefore, these findings support the recommendation of examining future datasets with both unmodulated and Jacobian-scaled modulated

images. Including both quantities could provide a more complete picture of true cortical differences in the populations of interest.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: http://schizconnect.org/.

AUTHOR CONTRIBUTIONS

JT and KR-M designed the study. JT and VC acquired the data. KR-M and EZ analyzed the data. KR-M wrote the article, which all authors reviewed. VC consulted on the interpretation. All authors approved the final version to be published and can certify that no other individuals not listed as authors have made substantial contributions to the manuscript.

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Female Rats Are Resistant to Cognitive, Motor and Dopaminergic Deficits in the Reserpine-Induced Progressive Model of Parkinson's Disease

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Parkinson's disease (PD) is the second most common neurodegenerative disease. The main symptoms are motor signs such as resting tremor and difficulty in initializing movements. Non-motor alterations, such as cognitive deficits, can precede the motor symptoms. PD is more frequent in men than women. The mechanisms related to this difference are not completely understood. There is evidence that females present distinct characteristics in dopaminergic function compared to males. While the severity of motor impairments is often compared between sexes, little is known about sex differences in the prodromal stage. Most animal models of PD present acute severe motor impairment, which precludes the study of non-motor symptoms. Our research group have proposed an adaptation of the classic reserpine protocol, using low doses in a chronic treatment. This method allows the observation of progressive motor impairment as well as premotor deficits. Here we investigate possible behavioral and neuronal sex differences in the effects of the repeated treatment with a low dose of reserpine in rats. Male and female Wistar rats received 10–15 injections of reserpine (0.1 mg/kg) or vehicle, on alternate days. We followed-up the estrous cycle phases and conducted motor and cognitive assessments (catalepsy, open field, oral movements and object recognition tests). The euthanasia occurred 48 h after the 10th or 15th injections, with the collection of blood for the quantification of sex hormones and brains for tyrosine hydroxylase (TH) immunohistochemistry in the substantia nigra pars compact (SNpc). Reserpine induced progressive catalepsy, involuntary oral movements and cognitive deficits in male rats. The behavioral effects of reserpine were attenuated (motor) or absent (cognitive) in females. Reserpine decreased TH immunoreactivity in males, but not in females. Estrogen levels in females negatively correlated with catalepsy duration. Our findings show that females present a delay and/or a prevention in the reserpine-induced motor alterations in the progressive PD model, compatible with the lower

prevalence of this disease in women. Further, females were protected from the deficit in object recognition at the prodromal stage. The absence of reserpine-induce decrease in TH immunoreactivity suggests that differences in dopaminergic function/plasticity are related to this protection in female sex.

Keywords: parkinsonism, motor impairment, cognitive deficits, sexual dimorphism, tyrosine hydroxylase, estrogen

INTRODUCTION

Parkinson's disease (PD) is the most common motor disorder and the second most common age-related neurodegenerative disease (Tysnes and Storstein, 2017). The disease is mainly characterized by motor features such as resting tremor, bradykinesia, rigidity, and postural instability (Moustafa et al., 2016), which are associated with loss of dopaminergic nigrostriatal neurons (Alexander, 2004).

PD also includes non-motor symptoms, such as autonomic dysfunctions, cognitive abnormalities, psychiatric symptoms (anxiety, depression), and sleep disorders, some of which may precede clinical diagnoses (Brown and Marsden, 1990; Chaudhuri and Schapira, 2009). Cognitive impairments are frequently reported in PD patients, particularly working and spatial memory deterioration (Gillies et al., 2004), attentional deficits (Benedetti et al., 2001), and recognition memory impairment (Wirdefeldt et al., 2011). Moreover, the cognitive impairments may be among the earliest symptoms in PD patients (Chaudhuri and Naidu, 2008).

The incidence of PD is 1.5–2.0 times higher in men than in women (Van Den Eeden et al., 2003; de Lau and Breteler, 2006; Wirdefeldt et al., 2011; Tysnes and Storstein, 2017). Furthermore, there is a delay in the symptoms onset in females, which is possibly related to a neuroprotective effect of estrogen on the nigrostriatal dopaminergic system (Gillies et al., 2004) or to sex differences in the physiology of dopaminergic neurotransmission (Haaxma et al., 2007). Clinical studies have demonstrated that the lowest severity of motor symptoms of PD in women was correlated with a higher period of estrogen exposure along lifetime. In addition, hormonal decrease or deprivation increases the risk of developing PD (Benedetti et al., 2001; Haaxma et al., 2007).

On the other hand, sex differences in prevalence and prognosis of non-motor symptoms in PD remain controversial. Non-motor symptoms can often reduce quality of life even more significantly than motor alterations (Todorova et al., 2014). Previous studies reported that women show higher predisposition to develop fatigue, constipation, pain, and depression, whereas male PD patients present more serious cognitive deficits (Scott et al., 2000; Solimeo, 2008; Martinez-Martin et al., 2012).

The mechanisms underlying sexual differences in PD, particularly regarding non-motor symptoms, have received little attention. In this regard, some authors pointed out that estrogen is neuroprotective. The protective effect of estradiol might be mediated by a suppressive effect on the dopamine transporter (DAT) function (Watson et al., 2006; McArthur et al., 2007). However, whether or not estradiol protects the dopaminergic pathways in experimental PD appears to vary according some experimental conditions (e.g., neurotoxin used, the severity of the lesion, the strain and species of rodent and the treatment regimen) as well as sex (McArthur et al., 2007). For example, in a previous study Bispo et al. (2019) have shown that ovariectomized females have decreased catalepsy compared to intact male and female rats, although showing a loss in TH + cells in SNpc.

The repeated low-dose reserpine protocol has been used as a model of progressive parkinsonism, showing phenomenological (progressive motor alterations) and construct (reduced dopaminergic parameters, increased oxidative stress, and augmented alpha-synuclein expression) similarities with PD (Fernandes et al., 2012; Santos et al., 2013; Leão et al., 2015, 2017, 2021; Ikram and Haleem, 2019). Unlike neurotoxin models, which lead to acute severe motor impairment, the low-dose repeated reserpine protocol allows the observation of progressive motor impairments as well as premotor deficits such as cognitive prejudice, anxiety and nociception alterations (Santos et al., 2013; Leal et al., 2019; Cintra et al., 2021; Dos Santos et al., 2021). This is of relevance when studying PD sex differences because women seem to have a later onset of the motor symptoms, while non-motor prodromic features are poorly understood. Thus, the present study aimed to evaluate sex differences on motor, cognitive, and neurochemical alterations in rats submitted to the low-dose reserpine-induced progressive model of PD.

MATERIALS AND METHODS

Animals

Female (n = 40) and male (n = 20) Wistar rats (7–8 months old) were used in this study. All animals were housed in groups of 4 per cage (30 cm × 42 cm × 16 cm) under controlled airflow, acoustic isolation, and temperature at 22 ± 1°C with a 12 h light/12 h dark cycle (light on at 7:30 a.m.). All animals had free access to water and food. Animals used in this study were handled according to the Brazilian law for the use of animals in research (Law Number 11,794) and all procedures were approved by the local ethics committee (protocol number 3683250517). All efforts were made to minimize animal pain, suffering or discomfort and to reduce the number of animals used.

Drug Treatment and General Procedures

Reserpine (Res, Sigma Chemical Co., United States) was dissolved in glacial acetic acid (1%) and then diluted to the correct concentration with distilled water. The vehicle solution (Veh) consisted of the same amount of acetic acid and water as in the

reserpine solution. Rats were randomly assigned to one of four groups: Female-Veh (n = 20), Male-Veh (n = 10), Female-Res (n = 20), and Male-Res (n = 10). Animals received subcutaneous injections of vehicle (Veh) or 0.1 mg/kg of reserpine (Res) at a volume of 1 mL/kg body weight, every 48 h for 20 or 30 days of treatment, completing 10 or 15 injections, respectively. Animals were euthanized 48 h after the 10th or 15th injections (**Figure 1**).

Across treatment, animals were submitted to the following procedures: (1) estrous cycle monitoring (daily); (2) catalepsy test (before the first injection and daily across treatment); (3) assessment of oral movements before the first injection and 48 h after the 4th, 10th, and 15th injections; (4) evaluation of open field behavior 24 h after the 4th, 10th, and 15th injection; (5) novel object recognition test after the 4th and 10th. If the procedure was held in a treatment day, it was conducted before the injection (from 12:00 a.m. to 14:00 p.m.). Experimental design is shown in **Figure 1**.

In order to evaluate the estrous cycle, vaginal smears were collected by vaginal lavage using saline at room temperature. The vaginal smears collected were placed immediately on the glass slide, colored with methylene blue and analyzed under the optical microscope in 10× magnification, following the methods described by Marcondes et al. (2002).

Behavioral Tests

Catalepsy Test

The catalepsy behavior was assessed by placing the animal's forepaws on a horizontal bar positioned 9 cm above the bench surface while the hind paws rested on the bench. The animals were free to move after they were placed in this position by the experimenter. Catalepsy was defined as an immobile posture (keeping both forepaws on the bar, see **Supplementary Video 1**). The latency to withdraw one or both forepaws from the bar was measured up to a maximum of 180 s (Sanberg et al., 1988). Three trials for animal in each observation day were carried out and the results were analyzed considering the mean value of these trials. In addition to the daily analysis of catalepsy curve, analyzes were performed in phases, considering means of 5 observations: basal (without injection), prodromal phase (1st∼5th), early motor phase (6th∼10th) and late motor phase (11th∼15th).

Vacuous Chewing Test

The animals were individually placed in a wire cage (20 × 20 × 15 cm). Mirrors were positioned under and behind it to allow behavioral quantification when animal faced away from the observer. The number of vacuous chewing movements (mouth openings in the vertical plane not directed toward physical material, see **Supplementary Video 2**) was measured continuously for 10 min.

Open Field Test

The animals were submitted to the open field in order to evaluate spontaneous locomotor activity. The apparatus was a circular arena (67 cm in diameter) surrounded by a 40 cm high wall, made of wood and painted black. Animals were placed in the center of the apparatus for free exploration for 5 min. Distance traveled in the whole arena (in meters) was evaluated. The sessions were recorded by a digital camera and the behavioral parameters were registered by Any-maze® (Stoelting. Co., IL, United States).

Novel Object Recognition Test

The novel object recognition (NOR) task was carried out in the same arena used in the open field test. In each day of test two pairs of objects were used randomly among animals. The objects used were all made of plastic material and filled with cement to ensure that animals could not displace them. The objects differed in height, width, color and shape. A previous experiment demonstrated that rats do not show spontaneous preference for any of these objects. In the training session, the animals were exposed to two copies of an object for 5 min. The same procedure was carried out 1 h later (test session), except that one of the objects was replaced for a new one. Different sets of objects were used on the 20th day (10th injection) to avoid a possible recognition of objects used on the 8th day (4th injections). The apparatus and objects were cleaned with a

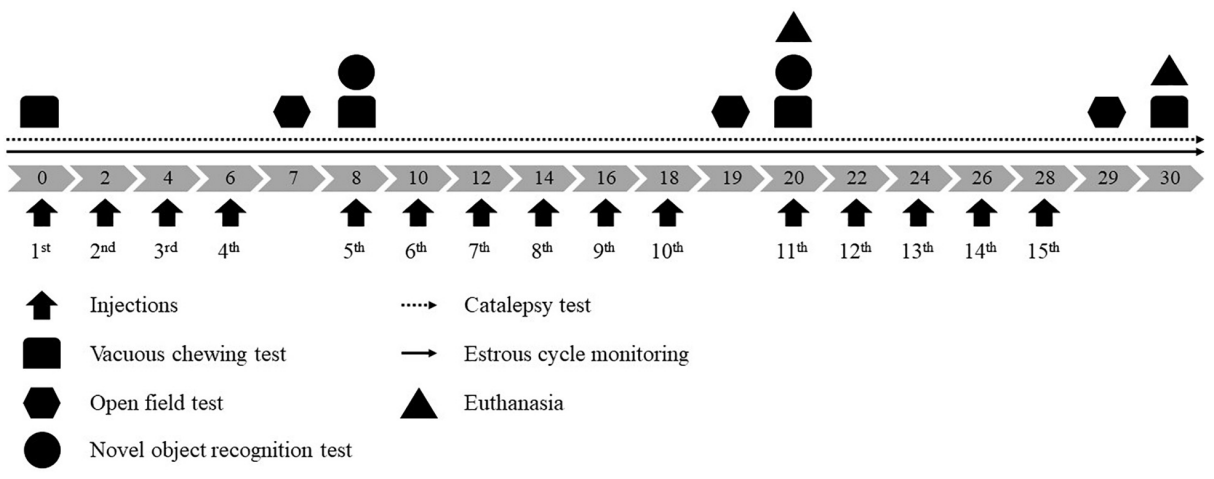

FIGURE 1 | Experimental design. Gray arrows indicate days of treatment and black arrows pointed up indicate vehicle or 0.1 mg/kg reserpine s.c. injections.

5% alcohol solution after each behavioral session. The sessions were recorded by a digital camera and the behavioral parameters were registered by Any-maze®. The time spent exploring each object was measured in both sessions. Exploration behavior was defined by touching with forepaws or nose, sniffing and biting the objects. Only animals that showed total object exploration greater than 3 s during the training were included in the analysis. The discrimination index was calculated as exploration difference (old – new object)/total objects exploration (old + new object).

Tissue Processing and Tyrosine Hydroxylase Immunohistochemistry

Upon completion of behavioral procedures, animals were pre-anesthetized with fentanil (0.5 mg/kg, i.p.) and acepran (1.0 mg/kg, i.m.). After 5 min they were deeply anesthetized with an intraperitoneal injection of 9.0 mg/kg ketamine chloridate and xylazine and transcardially perfused with 200–250 mL phosphate-buffered saline (PBS), pH 7.4, containing 0.2% heparin, followed by 200 mL PBS with 4% paraformaldehyde 0.1 M. The brains were removed from the skull, post fixed in the same fixative solution and stored at 4°C. After 24 h, we transferred brains to a solution containing sucrose 30% 0.1 M PBS, at 4°C. Each brain was fixed in Tissue-Tek® (Sakura, Japan) at −20°C. Then, we serially sliced the brains in the coronal plane into 50 μm thick sections with a cryostat microtome (Leica, Germany) at a temperature of −20°C. Following tissue processing, we performed immunohistochemistry for TH, using a free-floating protocol. Sections were washed four times with PBS (pH 7.4) for 5 min each and consecutively washed with 0.03% H_2O_2 solution for 20 min to reduce endogenous peroxidase activity. For detection of TH, sections were incubated with rabbit anti-tyrosine hydroxylase polyclonal antibody (cat # AB152 Chemicon, United States, 1:5,000) diluted in triton x-100 0.4% and PBS with 2% albumin serum, for 18–24 h at room temperature. Afterward, sections were incubated with goat biotinylated anti-rabbit IgG (Vector Labs, United States, 1:1,000) diluted with triton x-100 0.4% NaCl and PBS for 2 h at room temperature, followed by washing steps, and incubated with avidin-biotin-peroxidase solution (ABC Elite kit, Vector Labs, Burlingame, United States) for another 2 h. The reaction was developed by adding of 3,3-diaminobenzidine (DAB—Sigma-Aldrich, United States) and 0.01% H_2O_2 0.1 M phosphate buffer solution for 3 min. Then, we left sections to dry, dehydrated in a graded alcohol series, cleared in xylene, and coverslipped with Entellan (Merck). All sections were immunostained concomitantly, to minimize possible background differences between samples. Sections were examined under brightfield illumination with an optical microscope (Nikon Eclipse 80i), attached with a motorized stage (MBF Bioscience) and CCD camera (Nikon, DXM-1200) to record images. In order to estimate the number of TH + cells in SNpc, we analyzed 8–12 sections of each animal, equally distributed at the rostral, medium and caudal levels of the structure. All TH + cells of SNpc on each section were registered. The mean of all measures was calculated, and the data were normalized by the respective control.

Estrogen Dosage

During the transcardiac perfusion, the blood was collected with a coagulation activator and separator gel. Posteriorly, it was centrifuged at 5,000 rpm for 10 min at 4°C, the pliers were stored at −80°C until the assays. The determination of estradiol concentrations in plasma was performing using electroimmunoluminescence method (Tiskievicz et al., 2011) in the Hermes Perdini Analysis Laboratory.

Data Analysis

Data normality and homogeneity of variances were tested by the Shapiro-Wilk and Levene's tests, respectively. Behavioral data was grouped in two sets of analysis: animals that received 10 and 15 injections. The average of 2 days of catalepsy behavior, catalepsy phases, oral movements and locomotion in the open field were analyzed by two-way repeated-measure ANOVA followed by Sidak's *post hoc* test. Discrimination indexes in NOR and percentage of occurrence of estrous cycle phases were analyzed by two-way ANOVA followed by Sidak's *post hoc* test. Non-parametric data from TH + quantification and the hormonal levels were analyzed by the Mann-Whitney *U*-test in each group separately. In these cases, Bonferroni correction was carried out and alpha value was set at 0.025. Spearman's rank correlation coefficient was used to investigate a possible association between hormonal levels and motor performance in the catalepsy test. Correlations in vehicle and reserpine groups were investigated separately, considering the different treatment durations. Results were expressed as mean ± SEM and $p < 0.05$ was considered to reflect significant differences.

RESULTS

Catalepsy Test

A two-way repeated-measure ANOVA for all animals until the 10th injection of Veh or Res revealed significant effects of time [$F_{(10,\ 560)} = 61.014$; $p < 0.001$], treatment (Veh and Res) [$F_{(1,\ 56)} = 16.740$; $p < 0.001$], and interactions between time and treatment [$F_{(10,\ 560)} = 16.962$; $p < 0.001$], time and sex (Male and Female) [$F_{(10,\ 560)} = 3.278$; $p = 0.021$] and time, treatment and sex [$F_{(10,\ 560)} = 3.307$; $p = 0.021$]. Another two-way repeated-measure ANOVA (only with animals that received 15 injections of Veh or Res) revealed significant effects of time [$F_{(15,\ 390)} = 15.918$; $p < 0.001$], treatment [$F_{(1,\ 26)} = 22.356$; $p < 0.001$], sex [$F_{(1,\ 26)} = 4.446$; $p = 0.045$] and interactions between time and treatment [$F_{(15,\ 390)} = 10.951$; $p < 0.001$] and sex and treatment [$F_{(1,\ 26)} = 4.605$; $p = 0.041$]. The Sidak's *post hoc* test revealed significant increases in the duration of catalepsy behavior in Male-Res group compared to Male-Veh from the 6th to the 15th measures. Female-Res group exhibited significant increases in the duration of catalepsy compared to Female-Veh in the 6th, 8th, 9th, 10th, and 15th measures. In addition, male-Res showed significant increases in the time on the bar compared to Female-Res on the 8th, 9th, 10th, 12th, and 13th measures (**Figure 2A**).

We also analyzed the catalepsy progression in means of 5 injections in basal (without injections of Veh or Res), prodromal

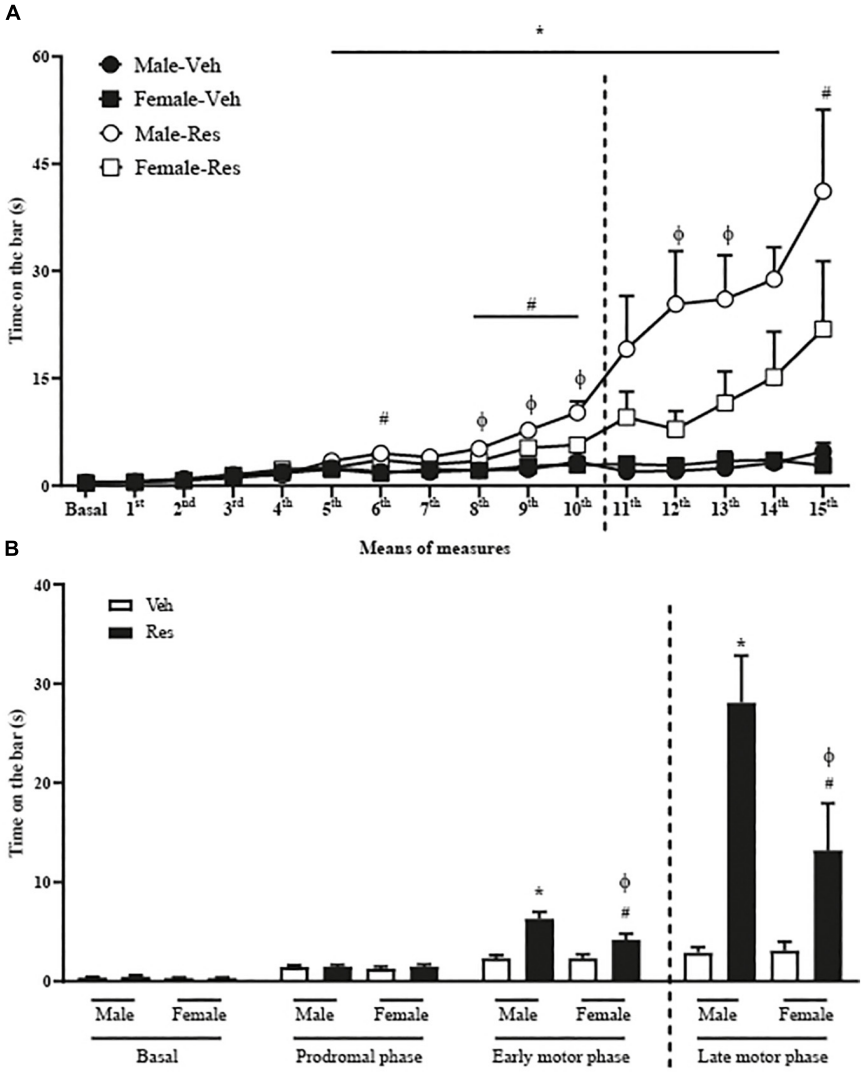

FIGURE 2 | Effects of repeated treatment with 0.1 mg/kg reserpine (Res) or vehicle (Veh) on catalepsy behavior in female and male rats throughout the treatment **(A)** and grouped in phases **(B)** basal (without injection of Res or Veh), prodromal (1∼5 injections), early motor (6∼10 injections), and late motor (10∼15 injections). Dashed lines indicate the euthanasia of half the animals. *$p < 0.05$ comparing Male-Res and Male-Veh, #$p < 0.05$ comparing Female-Res and Female-Veh and ϕ$p < 0.05$ comparing Male-Res and Female-Res (Two-way repeated-measure ANOVA followed by Sidak's *post hoc* test). Values were expressed by mean ± SEM. Individual data are displayed in **Supplementary Tables 1, 2**).

(1st∼5th injections), early motor (6th∼10th injections) and late motor (11th∼15th injections) phases. Two-way repeated-measure ANOVA (considering all animals until the 10th injection of Veh or Res) revealed significant effects of phases (basal, prodromal and early motor phase) [$F_{(2,\ 112)} = 122.805$; $p < 0.001$], treatment (Veh or Res) [$F_{(1,\ 56)} = 17.028$; $p < 0.001$], and interactions between time and treatment [$F_{(2,\ 112)} = 27.013$; $p < 0.001$] and time, treatment and sex [$F_{(2,\ 112)} = 4.139$; $p = 0.039$].

The analysis of the phases of catalepsy by two-way repeated-measure ANOVA (considering only animals that received 15 injections) showed significant effects of phases [$F_{(3,\ 78)} = 31.261$; $p < 0.001$], treatment [$F_{(1,\ 26)} = 22.362$; $p < 0.001$], sex [$F_{(1,\ 26)} = 4.556$; $p = 0.042$], and interactions between phases and treatment [$F_{(3,\ 78)} = 20.288$; $p < 0.001$] and sex and treatment [$F_{(1,\ 26)} = 4.635$; $p = 0.041$]. The Sidak's *post hoc* test revealed that Male-Res show increased time in catalepsy duration compared to Male-Veh and Female-Res on early and late motor phases (**Figure 2B**).

Vacuous Chewing Movements

A two-way repeated-measure ANOVA (considering all animals until the10th injection of Veh or Res) revealed significant effects of the interactions between time (basal, 4th and 10th injections) and treatment (Veh or Res) [$F_{(2,\ 66)} = 3.807$; $p = 0.027$] and treatment and sex [$F_{(1,\ 33)} = 5.107$; $p = 0.031$].

Another two-way repeated-measure ANOVA (considering only animals that received 15 injections) revealed a significant

effect of time [$F_{(3, 42)} = 3.767$; $p = 0.018$] and interaction between time and sex [$F_{(3, 42)} = 4.274$; $p < 0.010$].

The Sidak's *post hoc* test exhibited a significant effect of Res in Male animals in the 3rd and 4th measures (10th and 15th administrations, respectively). There was also a sex difference from the 2nd until the last measure (4th, 10th, and 15th injections). Reserpine-treated females did not show an increase in the vacuous chewing (**Figure 3**).

Open Field Test

A two-way repeated-measure ANOVA (considering all animals that went through OF 48 h after the 4th and 10th injections of Veh or Res) revealed significant effects of time [$F_{(1, 36)} = 47.821$; $p < 0.001$], treatment [$F_{(1, 36)} = 6.378$; $p = 0.016$], sex [$F_{(1, 36)} = 17.190$; $p < 0.001$] and interaction between time and treatment [$F_{(1, 36)} = 9.571$; $p = 0.004$].

Another two-way repeated-measure ANOVA (considering only animals that went through OF 48 h after the 4th, 10th, and 15th injections) exhibited significant effects of time [$F_{(2, 32)} = 64.913$; $p < 0.001$], treatment [$F_{(1, 16)} = 22.256$; $p < 0.001$], sex [$F_{(1, 16)} = 9.475$; $p = 0.007$], and interaction between time and treatment [$F_{(2, 32)} = 34.727$; $p < 0.001$] and time, treatment and sex [$F_{(2, 32)} = 8.018$; $P = 0.002$].

The Sidak's *post hoc* test revealed a significant increase of locomotion of Female-Veh in the three measures. In addition, 48 h after the 10th and 15th injections, the Sidak *post hoc* test showed Res decreased locomotion in both sexes (**Figure 4**).

Novel Object Recognition

The NOR analysis included only animals that obtained a total exploration time greater than 3 s in the training phase. A two-way ANOVA for the discrimination index considering the first and second measures (48 h after the 4th and 10th injections of Veh or Res) exhibited a significant effect of the interaction between treatment and sex in the first (after the 4th injection of Veh or Res) [$F_{(1, 34)} = 4.302$; $p = 0.046$] and second (after the 10th injection of Veh or Res) [$F_{(1, 30)} = 6.947$; $p = 0.013$] measures. The Sidak's *post hoc* test revealed significant effects of Res in Male in both tests. On the other hand, females did not show the same effect, and differed from males treated with Res (**Figure 5**).

Tyrosine Hydroxylase Immunohistochemistry

Mann-Whitney *U*-test did not show differences in TH + levels between vehicle and reserpine for females and males after ten injections of Res ($U = 13.000$; $p = 0.917$, and $U = 9.000$; $p = 0.905$, respectively). On the other hand, fifteen injections of reserpine reduced TH expression of Male-Res when compared to Male-Veh ($U = 0.000$; $p = 0.008$). Female-Res did not show differences in TH expression compared with Female-Veh ($U = 5.000$; $p = 0.286$) (**Figure 6**).

Evaluation of Estrous Cycle and Estradiol Levels

In order to verify the influence of the estrous cycle throughout the treatment, we monitored the estrous cycle phases throughout the protocol. The prevalence of each cycle phase in each day of the protocol was determined for each female group. A two-way ANOVA applied to the percentage of occurrence of the cycle stage between treatments showed only a significant effect of cycle phases [$F_{(3, 240)} = 42.194$; $p < 0.001$], probably because the duration of each phase is physiologically different. There was a

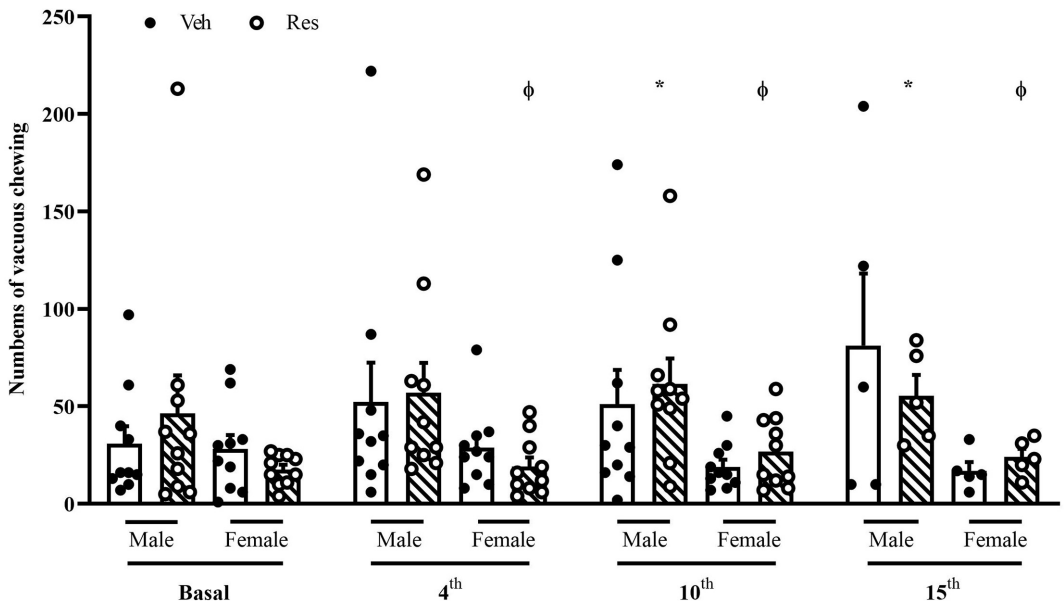

FIGURE 3 | Effects of repeated treatment with 0.1 mg/kg reserpine (Res) or vehicle (Veh) on the vacuous chewing (VCM) behavior in female and male rats. *$p < 0.05$ comparing Res and Veh and ϕ$p < 0.05$ comparing Male-Res and Female-Res (Two-way repeated-measure ANOVA followed by Sidak's *post hoc* test). Values were expressed by mean ± SEM. Black and circles represent individual data of Veh and Res animals, respectively.

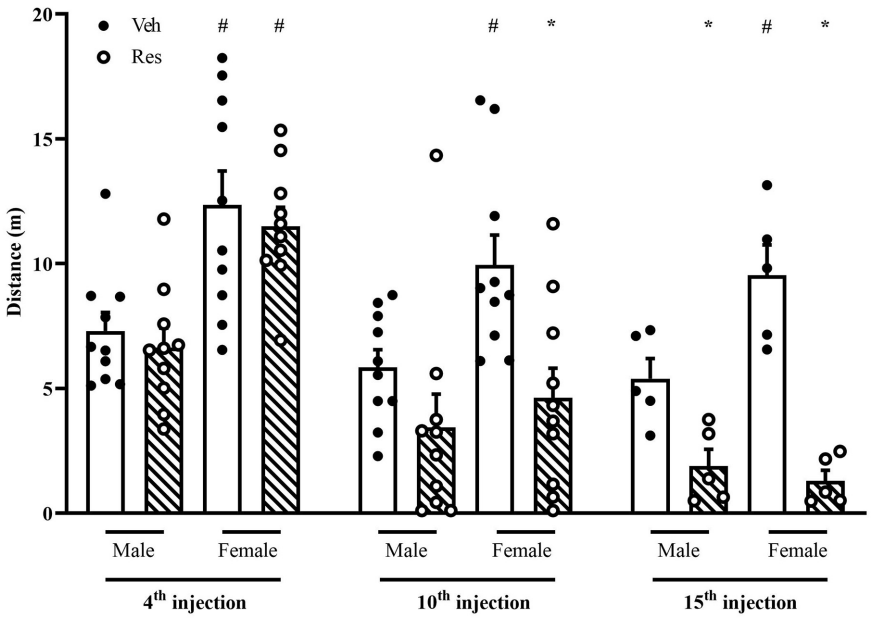

FIGURE 4 | Effects of repeated treatment with 0.1 mg/kg reserpine (Res) or vehicle (Veh) on locomotion in the open field in female and male rats. $^{*}p < 0.05$ comparing RES and Veh. $^{\#}p < 0.05$ comparing males and females with the same treatment (Two-way repeated-measure ANOVA followed by Sidak's *post hoc* test). Values were expressed by mean ± SEM. Black and circles represent individual data of Veh and Res animals, respectively.

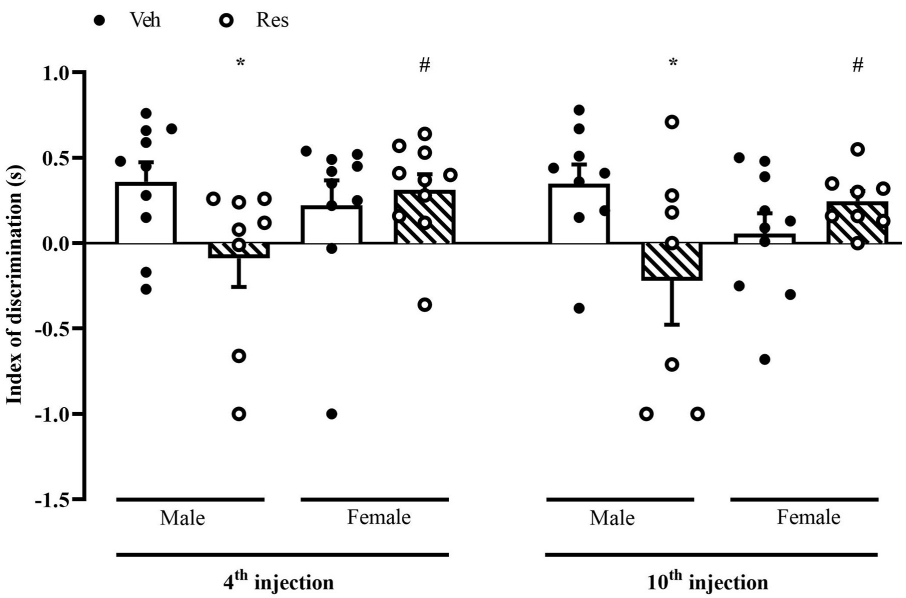

FIGURE 5 | Effects of repeated treatment with of 0.1 mg/kg reserpine (Res) or vehicle (Veh) on object recognition memory in female and male rats. $^{*}p < 0.05$ comparing Res and Veh $^{\#}p < 0.05$ comparing Male-Res and Female-Res (Two-way ANOVA followed by Sidak's *post hoc* test). Values were expressed by mean ± SEM. Black and circles represent individual data of Veh and Res animals, respectively.

lower overall occurrence of the metaoestrous phase (**Table 1** and **Figure 7**). There were no differences regarding treatment.

A non-parametric analysis (Mann-Whitney test) did not show differences in estrogen levels in vehicle and reserpine females after 10 or 15 injections (U = 12,000; p = 0.0916, and U = 16.000; p = 0.296, respectively, **Table 2**). A Spearman's rank-order correlation was run to investigate a possible association between estradiol levels and motor performance (last catalepsy measure) for each group at both treatment lengths. There was a strong negative correlation between estradiol and catalepsy immobility for reserpine-treated group at the 15th injection [$r_{s(6)} = -0.928$; $p = 0.008$], but not at the 10th injection [$r_{s(5)} = -0.500$; $p = 0.391$]

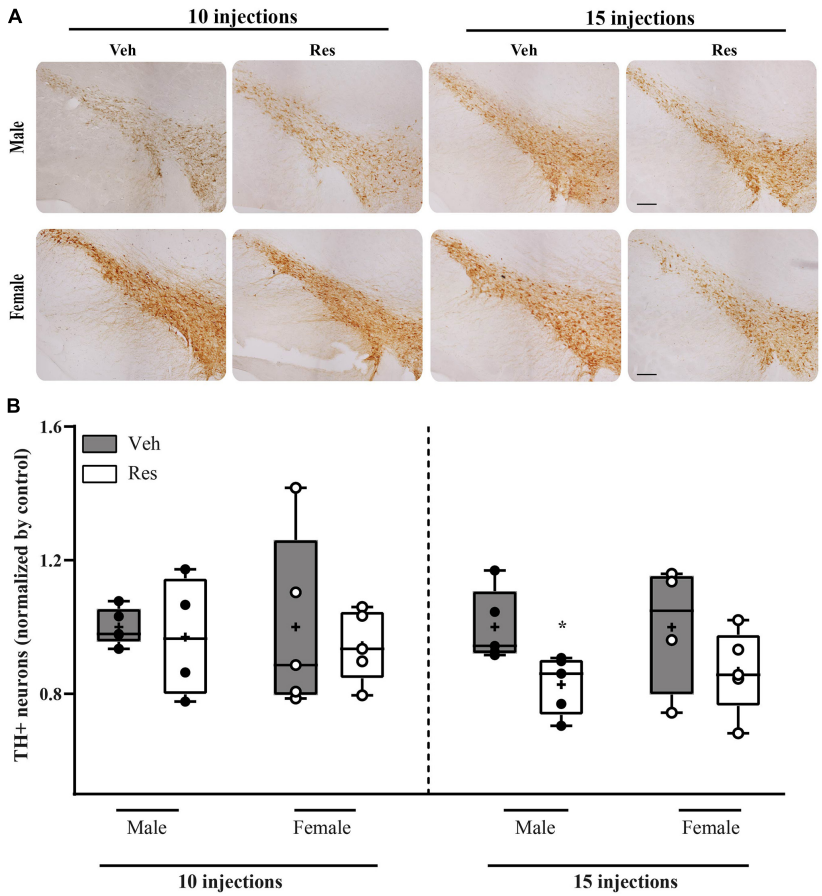

FIGURE 6 | Effects of repeated treatment with 0.1 mg/kg reserpine (Res) or vehicle (Veh) on the number of TH immunoreactive cells in substantia nigra pars compacta in female and male rats after 10 or 15 injections. **(A)** Representative photomicrographs of brain coronal sections of substantia nigra pars compacta. Scale bar: 100 μm. **(B)** Quantification of TH immunoreactive cells. Horizontal lines in the middle of each box indicates the median TH levels, while the top and bottom borders of the box mark the 25th and 75th percentiles, respectively. Black and circles represent individual data of Veh and Res animals, respectively. *$p < 0.05$ comparing Res and Veh (Mann-Whitney *U*-test). Data of individual brain sections are displayed in **Supplementary Table 3**.

or vehicle-treated animals at the 10th [$r_{s(5)} = -0.564$; $p = 0.322$] and 15th [$r_{s(8)} = 0.204$; $p = 0.629$) injections (**Figure 8**).

DISCUSSION

In this work, we investigated sex differences in motor, cognitive and neurochemical alterations at different stages of repeated low-dose reserpine administration, a progressive animal model of PD (Santos et al., 2013; Leão et al., 2015, 2017; Peres et al., 2016; Campelo et al., 2017; Lins et al., 2018; Bispo et al., 2019).

TABLE 1 | Percentage of occurrence of each phase of the estrous cycle during treatment with 0.1 mg/kg reserpine (Res) or vehicle (Veh).

	Veh	Res
Diestrous	27.10 ± 2.12	32.58 ± 2.41
Proestrous	20.64 ± 1.75	20.48 ± 2.22
Estrous	38.87 ± 1.98	30.48 ± 1.68
Metaestrous	13.39 ± 1.60	16.45 ± 1.59

Values are expressed in means of percentage of animals in each phase across treatment ± SEM. (Two-way ANOVA revealed only effect of phases).

Corroborating previous studies, we observed that reserpine treatment induced a progressive motor impairment in male rats (Fernandes et al., 2012; Santos et al., 2013; Leão et al., 2017; Lins et al., 2018; Bispo et al., 2019). Interestingly, male rats showed more susceptibility to these motor alterations than female rats. Indeed, our results showed that males had a greater increase in catalepsy duration and vacuous chewing movements when compared to females. Conversely, the decrement in open field spontaneous activity was similar for both sexes. Further, we observed a sex difference in the short-term memory task: only RES-treated male rats showed a reduction in the discrimination index, which was observed prior to the motor deficits onset, in the prodromal phase, and remained throughout the protocol. Moreover, the low-dose repeated reserpine administration caused a decrease in TH immunoreactivity in SNpc only in males. Decreased estrogen levels correlated with increased immobility in the catalepsy test. Overall, we showed sex

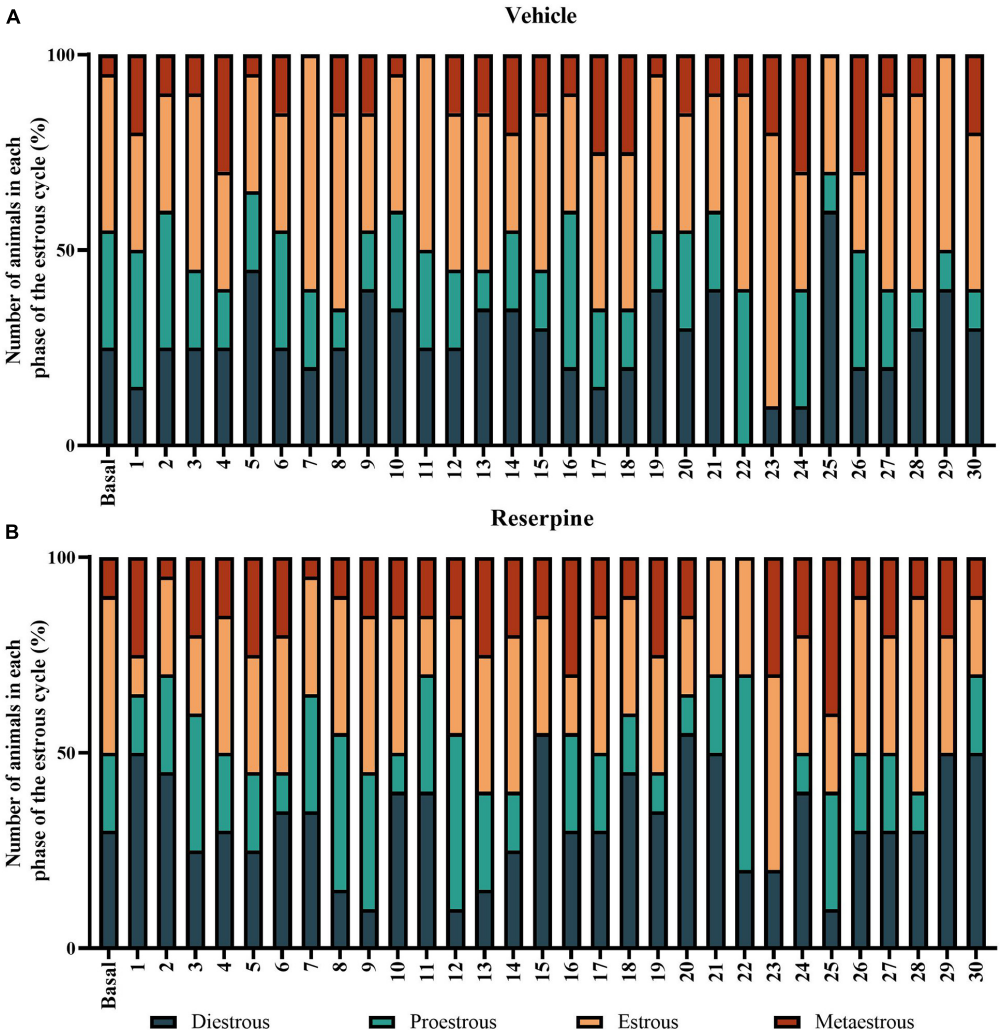

FIGURE 7 | Prevalence of the phases of estrous cycle throughout the 30-day repeated treatment with vehicle **(A)** or 0.1 mg/kg reserpine **(B)** in female rats. Values were expressed by percentage of animals in each phase in each day of the protocol.

differences in the development of repeated reserpine-induced parkinsonism, highlighting alterations that occur in non-motor and motor phases, which are poorly understood in terms of sex differences in PD.

Studies have investigated sex differences in the 6-OHDA (Cass et al., 2005; Field et al., 2006; Zarate et al., 2021) and MPTP (Ookubo et al., 2009; Antzoulatos et al., 2010) animal models of PD. However, these protocols promote acute brain injury and rapid instauration of severe motor impairment, which hinders the evaluation of differences in motor deficits progressiveness and non-motor assessments (Saunders-Pullman et al., 1999; Tsang et al., 2000; Datla et al., 2003). Our results showed that the low-dose reserpine injections induced a progressive motor deficit in both male and female rats. Our findings revealed a greater motor impairment in male rats, corroborating previous findings in which catalepsy onset occurred 48 h after 6–8 reserpine injections (Fernandes et al., 2012; Santos et al., 2013; Campelo et al., 2017; Leão et al., 2017). On the other hand, Res-treated females showed a delayed onset of motor deficits. This outcome corroborates a previous study conducted with male and female mice (Leão et al., 2015).

TABLE 2 | Serum estradiol levels after 10 or 15 injections of repeated treatment with 0.1 mg/kg reserpine (Res) or vehicle (Veh) in female Wistar rats.

	Veh	Res
10 injections	13.16 ± 4.68	9.20 ± 1.05
15 injections	11.94 ± 4.02	7.73 ± 1.84

Data are expressed as mean of estradiol levels (pg/mL) and SEM (Mann-Whitney test).

Besides the lower susceptibility to increased catalepsy, female rats were not susceptible to the oral dyskinesia (evaluated by the vacuous chewing movements) induced by repeated treatment with a low dose of reserpine, which was present in males. Previous studies shown increased VCM response in males with

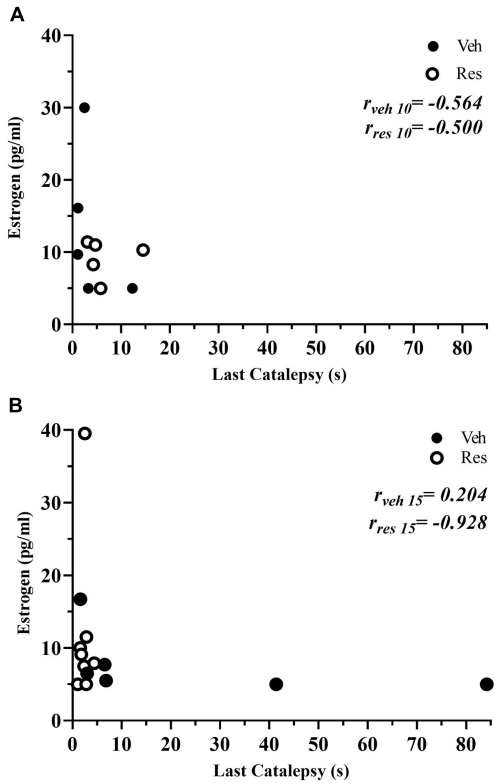

FIGURE 8 | Scatterplots depicting the correlations between the serum estradiol levels and duration of catalepsy of vehicle and reserpine groups that received 10 **(A)** and 15 injections **(B)**.

the same protocol used here (Fernandes et al., 2012; Peres et al., 2016; Leão et al., 2017). However, one prior study reported increased reserpine-induced VCM response in female mice (Silva et al., 2002) after an acute injection of 1.0 mg/kg reserpine. In this regard, these studies proposed several hypotheses to explain the mechanisms underlying VCM, including dopamine hypofunction. Notably, reserpine promotes dopamine depletion by VMAT2 inhibition and, possibly modulation of dopamine receptor expression in several brain areas. In addition, reserpine increases dopamine autooxidation and oxidative catabolism by MAO, leading to oxidative stress (Abílio et al., 2003; Fuentes et al., 2007; Reckziegel et al., 2013). Accordingly, oral dyskinesia is also related to oxidative stress and is attenuated by antioxidant compounds (Abílio et al., 2003). In this way, it is possible that female rats would have a greater protection against oxidative stress, since they showed higher antioxidant enzyme activity than that observed in males (Siani et al., 2017; Tenkorang et al., 2018; Varmazyar et al., 2019).

Concerning spontaneous locomotion in the open field, our results did not show a sex difference for locomotor deficits in the Res-treated rats. Despite both female groups (Veh and Res), 48 h after the 4th injection, presented a higher locomotor activity when compared with respective male groups, as reported before (Cronan et al., 1985; Ribeiro et al., 2010), our findings showed that both sexes significantly reduced locomotion throughout the experiment, probably due to habituation. Nevertheless, Res-treated rats, irrespective of sex, presented a significant decrease in motor activity 48 h after the 10th and 15th injections. In this regard, other studies have described similar effects for males (Santos et al., 2013; Leão et al., 2017; Lins et al., 2018; Bispo et al., 2019) and females (Bispo et al., 2019). Although there is a visually more pronounced reduction in locomotion in reserpine females than in males in the second open field session, statistical analysis did not show a significant interaction between time (repeated sessions) and sex. In addition, a comparison between percent of locomotion reduction between groups revealed only an effect of treatment (data not shown). The absence of a protection against Res-induced decrement in spontaneous locomotion in females may occur because this behavior does not depend purely on motor skills. Instead, it encompasses factors such as motivation to explore, anxiety-like behavior and habituation after repeated exposures, among others (Walsh and Cummins, 1976; Choleris et al., 2001).

Regarding non-motor alterations, which often appear before motor symptoms, cognitive deficits are highly prevalent in PD (Roheger et al., 2018). The progressive parkinsonism induced by low dose-reserpine is a valuable tool that enables the evaluation of non-motor impairment, including emotional and cognitive deficits, during early stages of parkinsonism development in rodents (Santos et al., 2013; Campelo et al., 2017; Leal et al., 2019). In a prior study, Santos et al. (2013) reported impaired short-term object recognition memory before motor deficits in male rats (48 h after the 4th injection of reserpine). In a similar way, our results showed that Res-treated male presented memory deficits 48 h after the 4th and 10th injections. Importantly, Res-treated female rats did not show object recognition memory deficits. The present study evaluated only one memory test. Thus, whether this sex difference is applicable for cognitive function as a whole remains to be investigated. Beneficial effects of estrogen on memory could be related to neuronal survival, neurotransmission modulation, increased synaptogenesis, and protection against oxidative stress damage (Behl et al., 1995; Maggi et al., 2004; Shulman, 2007; Matheus et al., 2016; Hsieh et al., 2017). In PD, clinical studies have reported that men are more susceptible to cognitive deficits, while in women there is a lower prevalence of cognitive impairment (Cereda et al., 2016; Reekes et al., 2020).

In our study, we showed a reduction in TH immunoreactivity in the substantia nigra 48 h after the 15th reserpine injection in males. However, female rats presented a lower susceptibility to the deleterious effects of reserpine on DA neurons, which reinforces the evidence regarding the protective effects of estrogen. A possible explanation concerning estrogen effects in females includes the neuroprotective effects on DA neurons (Hyman et al., 1991) as well as the maintenance of neural activity upregulated by BDNF (Cosgrove et al., 2007). Interestingly, a previous report from our group showed that repeated low-dose reserpine administration promotes a reduction in BDNF in the hippocampus of male mice (Campelo et al., 2017). Accordingly, the reduction of BDNF in the blood of PD patients of both sexes was demonstrated Wang et al. (2016) and a meta-analysis reported a more prominent reduction of BDNF in men (Rahmani et al., 2019).

It should be noted that the reserpine-induced reduction in TH could be related to a feedback inhibition of TH expression in DA neurons. In this respect, besides TH decrement in the nigrostriatal pathway, reserpine also causes increase in oxidative stress parameters (Fernandes et al., 2012; Silva-Martins et al., 2021), increase in alpha-synuclein expression (Leão et al., 2017), alterations in axonal ultrastructure (Leal et al., 2019) and increase of neuroinflammation parameters (unpublished data). However, despite the several indicators that repeated reserpine induces some extent of neuronal damage compatible with Parkinson's disease, it is important to mention that the TH decrement was partially recovered after prolonged withdrawal (in male rats—Santos et al., 2013). Thus, the repeated reserpine model comprises important validations as an animal model of progressive parkinsonism, but the extent of neuronal damage and the presence of neuronal loss are yet to be determined. Regardless, our study shows a protection against behavioral impairment and TH decrement in female subjects, which can be related to estrogen neuroprotective effects such as anti-inflammatory, antioxidant, neurotrophic actions, among others (see Dluzen, 2000 for a review). Alternatively, there are sex differences in the basal physiology of dopaminergic neurotransmission, possibly mediated by estrogen (Watson et al., 2006; Haaxma et al., 2007; McArthur et al., 2007) which can counteract the effect of reserpine on dopamine turnover. Specifically, estradiol may increase dopamine release in the striatum (Becker, 1990; Morissette et al., 2008) and increase the DA turnover by modulating the DA transporter (DAT, McArthur et al., 2007).

Our results are in accordance with the lower incidence of PD in women (Gillies et al., 2014) and reinforces the valuable contribution of this model in evaluating different characteristics associated with PD, especially regarding sex differences in clinical motor and non-motor symptoms of the prodromal phase.

The results related to the estrous cycle evaluation revealed a counter-balanced distribution of the female rats across phases throughout the experimental design (**Figure 7**). As mentioned, several studies have suggested that sex hormones, mainly estrogen, play an essential role in these sex differences in animal models of PD. In this regard, after the 15th injection of the treatment regimen, we observed that reserpine-treated females present the estradiol levels inversely associated with motor performance in the catalepsy task. In other words, female rats with the lowest estradiol levels showed the highest duration of catalepsy. In contrast, for the vehicle group, we did not observe an association between hormonal levels and motor performance along the experimental protocol. These data corroborate previous studies showing that estrogen treatment (Saunders-Pullman et al., 1999; Tsang et al., 2000; Rajsombath et al., 2019) and prolonged natural exposure to endogenous estrogens (Saunders-Pullman et al., 1999) reduced the symptoms and risk of developing PD. In addition, physiological levels of estrogen, as well as exogenous reposition of this hormone protect females against nigrostriatal injury (Yu and Liao, 2000; Datla et al., 2003; Dluzen and Horstink, 2003). In the MPTP model, ovariectomized females mice showed a higher striatal DA depletion compared with gonadal intact females (Dluzen et al., 1996). In female rats, the replacement of estradiol levels reversed this DA depletion in studies using the 6-OHDA model (Murray et al., 2003), although the physiological mechanisms are not yet elucidated.

As mentioned, there is evidence that estrogens act as a neuroprotective factor for dopaminergic neurons in several animal models of PD, such as induced by 6-OHDA (Tamas et al., 2005) and MPTP (Ookubo et al., 2009; Antzoulatos et al., 2010). However, it has been reported that the effects of these neurotoxins may depend upon the specific stage of the estrous cycle the females are in when injected the toxin (Yu and Liao, 2000). For example, an extended loss of DA neurons occurs in phases of low estrogen levels (Yu and Liao, 2000). In this sense, due to the progressive nature of the PD model used here, our results have shown a protection against reserpine-induced alterations during the early stage in females, when there are still regular estrous cycles.

In summary, we report a sexually dimorphic profile of parkinsonism progression induced by reserpine, consistent with the clinical literature and acute animal models of PD using neurotoxins. We also showed a correlation between low levels of estrogen and increased catalepsy, suggesting a protective role of this hormone. However, further studies are required to elucidate the specific role of sex hormones on the behavior and neurochemical mechanisms related to sexual dimorphism in the development of reserpine-induced parkinsonism.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Comissão de Ética no Uso de Animais—UNIFESP.

AUTHOR CONTRIBUTIONS

AL conducted the experiments and data analysis. AL, YM, and RS wrote the manuscript. VB, DC, NG, LL-S, MB, MS, and GM participated in experiments, data collection, and data analysis. YM, JS, and RS designed the study. JS contributed in writing. RS coordinated the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.757714/full#supplementary-material

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PERSPECTIVE
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Theoretical Perspective on an Ideomotor Brain-Computer Interface: Toward a Naturalistic and Non-invasive Brain-Computer Interface Paradigm Based on Action-Effect Representation

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Recent years have been marked by the fulgurant expansion of non-invasive Brain-Computer Interface (BCI) devices and applications in various contexts (medical, industrial etc.). This technology allows agents "to directly act with thoughts," bypassing the peripheral motor system. Interestingly, it is worth noting that typical non-invasive BCI paradigms remain distant from neuroscientific models of human voluntary action. Notably, bidirectional links between action and perception are constantly ignored in BCI experiments. In the current perspective article, we proposed an innovative BCI paradigm that is directly inspired by the ideomotor principle, which postulates that voluntary actions are driven by the anticipated representation of forthcoming perceptual effects. We believe that (1) adapting BCI paradigms could allow simple action-effect bindings and consequently action-effect predictions and (2) using neural underpinnings of those action-effect predictions as features of interest in AI methods, could lead to more accurate and naturalistic BCI-mediated actions.

Keywords: non-invasive brain-computer interface, ideomotor, action-effect prediction, intention decoding, human voluntary action

INTRODUCTION

Our ability to interact with our environment seems limitless. We can learn to use keyboards—in the office using 10 fingers, at home using only our thumb on the touchscreen of our phone. We can learn to play violin, to drive a car, to do heart surgery, and so on. According to the ideomotor principle of action control, such intention-based actions are performed to produce internally pre-specified and desired effects in the environment (see James, 1890; Stock and Stock, 2004). In this respect, any motor action would result in, or rather from, anticipating its perceptual consequences (Greenwald, 1970; Le Bars et al., 2016).

Pushing the frontiers of natural motor actions, recent advances in neuroscience and engineering are enabling human beings to directly act upon the environment with "thoughts" through Brain-Computer Interfaces (BCI). In a typical non-invasive BCI system, the user's neural activity is recorded via brain imaging techniques (e.g., EEG, fNIRS, fMRI), before being decoded with computational and Artificial Intelligence (AI) methods. This last phase allows the translation

of the brain signals into digital commands that are understandable by the connected device(s) (e.g., a computer, a robot etc.).

Besides the obvious benefit of BCIs for patients suffering from motor impairments, the dramatic expansion of this technology (see Douibi et al., 2021) raises important questions regarding the *disembodied* nature of resulting actions (Steinert et al., 2019). Notably, one might wonder whether it is even possible to qualify BCI-mediated actions as real human actions, given the potential reduction of sense of agency or responsibility it might cause in users (see Limerick et al., 2014; Rainey et al., 2020). Moreover, it is worth noting that most of non-invasive BCI paradigms aim to enable "acting with thoughts" but do not necessarily respect fundamental aspects of neuroscientific models of human actions, especially regarding the perceptual counterpart of action, which remains barely considered in BCI-mediated actions (see Wang et al., 2019).

In the current article, we attempted to conciliate the neuroscientific models of human actions with non-invasive BCI methods by proposing an innovative and more naturalistic BCI paradigm that would notably take advantage of the ideomotor principle.

In the first section, we summarized the most important evolutions of the ideomotor theory and its alternatives, which all emphasize the importance of action-effect prediction in human action.

In the second section, we reviewed the actual main kinds of non-invasive BCIs and we discussed their limitations in the light of previous motor action models.

Finally, we proposed a new experimental BCI paradigm directly inspired by the ideomotor principle. We believe that (1) adapting BCI paradigms could allow **simple action-effect bindings** and consequently **action-effect predictions** and (2) using neural underpinnings of **those action-effect predictions** as features of interest in Artificial Intelligence (AI) technics, could lead to more accurate and naturalistic BCI-mediated actions.

THE IDEOMOTOR PRINCIPLE: ORIGINS, EVOLUTIONS AND ALTERNATIVES

Recent decades gave rise to a school of thought, which postulates that, in our brain, perceiving our environment and acting upon it is a unified process. Notably, in line with conditioning theories, the *ideomotor* and *common coding* principles claim that if actions and their effects are repeatedly contingent, actions would end up in being coded in terms of the effects they evoke in the environment (e.g., Prinz, 1997; Hommel et al., 2001; Waszak et al., 2012).

Such sensorimotor contingencies and action-effect mapping could rely on reinforcement learning (e.g., Colzato et al., 2007; Muhle-Karbe and Krebs, 2012) or active inference (e.g., Friston et al., 2016) processes. Indeed, those models notably emphasize on the fact that action-effect bindings are continuously updated through experience.

In the Ideomotor framework, action is thus conceived of as perceptual states. Not present perceptual states, but *future* perceptual states. In other words, a voluntary action is supposed to be primarily driven by the anticipated representation of its expected effect or outcome (see Shin et al., 2010 for a comprehensive review).

At a neural level, different theories have linked this action-effect prediction to distinct patterns of brain activations such as the *cancelation* (see Wolpert et al., 1995), the *preactivation* (see Waszak et al., 2012), or the *sharpening* (see Kok et al., 2012) of activity in sensory and perceptual brain areas, which are usually related to the action (see **Figure 1**).

On the one hand, cancelation of activity in expected sensory areas would result from a *forward model* in the motor system that allows the prediction of action-outcomes. In fact, this neural cancelation related to perceptual expectations would keep the agent (i.e., the individual performing the action) maximally sensitive to unexpected or important outcomes, to optimize learning or planning of new actions (see Wolpert et al., 1995; Stanley et al., 2007). The cancelation account perfectly matches with the well-known phenomenon of *sensory attenuation*, which corresponds to a decrease in neural activity (e.g., Baess et al., 2011; Klaffehn et al., 2019) or perception performances (e.g., Cardoso-Leite et al., 2010), and which is commonly associated to the processing of expected outcomes.

On the other hand, the preactivation theory states that action-effect prediction would result from an early enhancement of activity in perceptual areas, which are typically involved in the sensory processing of the action outcome, even though it has not occurred yet in the environment (see Waszak et al., 2012; Roussel et al., 2013, 2014). Previous experiments have successfully demonstrated the existence of neural activation in the sensory units linked to the action, by using brain imaging techniques such as fMRI (e.g., Kühn and Brass, 2010; Kühn et al., 2011) and EEG (e.g., Hughes and Waszak, 2014). Thus, from this point of view, sensory attenuation would result from a more complex discrimination between the observed action-effect and the pre-activation of the predicted effect that occurs before, relative to when no prediction—or misprediction (see Hsu et al., 2015)—is present.

Halfway between the two last principles of *cancelation* and *preactivation*, the *sharpening* account (Kok et al., 2012) argued that sensory expectation would rather result from a suppression of activity in perceptual units that are not supposed to be involved in the processing of forthcoming effects, while neural activity in expected perceptual areas would remain high in parallel. Further experimental evidence of this model was provided recently via fMRI (Yon et al., 2018).

Overall, these neuroscientific theories related to motor action converge toward the two following notions. First, the perceptual component of action is essential and inseparable from motor control, which is notably observable through the action-effect prediction mechanism. Second, the action-effect prediction necessarily relies on the modulation of activity in perceptual brain areas, as synthesized in **Figure 1**.

However, the precise temporal dynamics of this anticipated action-effect representation is still under debate (see Waszak et al., 2012; Desantis et al., 2014). For instance, some experimental evidence suggested that action-effect representation was activated

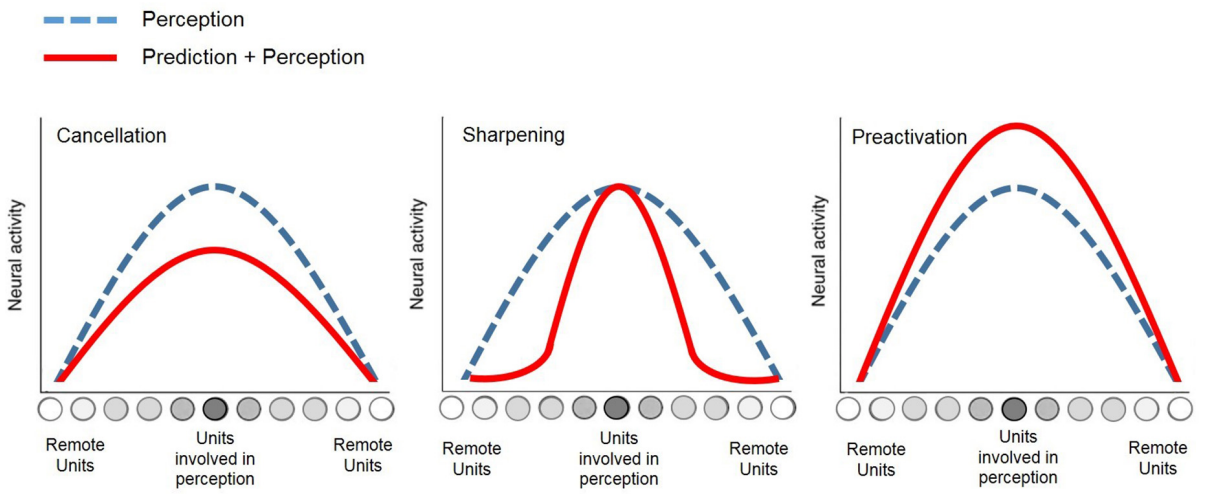

FIGURE 1 | Schematic of neural patterns underlying action-effect prediction relative to perception.

relatively late, during the action planning step (e.g., Ziessler and Nattkemper, 2011), while other studies—in line with the strong version of the Ideomotor principle—demonstrated that it should occur earlier, during the action selection stage, and before the action initiation (e.g., Paelecke and Kunde, 2007; Le Bars et al., 2016; Dignath et al., 2020).

To our knowledge, no existing BCI paradigm is congruent with the strong Ideomotor view, by using the EEG markers related to the perceptual prediction occurring ***before*** the BCI-mediated action.

ACTUAL NON-INVASIVE BRAIN-COMPUTER INTERFACE PARADIGMS

Three main kinds of non-invasive BCI paradigms are commonly described in literature, depending on the agent's task and the patterns of brain activity that are consequently generated (see Kögel et al., 2019). First, *passive BCIs* rely on brain activity that is not voluntarily produced by the user, to monitor his/her neurocognitive or affective state and adequately adapt the environment or issue a warning (e.g., Zander and Krol, 2017). Second, *reactive BCIs* are based on brain activity changes reflecting the agent's voluntarily focused attention on a specific external stimulus (e.g., Guger et al., 2012). Third, *active BCIs* require the user to apply intentionally a particular mental strategy, such as motor imagery (MI) that usually implies imagining a limb movement without actually performing it (MI-BCI; e.g., Salvaris and Haggard, 2014).

Although these three BCI paradigms eventually lead to outputs or new events in the users' environment, only reactive and active BCI paradigms allow the agents to *intentionally* perform those changes, by linking a prior intention to act, to a final effect or consequence (Metzinger, 2013; Steinert et al., 2019). Assuming that any voluntary motor action relies on the implementation of the agent's intention(s) (see Pacherie, 2008), we have thus specifically scrutinized active and reactive BCI-mediated actions through the prism of the motor action models we briefly described in the previous section.

Reactive BCI requires the user to intentionally focus his/her attention on external stimuli to potentially control an external device. In such a reactive paradigm, neural modulations are exogenously generated by specific stimulations provided by the BCI system (e.g., Höhne et al., 2011). Thus, EEG-based BCIs rely on visual or auditory evoked potentials such as the P300 event-related potential (ERP) that occurs 300 ms after an important event (see Jin et al., 2012; Lin et al., 2018), the error-related potential (ErrP) that occurs around 200 ms after a mistake (e.g., Dal Seno et al., 2010; Yousefi et al., 2018) or steady state evoked potentials (SSEP) that are induced by oscillating stimuli (e.g., Chen et al., 2017). Interestingly, some of these reactive settings do rely on neural prediction mechanisms. Notably, P300 and ErrP are, respectively, observed when a rare event (i.e., an event inducing "surprise") or an erroneous effect (i.e., the actual effect was mispredicted) is presented. For instance, reinforcement learning based BCIs, which use neural error responses (e.g., ErrP signals) as reward feedbacks on the agent's action, offer the potentiality to get autonomous paradigms that would dynamically adapt in case of erroneous classifications (e.g., Marsh et al., 2015; Wirth et al., 2019).

However, in such reactive BCIs, the used brain potentials do not constitute *direct* but rather *retrospective* markers of neural prediction, since they occur *after* the event or the action execution, as the result of the comparison process occurring between the internal model of forthcoming events (prediction) and the actual sensory effect/event (see Wolpert et al., 1995; Hsu et al., 2015).

Counter to reactive BCI, active BCI is *self-paced* and allows the user to endogenously/voluntarily control his/her brain activity—and consequently the external connected device (e.g., robot or computer)—at any time, without being tied to a stimulus (see

Rao, 2013). In particular, active BCI mainly relies on motor imagery (MI), which can correspond to a visual or a kinesthetic representation of the motor action (see Neuper et al., 2005). Indeed, MI produces neural activity that is spatiotemporally similar to the activity generated during the actual movement even though it is smaller in amplitude (see Pfurtscheller and Neuper, 1997; Miller et al., 2010). Specifically, MI is linked to recognizable EEG brainwave patterns (see Wierzgała et al., 2018) such as a decrease in the frequency bands μ (8–12 Hz) and β (18–30 Hz) corresponding to Sensorimotor Rhythms (SMR). Notably, SMR decrease occurs in the brain hemisphere that is contralateral to the limb "imagined" movement. One might presume that the use of brain mechanisms that are at play in motor execution should be sufficient to ensure a certain theoretical proximity between MI-BCI mediated actions and natural motor actions. However, the representation of perceptual action goal/outcome remains barely considered in such paradigms, and only a few researchers have investigated the importance of implementing action feedbacks to BCIs (e.g., Quick et al., 2020). From a broader perspective, the mental act that is executed through the MI-BCI is frequently disconnected from the final purpose or the proprioceptive effect of that action (see Beursken, 2013; Jeunet et al., 2016). This could notably explain the high illiteracy rate—corresponding to the inability to control the BCI system—that is commonly observed in participants (see Lee et al., 2019).

Thus, on the one hand, many reactive BCIs are based on *retrospective* markers of sensory prediction occurring *after* a particular event (e.g., P300 or ErrP). On the other hand, most of active BCIs rely on neural activity associated to sensorimotor commands, without considering the importance of the representation of that motor action in terms of its perceptual consequences.

TOWARD AN IDEOMOTOR BRAIN-COMPUTER INTERFACE

Preliminary Experimental Cues

Numerous experiments have highlighted the importance of adding simple sensory and proprioceptive "feedbacks" following the action performed via the BCI, to increase systems' accuracy and control (e.g., Omar et al., 2010; Suminski et al., 2010; Ramos-Murguialday et al., 2012; Tidoni et al., 2014), or improve users' experience (e.g., Wang et al., 2019). Such findings fit the *ideomotor view* stating that voluntary action is primarily performed to produce some anticipated or desired effects in the environment (e.g., Le Bars et al., 2019). From this perspective, one might also argue that these sensory feedbacks allow the agents to make perceptual representations of the BCI-mediated action, enhancing the system's ease of use. In line with this concept, kinesthetic BCIs—where participants have to make an "embodied" representation of the motor action associated with its sensations—have been found to be more efficient than MI-BCI based on external representations of the action (see Neuper et al., 2005).

Importantly, Aflalo et al. (2015) have tested an invasive active BCI based on neural signals related to *prior intentions* occurring *before* their translation into motor commands. They succeeded in identifying the general imagined goal (e.g., picking the glass on the table) from posterior parietal cortex neurons. This interesting paradigm demonstrates that a BCI relying on the neural activity occurring even before motor activations is possible.

Globally, the studies described above support the idea that a BCI based on a perceptual representation of action, earlier than the effective motor command/imagery (active BCI) or the stimulus onset (reactive BCI), could constitute an interesting way to make BCI-mediated action more naturalistic and accurate.

Decoding Perceptual Intentions

An essential requirement of reactive and active BCIs is to successfully decode the agent's intention. To this end, reactive BCIs use neural markers relative to attentional processes (SSEP, P300 etc.) while active BCIs systematically rely on motor intentions assessed via modulations in SMR (see Salvaris and Haggard, 2014). However, what if the agent's intention would also generate identifiable perceptual representations of desired effects in corresponding brain areas (e.g., specific activations in occipital lobes in case of visual representation), as stated by the ideomotor principle? Interestingly, besides the ideomotor field, perceptual activations linked to human intentions, and related to the goal, have already been observed in fMRI studies, notably in the occipital cortex (see Gilbert and Fung, 2018). Neely et al. (2018) also showed that modulations of neural activity in primary visual cortex could be *intentional* (i.e., goal-directed) in rodents.

Then, the ability to decode intentions without using pure motoric activations but rather perceptual representations of desired effects that would be less sophisticated than the complete goal (e.g., Aflalo et al., 2015), appears feasible. Moreover, we believe that such a paradigm would be more appropriate given the disembodied nature of BCI-mediated actions where the peripheral motor system is not supposed to be involved.

Proposal of a Novel Brain-Computer Interface Paradigm Based on a Century-Old Concept

Similarly to ideomotor experiments, an ideomotor BCI paradigm would necessarily require a first acquisition stage, aiming to link each specific action to specific sensory effects. For instance, performing the action A1 would result in the effect E1, while performing the action A2 would generate the effect E2 (see Shin et al., 2010). However, given the low spatial resolution and Signal/Noise Ratio of EEG method, not every sensory effect representation would be appropriate for EEG tracking. Then, properties of sensory feedbacks must be carefully selected to allow the identification of their anticipated representation at a cortical level. Previous experiments have shown that certain visual effects, such as flickering stimuli (see Dignath et al., 2020) or houses and faces (see Hughes and Waszak, 2014), were linked to discernible neural modulations in the occipital and temporal cortex, before their onset (i.e., before the start of the visual stimulation). Moreover, the expectation of auditory effects (i.e., 400-, 600-, and 800-Hz sinusoidal wave tones of 200-ms duration)—normally associated to certain actions—has also

been shown to generate an increase of activity in the auditory cortex, in the absence of actual auditory stimulation (see Kühn and Brass, 2010). Using equivalent stimuli (i.e., visual patterns or auditory tones) as action-effects during the acquisition phase (see **Figure 2A**) might allow the use of *anticipated* neural modulations in corresponding perceptual areas as proxies of *perceptual intentions*.

Interestingly, during this acquisition step, the agent could either perform real motor actions (e.g., pressing a key with a finger) or BCI-mediated actions (e.g., focusing his visual attention on a flickering target or imagining a limb movement) in order to generate the specific effects. We believe that both action types would lead to action-effect binding if a prior intention to act exists (see Caspar et al., 2021). However, intentional binding between actions and effects will certainly be higher in natural motor actions, notably because accuracy and latency are not optimal in BCI systems (see Limerick et al., 2014). For this reason, we suggest to first test the potentiality of an ideomotor BCI with a motoric acquisition phase, as shown in **Figure 2A**. Then, it would be fascinating to analyze whether simple sensory feedbacks of BCI-mediated actions could also lead to neural modulations in corresponding perceptual areas, before or during the "mental

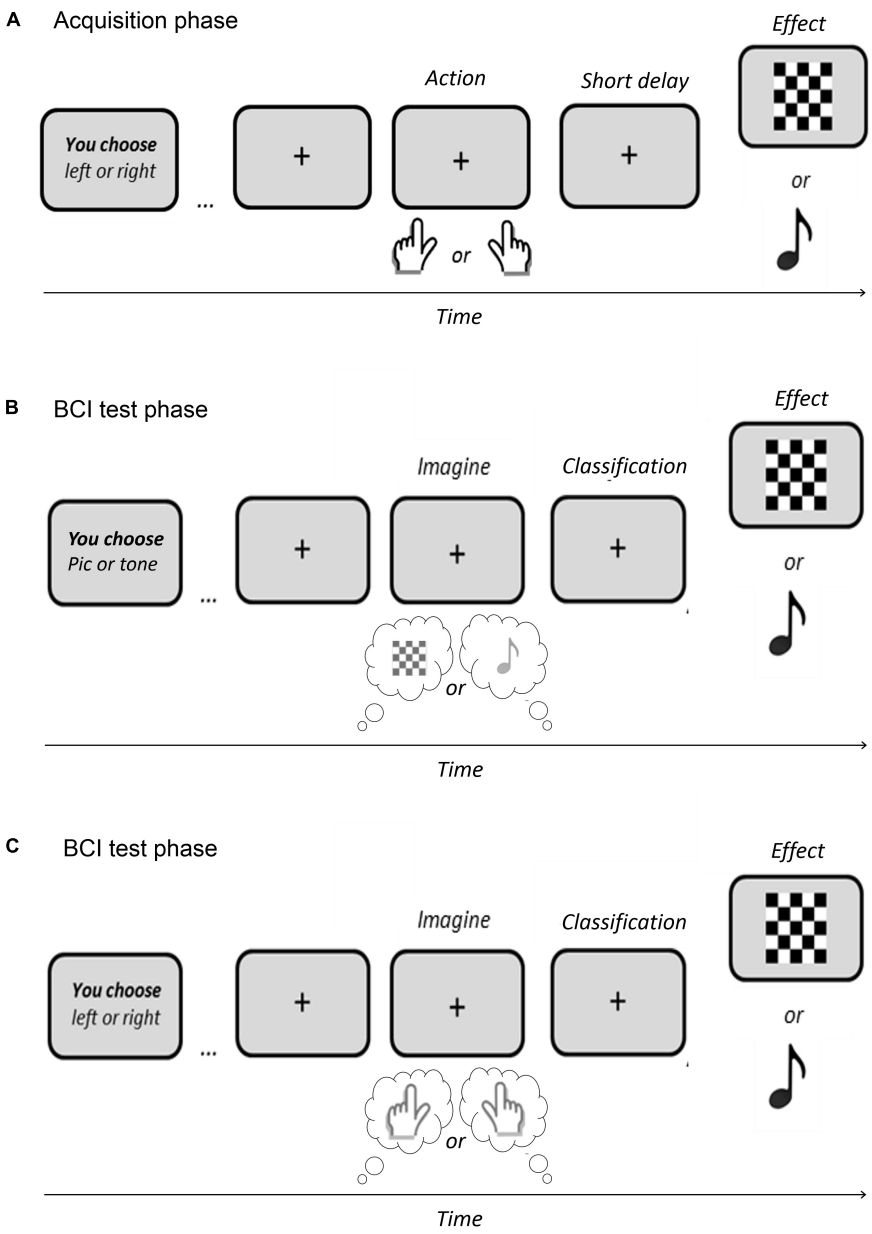

FIGURE 2 | Example of an Ideomotor BCI paradigm. **(A)** Acquisition phase aiming to link simple actions with specific effects (e.g., right key press → visual effect vs. left key press → auditory tone). **(B)** Test phase of a BCI based on the user's active representation of the effect. **(C)** Test phase of a BCI based on the user's active representation of the action.

act" (e.g., imagining a limb movement or focusing on a specific flickering target).

Assuming that the mapping between actions and effects would be effective at the end of the acquisition phase, further similar trials would also serve to train and calibrate AI models aiming to decode the "perceptual intention" that should be identifiable *before* action execution (e.g., Waszak et al., 2012; Dignath et al., 2020).

Then, the ability to accurately decode perceptual intentions could lead to two different BCI applications and tasks:

First, as an independent ideomotor BCI where the agent would be instructed to focus on the intended sensory effect, that is, on the perceptual outcome of the action, or more generally on the global action that led to this outcome, as represented in **Figures 2B,C**, respectively. In the first case, the user's task (i.e., thinking about the action outcome) should be easier than kinesthetic paradigms that require an embodied representation of the action. More importantly, in both cases, the neural markers used for AI models will be spatially and temporally different from the ones that are currently used in existing BCI paradigms: they should correspond to modulations of activity in the perceptual brain areas involved during action-effect perception, and should be activated *before* the occurrence of the actual outcome or action.

Second, Ideomotor BCI could serve as a hybrid BCI where the user would be instructed to perform another BCI task (MI or Attentional focus) while the decoded perceptual intention would be used in conjunction with other typical neural makers (e.g., SMR or SSVEP), to make the global BCI faster and more accurate. In this situation, the user's main task will correspond to usual reactive or active BCI paradigms.

In both cases, we believe that the adaptation of BCI paradigms in order to elicit perceptual representations of BCI-mediated actions should improve their technical performance and users' experience.

DISCUSSION

In this article, we proposed an experimental framework, in line with the ideomotor principle, to test a new type of non-invasive BCI that would allow to perform more naturalistic BCI-mediated actions.

This perspective depends entirely on the ability to decode efficiently the users' *perceptual intentions*, by using neural modulations related to action-effect predictions occurring *before* the action (e.g., Kühn et al., 2010; Waszak et al., 2012; Dignath et al., 2020). This point is not trivial for at least two reasons. First, due to the lack of a unified theory regarding the neural patterns underlying action-effect prediction, which could for instance correspond to a *cancelation* (Wolpert et al., 1995), a *preactivation* (Waszak et al., 2012), or a *sharpening* (Yon et al., 2018) of activity in the perceptual units concerned. Second, because the standard method for brain activity recording in non-invasive BCIs, namely EEG, has a low spatial resolution, which prevents from a precise discrimination between different sources of activations in perceptual units. Thus, further studies must harness adequate paradigms to allow perceptual intentions modeling, notably by using sensory feedbacks that would elicit discriminable EEG patterns during action-effect prediction and that would also be discriminable from brain activations resulting from the BCI task itself.

Theoretically, an independent ideomotor BCI would then constitute a novel BCI paradigm located in-between active, reactive and passive paradigms, being endogenously controlled as active paradigms, i.e., the agent would have to think about the perceptual feedback of the BCI-mediated action, but relying on sensory stimuli, i.e., the action-effects, as reactive paradigms. One might also propose to use this ideomotor paradigm in combination with other BCI methods (e.g., MI-BCI). Given the automatic nature of the action-effect prediction process (see Kunde, 2004; Le Bars et al., 2016), the agent would not be instructed to make a conscious representation of the intended feedback, even though EEG markers of action-effect prediction would be used as additional features to improve the BCI accuracy. In this case, the hybrid ideomotor BCI would be closer from passive BCI paradigms but would still rely on sensory stimuli, as represented in **Figure 3**.

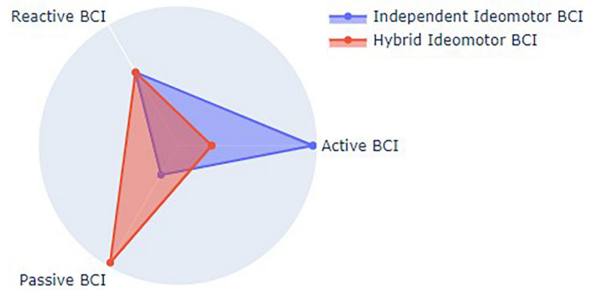

FIGURE 3 | Theoretical positioning of Ideomotor BCI types relative to existing BCI paradigms.

In conclusion, an ideomotor BCI would allow to get closer from natural action by considering the perceptual facet of human action through action-effect prediction. This should improve both users' experience and systems accuracy. Importantly, an effective BCI paradigm—that would be based on perceptual representation of action would definitely endorse and concomitantly renew the old-fashioned ideomotor theory.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SL and FW developed the idea and the research proposal which were described in the current article. SL wrote the manuscript with supervision from SC and FW. All authors contributed to the article and approved the submitted version.

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The Effect of Noisy Galvanic Vestibular Stimulation on Learning of Functional Mobility and Manual Control Nulling Sensorimotor Tasks

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Galvanic vestibular stimulation (GVS) is a non-invasive method of electrically stimulating the vestibular system. We investigated whether the application of GVS can alter the learning of new functional mobility and manual control tasks and whether learning can be retained following GVS application. In a between-subjects experiment design, 36 healthy subjects performed repeated trials, capturing the learning of either (a) a functional mobility task, navigating an obstacle course on a compliant surface with degraded visual cues or (b) a manual control task, using a joystick to null self-roll tilt against a pseudo-random disturbance while seated in the dark. In the "learning" phase of trials, bilateral, bipolar GVS was applied continuously. The GVS waveform also differed between subjects in each task group: (1) white noisy galvanic vestibular stimulation (nGVS) at 0.3 mA (2) high-level random GVS at 0.7 mA (selected from pilot testing as destabilizing, but not painful), or (3) with the absence of stimulation (i.e., sham). Following the "learning" trials, all subjects were blindly transitioned to sham GVS, upon which they immediately completed another series of trials to assess any aftereffects. In the functional mobility task, we found nGVS significantly improved task learning (p = 0.03, mean learning metric 171% more than the sham group). Further, improvements in learning the functional mobility task with nGVS were retained, even once the GVS application was stopped. The benefits in learning with nGVS were not observed in the manual control task. High level GVS tended to inhibit learning in both tasks, but not significantly so. Even once the high-level stimulation was stopped, the impaired performance remained. Improvements in learning with nGVS may be due to increased information throughput resulting from stochastic resonance. The benefit of nGVS for functional mobility, but not manual control nulling, may be due to the multisensory (e.g., visual and proprioceptive), strategic, motor coordination, or spatial awareness aspects of the former task. Learning improvements with nGVS have the potential to benefit individuals who perform functional mobility tasks, such as astronauts, firefighters, high performance athletes, and soldiers.

Keywords: stochastic resonance, white noise, retention, locomotion, orientation perception

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INTRODUCTION

Galvanic vestibular stimulation (GVS) is a non-invasive tool by which electrical stimulation can be applied to the vestibular system through transcutaneous current applied through electrodes placed at the mastoid processes (behind the ears) (Utz et al., 2010). Previous studies have suggested GVS to be a potential tool for enhancing vestibular performance (Mulavara et al., 2011) and shown improvements in perception of small, passive self-motions (Galvan-Garza et al., 2018). Performance benefits following GVS treatment are often thought to occur due to stochastic resonance (SR), a phenomenon in which the response of a non-linear system to an input signal is benefited by the presence of a particular non-zero level of noise (Aihara et al., 2010). SR is broadly described as "noise benefit" (McDonnell and Abbott, 2009) and this benefit can be represented by a pseudo-bell shape performance curve as a function of the noise level added, with a peak in performance at some optimal noise level (Moss et al., 2004).

While a growing number of studies have demonstrated improvement of sensorimotor performance with GVS, few have explored the potential retention of effects on performance once GVS treatment has been removed. Keywan et al. (2020) found no evidence of improved roll tilt thresholds following GVS treatment. However, this study used passive application of nGVS to seated subjects prior to performing a balance task. A 2016 postural stability study found evidence of balance improvement after cessation of stimulation potentially due to vestibular neuroplasticity (Fujimoto et al., 2016). However, this study included only elderly adults (mean age 66.7 ± 0.4 years) and GVS stimulation was applied either passively between performance evaluations or actively but only during postural sway evaluation. Nooristani et al. (2019) raised concerns about Fujimoto's study design, namely that the lack of a control group prevents controlling for an ordering confound. In their replication of the Fujimoto study, adding a control group, they found no significant difference in improvement between nGVS and sham groups, suggesting that the improvement seen by Fujimoto et al. (2016) was due to a repeated measure learning effect.

Our study sought to evaluate retention of effects of GVS on performance by applying stimulation during the active learning of a task, rather than passive application before task completion. In doing so, the effect of GVS on both learning and retention of task performance can be explored. We also sought to explore the effect of GVS treatment on learning and retention in more complex and operationally relevant tasks, rather than postural sway. As such, subjects completed either a functional mobility or manual control task.

Functional mobility and manual control are essential performance tasks for both pilots and astronauts, which rely on healthy vestibular function for optimal performance. The future of crewed space exploration will involve long duration deep space missions that pose a cascade of physiological adaptations and potential risks to human health. Deleterious effects of spaceflight may also impair operational performance and task completion. Previous studies show that spaceflight causes an increased risk of vestibular dysfunction and spatial disorientation (Reschke and Clément, 2018; Clark, 2019), which pose significant risks for spacecraft control (Bloomberg, 2015). As such, improving vestibular signal detection and overall performance may assist astronauts to perform such tasks despite spaceflight induced maladaptation. GVS may have benefits for individuals on Earth as well, improving balance and performance, for example, in elderly individuals who otherwise may be at a higher risk for falls (Inukai et al., 2018a). Scientifically, GVS has been shown to impact some aspects of bodily awareness (Ferrè et al., 2013c), somatosensory perception (Ferrè et al., 2013a), and even sense of self (Ferrè et al., 2014), which may be relevant for these types of operational sensorimotor tasks.

In this study, two different applications of GVS during task learning were explored. The impact of noisy galvanic vestibular stimulation (nGVS) treatment on task learning as assessed in both the functional mobility and manual control tasks. nGVS has grown in popularity as a method for improving vestibular performance in areas such as static and dynamic balance (Goel et al., 2015; Stefani et al., 2020), postural sway (Inukai et al., 2018b), locomotor stability (Mulavara et al., 2015), manual control (Galvan-Garza, 2016), and roll tilt vestibular perception in direction recognition tasks (Galvan-Garza et al., 2018; Keywan et al., 2018). However, little research has been done to explore the effect of noisy GVS on the potential enhancement of performance when the treatment is applied during active learning of task or whether enhanced learning with GVS is retained once treatment has been removed.

This study also explored whether a high level disruptive GVS waveform could be used to improve sensorimotor learning. This type of GVS signal can cause postural instability (MacDougall et al., 2006) and has been used to mimic locomotor dysfunction and spatial disorientation (Moore et al., 2006, 2011). High levels of GVS can also impact spatial perception (Ferrè et al., 2013b). Learning in a disruptive vestibular signaling environment may prompt adaptation displayed by rapid learning and increased performance once such disruption has been removed. To our knowledge, no previous studies have explored utilizing high level randomized GVS to impact learning of sensorimotor tasks. Our selected parameters for both nGVS and high GVS conditions can be found in the Methods, and elaboration of those selections as they relate to our results can be found in the Discussion.

Tests of sensorimotor performance and learning typically have substantial inter-individual differences. This was expected to be true of the two sensorimotor tasks in this study as well. However, vestibular perceptual thresholds have been shown to explain individual differences in performance of manual control tasks, including in hypergravity (Rosenberg et al., 2018), as well as balance performance tasks (Bermúdez Rey et al., 2016; Karmali et al., 2017, 2021). Due to its relationship to the manual control task, we assessed roll tilt direction recognition (DR) thresholds in the subjects that subsequently performed manual control testing. These thresholds were then investigated to explore potential correlations between DR threshold and sensorimotor performance and learning on the manual control task.

As a preliminary investigation into the effects of GVS on sensorimotor task *learning*, we explored two GVS waveforms aimed at improving learning of operationally relevant

sensorimotor tasks. We hypothesized that nGVS may improve learning of both tasks by improving vestibular information processing, and that this enhanced performance may be retained even when stimulation was removed. We hypothesized that high GVS may be disruptive to performance, but that learning in this disruptive environment may result in improved performance once stimulation was removed. Finally, DR thresholds were explored as a potential method for predicting inter-subject variability on manual control task performance.

MATERIALS AND METHODS

This experiment design was approved by the University of Colorado at Boulder Institutional Review Board under protocol #20-0097 and all subjects signed a written informed consent form. Thirty-six healthy subjects were recruited for participation in this study (13F, ages 18–32, mean age 22.7 years). Subjects were prescreened and excluded from the study if they had a history of vestibular dysfunction or if they scored above the 90th percentile on the Motion Sickness Susceptibility Questionnaire (Reason, 1968), as we wanted to avoid the potential for motion sickness during our protocols in highly susceptible individuals. Subjects were randomly assigned to one of three treatment groups: sham GVS, nGVS, or High GVS. Subjects were also randomly assigned to one of two performance tasks: a functional mobility assessment or manual control assessment such that in total, six subject groups were formed (**Table 1**).

The experimental protocol consisted of two phases: learning trials and aftereffect trials. Learning trials were completed with the GVS treatment as randomly assigned to the subject (this could include sham GVS in which no current was applied). In aftereffect trials, any GVS current was deactivated, such that all subjects experienced the sham GVS treatment. Subjects were blind to the transition in treatment between learning and aftereffects trials.

Galvanic vestibular stimulation was administered using 2 × 2 inch sponge electrodes (Caputron) placed on the mastoid processes. Subjects were prepped by manually exfoliating the skin along the mastoid processes (behind the ears) using NuPrep exfoliant gel. The area was then cleaned using 70% isopropyl alcohol. Sponge electrodes were saturated with 7 ml of 0.9% sodium chloride sterile saline per manufacturer's instructions. Sponge electrodes were held in place against the mastoid process on each side of the skull and secured using an elastic headband.

TABLE 1 | Summary of subject demographics and distribution amongst treatment groups.

Group	Task	Treatment	Subjects	Age (year) mean ± SD	Sex
1	FMT	Sham	6	26.0 ± 5.6	2F
2	FMT	nGVS at 300 μA bipolar 0–30 Hz white noise	6	22.3 ± 2.9	2F
3	FMT	High-level GVS at 700 μA bipolar 1.5 Hz random noise	5	24.6 ± 3.6	1F
4	Manual control	Sham	7	21.3 ± 2.4	2F
5	Manual control	nGVS at 300 μA bipolar 0–30 Hz white noise	7	21.4 ± 2.0	5F
6	Manual control	High-level GVS at 700 μA bipolar 1.5 Hz random noise	5	21.0 ± 2.3	1F

The nGVS waveform was identical to that previously used to improve balance (Mulavara et al., 2011; Goel et al., 2015), locomotion (Mulavara et al., 2015), and vestibular perceptual thresholds (Galvan-Garza et al., 2018). Studies have shown high variability in subject optimal levels of noisy GVS stimulation for performance enhancement, with average optimal levels typically ranging between 100 and 500 uA (Iwasaki et al., 2014; Goel et al., 2015; Keywan et al., 2018). It appears that at approximately 300 uA (± peak to peak), most subjects have improved perceptual thresholds (Galvan-Garza et al., 2018), so we chose to use this single nGVS stimulus amplitude rather than attempt to personalize for each subject.

The High Level GVS was a deterministic, pseudo-random sum-of-sinusoids profile applied at 700 uA, which in pilot testing we found was disorienting and induced instability but caused little to no sensation on skin in order to blind the transition to sham between learning and aftereffect trials. Both GVS cues were zero-mean and generated by a current controlled stimulator (Soterix Medical Inc., Model 0810) which was customized to include the nGVS 0–30 Hz profile.

Functional Mobility Task

Subjects assigned to the functional mobility task (FMT) completed timed trials in an obstacle avoidance course (**Figure 1A**). This course is modeled after a similar course used at NASA's Johnson Space Center to assess functional mobility of spaceflight participants after their flight experience (Mulavara et al., 2010) and which we have used recently (Dixon and Clark, 2020). The course consists of a series of obstacles that require balance, spatial awareness, and motor control for successful completion. Our course had a 7.5 m long linear design comprised of 1.5 m of foam balance beam followed by a 6 m long inflated air track for reduced proprioceptive cues, ending with a three-step stair climb. Hurdle obstacles had to be stepped over or ducked under, with the latter adjusted to each subject's shoulder-height. Slalom hurdles were also adjustable in width spacing such that each subject could stand shoulder width in between each slalom.

Subjects were informed of the procedures for completing a trial through the course, where a trial is defined as starting seated at the beginning of the course, proceeding through each obstacle and up the stairs, turning around and proceeding through the obstacles in reverse order, and ending seated. Subjects were also informed that there were time penalties for incorrectly moving through the obstacles. Penalties included stepping off the balance beam, body contact with a hurdle in the extended portal or slalom, or using the handrails on the stairs to stabilize. Stepping off the air track at any point during the trial also resulted in a penalty. Subjects were informed that each occurrence of a penalty would add 5 s to their trial time. In data analysis, we found 2 s per incident to be a more suited penalty relative to the total average trial completion length. Subjects were given one practice

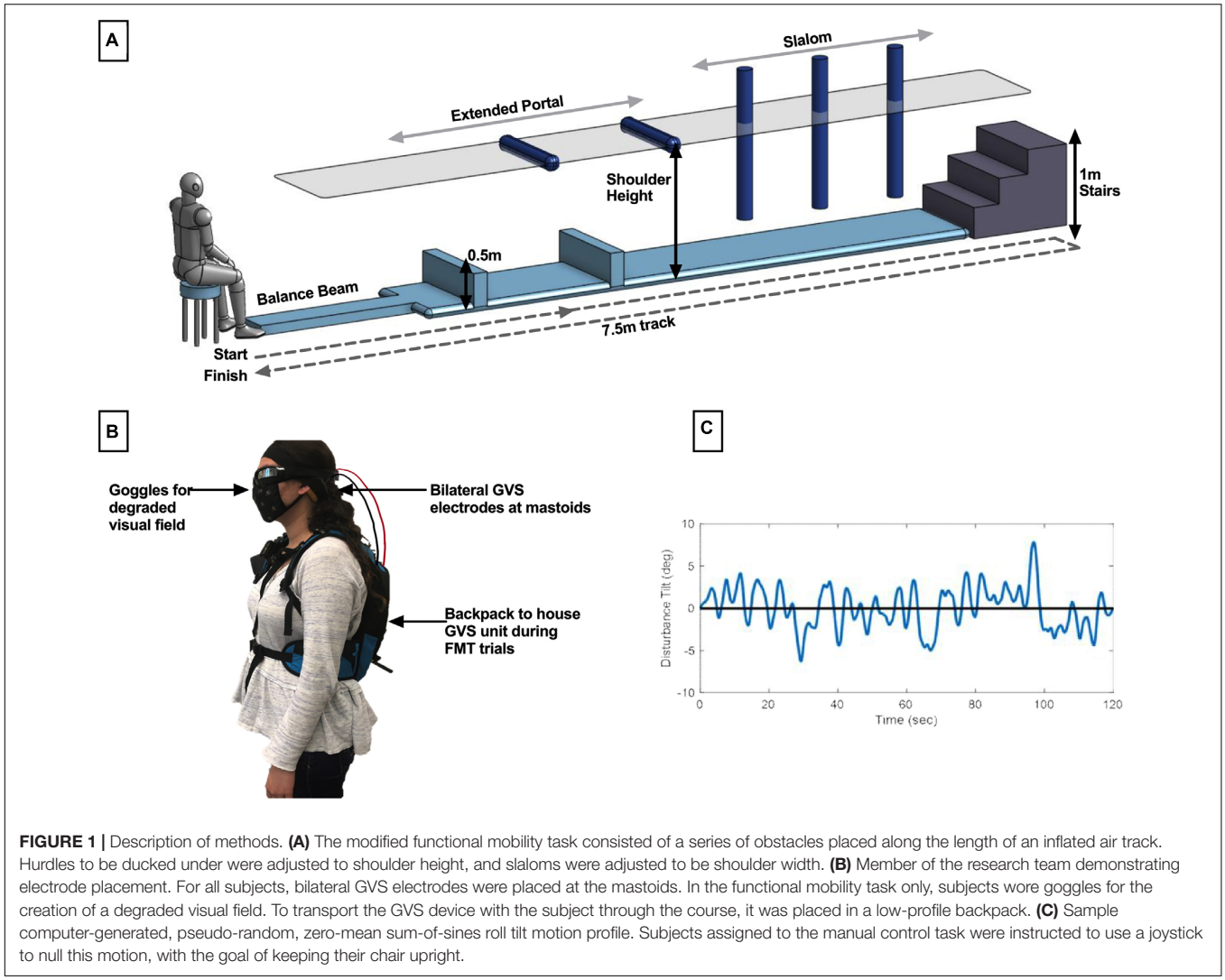

FIGURE 1 | Description of methods. **(A)** The modified functional mobility task consisted of a series of obstacles placed along the length of an inflated air track. Hurdles to be ducked under were adjusted to shoulder height, and slaloms were adjusted to be shoulder width. **(B)** Member of the research team demonstrating electrode placement. For all subjects, bilateral GVS electrodes were placed at the mastoids. In the functional mobility task only, subjects wore goggles for the creation of a degraded visual field. To transport the GVS device with the subject through the course, it was placed in a low-profile backpack. **(C)** Sample computer-generated, pseudo-random, zero-mean sum-of-sines roll tilt motion profile. Subjects assigned to the manual control task were instructed to use a joystick to null this motion, with the goal of keeping their chair upright.

trial, prior to the learning phase, to move through the course at their own pace without treatment to ensure they were familiar with the procedures and penalties. They were then instructed that their goal was to complete each trial as quickly as possible while minimizing penalties. The GVS unit was securely placed in a low-profile running backpack to move with subjects through the course (all subjects wore this throughout testing, regardless of whether it was a sham trial). The electrode cables were fed through an opening in the backpack and electrodes were held securely against the mastoids by an elastic headband as described above. Subjects also wore goggles to create a degraded visual field in order to reduce reliance on visual cues for completion of the course. The goggles were mirrored swim goggles with lower peripheral vision blockers (**Figure 1B**).

Subjects completed 12 FMT learning trials with GVS treatment and 8 aftereffects trials with sham GVS treatment. To reduce physical fatigue, subjects were given a 60 s break between each trial to rest. In order to blind the transition to sham, subjects were given a 90 s break instead of a standard 60 s break after every fourth trial, during which the operator would open the running backpack and check the GVS unit. On the third such break (after the 12th trial), the operator turned the GVS unit off without informing the subject.

Manual Control Task

Subjects assigned to the manual control task completed a roll tilt nulling task using a custom human-rated motion device [the Tilt Translation Sled (TTS), without the translation axis activated]. Subjects were secured with a 5-point harness and head restraint. Manual control performance was assessed in the dark with auditory white noise playing through subject headphones to minimize non-vestibular cues. Subjects were instructed that they would experience a series of randomized head-centered disturbance tilts in the roll axis. As they experienced these tilts, their task was to use their joystick to null perceived tilt by executing opposing joystick inputs (i.e., to keep their chair as close to upright as possible throughout the 120 s trial). Joystick deflections were measured with a potentiometer, averaged over 1 s, and commanded chair roll tilt with a proportional gain ($K = 2.5°$ of chair roll tilt per 1° of smoothed joystick deflection).

Roll tilt disturbances were computer-generated, pseudo-random, zero-mean sum-of-sines profiles (**Figure 1C**). The generated profile was inverted by sign, reversed in time, or both inverted and reversed to create four randomized profiles comprised of the same 120 s of motion but preventing pattern recognition from the subject throughout trial completion. The first and last 5 s of each profile was scaled so that the chair disturbance began and ended at upright. These scaled portions were excluded from analysis. Maximum roll tilt disturbance was ±7.8°. Maximum joystick command was ±9° of chair tilt to allow for overcorrection.

Subjects completed 8 learning trials with their assigned GVS treatment followed by 5 aftereffects trials with sham GVS treatment. This distribution of trials was selected to match the approximate 45-min testing duration of the functional mobility task design. Subjects were blind to the transition between learning and aftereffects trials in a similar manner as for the functional mobility assessment. Joystick commands, as well as actual chair tilt, were recorded at 50 Hz. Performance was characterized by root mean square error (RMSE) of the chair nulled tilt angle, such that smaller values correspond to better performance.

Direction Recognition Thresholds

Direction recognition thresholds were tested utilizing the TTS in roll tilt configuration. DR thresholds were only measured for manual control subjects, due to the relatively equivalent motion experience between the nulling task and roll-axis direction recognition. Roll-axis thresholds were not expected to be an effective prediction of inter-subject variability of performance on the multi-axis, multi-sensory FMT.

Subjects were seated and secured with a 5-point harness as well as a custom head restraint to keep the head en bloc with the body. Direction recognition thresholds were performed in the dark with auditory white noise to reduce non-vestibular cues. Operator commands were communicated through subject headphones. Roll tilt motions began at upright and consisted of a single-cycle sinusoid in angular acceleration, as is typically used in previous studies (Bermúdez Rey et al., 2016; Lim et al., 2017; Suri and Clark, 2020). The tilt profile was performed at 0.5 Hz frequency (2 s tilt duration). This tilt duration requires integration of otolith and semicircular canal cues (Lim et al., 2017; Suri and Clark, 2020). Subjects were tilted in the dark, and once the motion was complete while still tilted, were asked to verbally indicate which direction they perceived to be tilted, as well as their confidence in their answer. Confidence reports were provided in 5% increments, with 50% being a complete guess and 100% being complete certainty, however, the research on confidence adjusted thresholds is still ongoing thus are not further considered here. On each trial, tilt direction (i.e., left or right) was randomized, and tilt magnitude (in degrees) was adjusted using a 3 down 1 up staircase beginning at 6 degrees. Subjects completed 100 trials in order to produce a reliable threshold estimate (coefficient of variation of the estimate was theoretically ∼0.18) (Karmali et al., 2016). Subject responses were fit with a cumulative Gaussian psychometric curve (Merfeld, 2011). To accommodate the adaptive staircase, a bias-reduced generalized linear model (BRGLM) fit was performed with a probit link function (Chaudhuri and Merfeld, 2013), as is standard practice (Bermúdez Rey et al., 2016; Suri and Clark, 2020). The psychometric curve fit produces two parameters: μ – the vestibular bias (i.e., the stimulus at which the subject is equally likely to response "left" vs. "right," and σ – which is commonly defined as the 1-sigma threshold (Merfeld, 2011), we adopt here. Psychometric curve fits to produce roll tilt thresholds were performed in MATLAB. Thresholds were log-transformed for normality (Suri and Clark, 2020).

Data Analysis

For each performance task, we fit a piecewise model as a function of trial number, in order to capture the learning and aftereffect responses. An example is provided of a subject in the manual control task in **Figure 2**.

During the learning phase, an exponential decay was fit to capture the improvement in performance as trials progressed. In particular, we calculated the model fit corresponding to initial performance and final learning phase performance [L(1) and L_{end}, respectively], where L_{end} averaged performance in the final two learning phase trials [L(7) and L(8) for manual control, L(11) and L(12) for FMT], and L(1) was taken as an independent measure of initial performance. The exponential model of learning is a well-used standard for measuring improvement of task performance with practice (Heathcote et al., 2000; Ritter et al., 2013). However, some subjects demonstrated learning that was not well captured by an exponential model. To quantify the appropriateness of the learning phase exponential model fit, R^2 was calculated for each subject's data. For individuals with a model fit $R^2 > 0.45$, the exponential model was used to determine L(1) and L_{end} parameters. For subjects whose performance was not well represented by the exponential decay model ($R^2 < 0.45$), their raw trial performance was used to calculate L(1) and L_{end} parameters instead. Learning was defined as the difference in performance across the learning phase relative to the subject's initial performance during this phase, using the following equation:

$$\text{Learning Metric} = \frac{L(1) - L_{end}}{L(1)}$$

A high learning metric score indicates that the subject had a large improvement of performance from their initial performance (L_1) to their performance at the end of the learning phase (L_{end}), indicating more learning. If the learning metric is zero, no change in performance occurs during this phase.

During the aftereffect phase, average performance across all trials within that phase was computed (AE_{avg}) and compared to performance at the end of the learning phase (L_{end} as previously described). A retention metric was calculated, which corresponds to the average performance when GVS was removed relative to the subject's performance at the end of the learning phase, and was calculated using the following equation:

$$\text{Retention Metric} = \frac{L_{end} - AE_{avg}}{L_{end}}$$

If the subject's performance did not change from the end of the learning phase to the aftereffect phase (i.e., there was no change

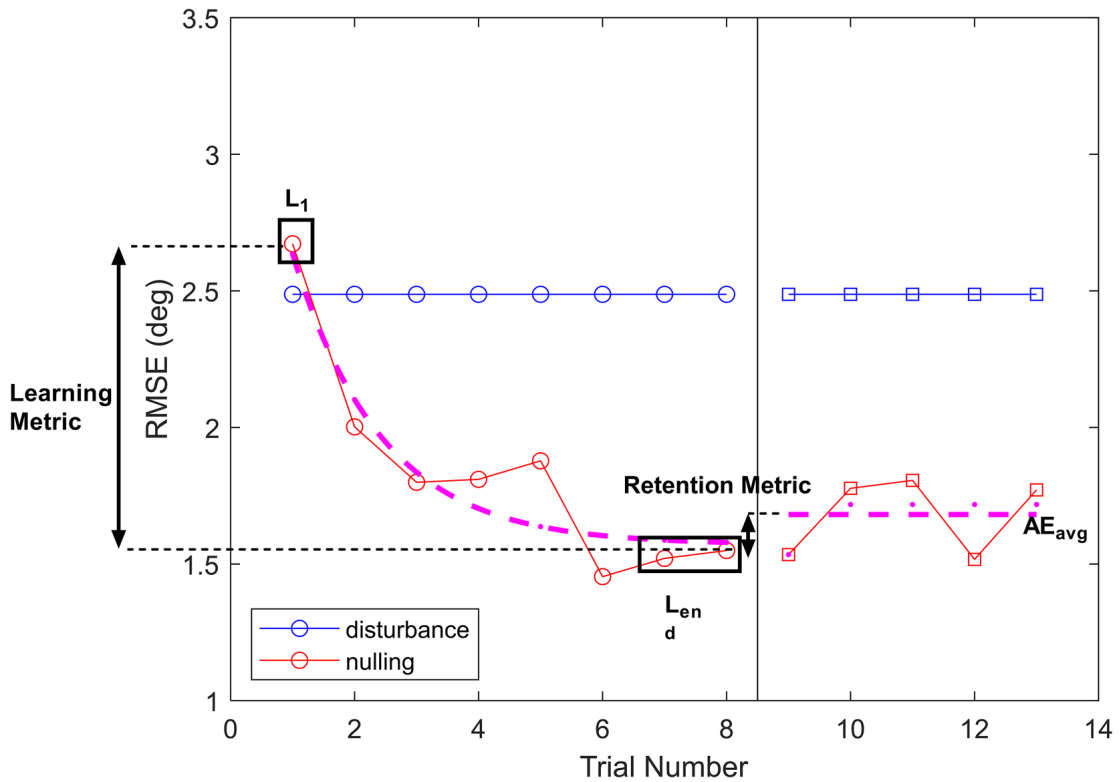

FIGURE 2 | Sample plot of performance for a subject in the manual control task. For both tasks, performance on each trial was plotted for the learning phase and the aftereffects phase. An exponential decay was fit to the learning phase, with L_1 and L_{end} values pulled from the model fits with $R^2 > 0.45$. For subject whose learning was not well captured by the model fit ($R^2 < 0.45$), raw performance on trials 1 or the average of trials 7 and 8 were used for L_1 and L_{end}, respectively. Average performance across the aftereffects phase was used as AE_{avg} in calculating the retention metric.

when the GVS was blinded turned off), the retention metric would be zero. If performance improved after GVS was turned off, the retention metric would be positive, while it would be negative if performance degraded (e.g., a subject lost some of the learning accomplished during GVS treatment).

RESULTS

Functional Mobility Task

In the functional mobility task, a one-way ANOVA showed a significant difference in learning metric across GVS treatment groups [$F(2,14) = 6.09$, $p = 0.013$]. Pairwise comparisons with a Bonferroni adjustments demonstrated that subjects receiving nGVS treatment showed a significant increase in learning of 171% compared to sham [$t(14) = 3.03$, $p = 0.027$], as well as a significant increase in learning of 184% compared to the high GVS condition [$t(14) = 2.97$, $p = 0.031$]. No significant difference in task learning was observed between sham and high GVS treatments [High GVS learning 5% lower than sham, $t(14) = 0.08$, $p > 0.99$ with Bonferroni adjustment]. These results can be seen in **Figure 3A**. Due to the low sample size in this investigation, non-parametric analysis was also performed. Kruskal–Wallis testing demonstrated global significance between learning metric across GVS treatment groups [$H(2) = 8.542$, $p = 0.007$]. *Post hoc* testing using Dunn's multiple comparisons showed a significantly higher learning metric in nGVS subject compared to sham ($p = 0.04$) as well as high GVS subjects ($p = 0.03$), but no significant difference in learning metric between sham and high GVS subjects ($p > 0.99$) (matching the parametric test conclusions in each case). For remaining analysis in this investigation, tests of normality and appropriate assumptions were used to select parametric vs. non-parametric analysis. Unless reported otherwise, results of non-parametric testing matched those of parametric testing.

Next, we considered the raw performance at the end of the learning phase (L_{end}), in which a one-way ANOVA showed a significant difference in performance across treatment groups [$F(2,14) = 5.46$, $p = 0.018$]. This analysis further demonstrates that subjects receiving nGVS treatment finished the learning phase with marked improvement compared to other treatment groups. nGVS subjects showed significantly faster trial completion than high GVS subjects, with a mean difference in trial time 5.91 s faster [$t(14) = 2.49$, $p = 0.024$], and marginal significance compared to sham subjects with a mean difference 4.53 s faster [$t(14) = 3.096$, $p = 0.08$]. No significant difference

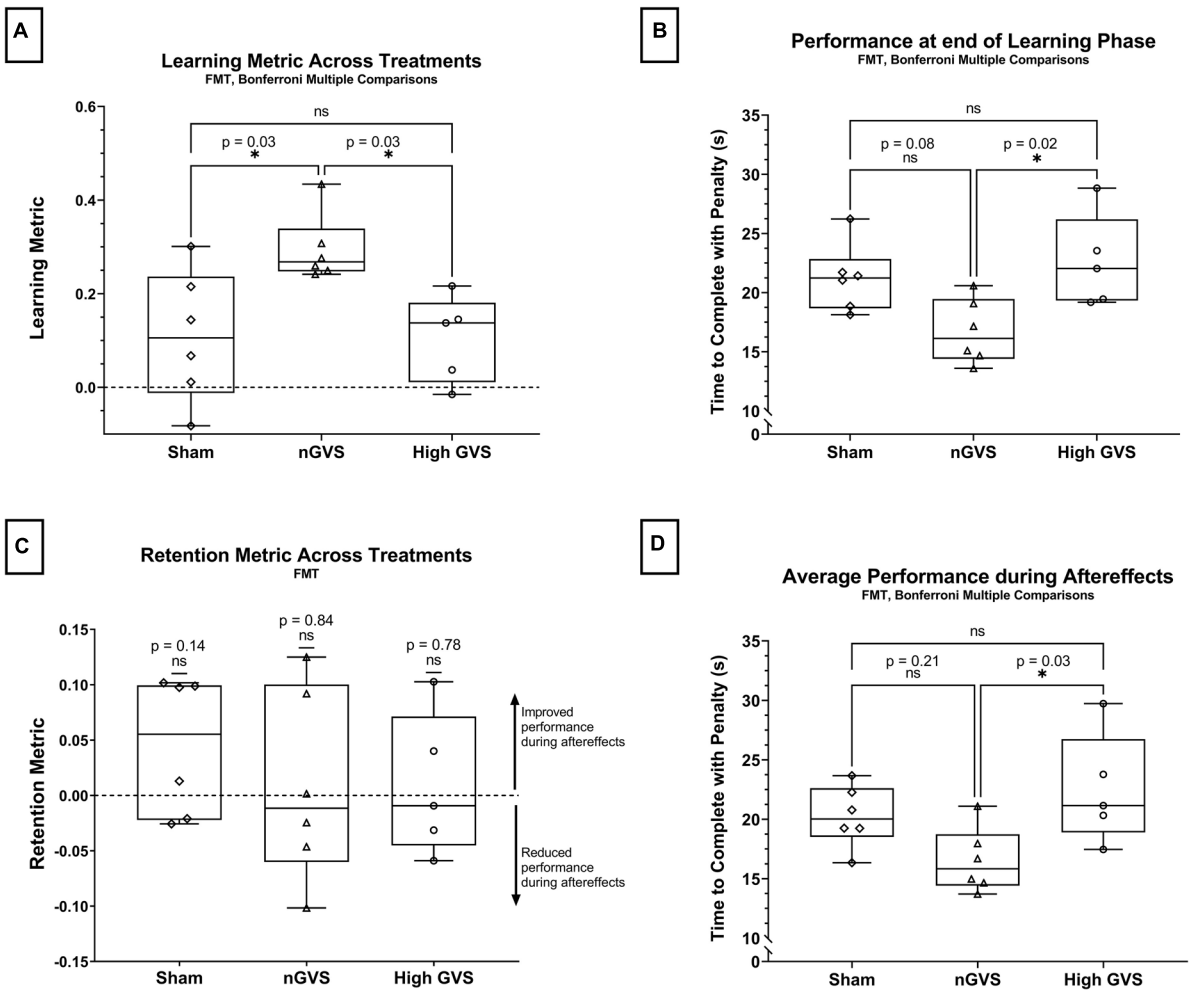

FIGURE 3 | FMT data analysis. The symbol * denotes significant comparisons at $\alpha = 0.05$. **(A)** Comparison of learning metric across treatments. **(B)** Comparison of time to complete the course (with penalties) at the end of the learning phase (L_{end}) across treatments. **(C)** Comparison of retention metric across treatments shows no significant change in performance between end of learning phase and average performance during aftereffects for any treatment. **(D)** Average time to complete the course (with penalties) during aftereffects (AE_{avg}) across treatment groups.

in performance at the end of the learning phase was seen between sham and high GVS subjects [$t(14) = 0.723$, $p > 0.99$] (**Figure 3B**).

We tested the retention metric across treatments against a theoretical mean = 0, which would indicate no change in performance between learning and aftereffects phases. Two-tailed *t*-tests showed no significant difference from zero for sham [$t(5) = 1.64$, $p = 0.14$], nGVS [$t(5) = 0.219$, $p = 0.84$], or high GVS [$t(4) = 0.302$, $p = 0.78$], demonstrating that change in performance during the learning phase was retained into the aftereffects phase when nGVS and high GVS treatments were transitioned to sham. These results also demonstrate that high GVS subjects showed no significant improvement in performance during the aftereffects phase as might be expected when removing the destabilizing treatment (**Figure 3C**).

Finally, analysis of averaged performance throughout the aftereffects phase was used to examine how subject performance differed beyond the learning phase. This analysis was performed to determine if nGVS subjects remained the fastest performance group even once treatment was removed. One-way ANOVA showed a significant difference between average aftereffects performance across treatment groups [$F(2,14) = 4.552$, $p = 0.03$]. *Post hoc* comparisons with a Bonferroni adjustment show that nGVS subjects remain significantly faster in trial completion than high GVS subjects, with a mean difference in trial time 5.97 s faster [$t(14) = 2.96$, $p = 0.031$], but no significant difference compared to sham subjects [mean difference 3.74 s faster in nGVS subjects, $t(14) = 1.943$, $p = 0.22$]. No significant difference in trial completion time was seen between sham and high GVS subjects [mean difference 2.23 s faster in sham subjects, $t(14) = 1.10$, $p = 0.86$] (**Figure 3D**).

Manual Control Task

In the manual control task, a one-way ANOVA approached but did not reach a significant difference in learning metric across GVS treatment groups [$F(2,16) = 3.19$, $p = 0.07$] (**Figure 4A**). nGVS subjects showed an average learning metric 10% higher

than subjects receiving sham and 59% higher than subjects receiving high GVS treatment.

Performance at the end of the learning phase (L_{end}) failed Shapiro–Wilk tests of normality for sham ($p = 0.014$) subjects, thus non-parametric test were used for hypothesis testing. Kruskal–Wallis testing showed a significant difference in performance at L_{end} across treatment groups [$H(2) = 6.01$, $p = 0.04$]. A pairwise *post hoc* Dunn test was significant for sham vs. high GVS ($p = 0.046$ but not sham vs. nGVS ($p > 0.99$) or nGVS vs. high GVS ($p = 0.27$) as seen in **Figure 4B**.

The retention metrics across treatments for this task were also tested against a theoretical mean = 0, as described above. Two-tailed *t*-tests showed no significant difference from zero for sham [$t(6) = 1.503$, $p = 0.18$], nGVS [$t(6) = 0.337$, $p = 0.75$], or high GVS [$t(4) = 0.390$, $p = 0.72$], demonstrating that change in performance during the learning phase was retained into the aftereffects phase when nGVS and high GVS treatments were transitioned to sham. These results also demonstrate that high GVS subjects in the manual control task also showed no significant improvement in performance during the aftereffects phase, again as might be expected (**Figure 4C**).

Average performance throughout the aftereffects phase (AE_{avg}) also failed Shapiro–Wilk tests of normality for sham ($p = 0.001$) and nGVS treatments ($p = 0.011$), thus non-parametric tests were used for hypothesis testing. A Kruskal–Wallis test showed a significant difference in performance at L_{end} across treatment groups [$H(2) = 8.49$, $p = 0.008$]. A pairwise *post hoc* Dunn test was significant for sham vs. high GVS ($p = 0.01$) but not sham vs. nGVS ($p = 0.55$) or nGVS vs. high GVS ($p = 0.27$) as seen in **Figure 4D**.

Roll Tilt Direction Recognition Thresholds for Manual Control Subjects

Simple linear regression was performed to investigate the relationship between roll tilt direction recognition thresholds and manual control performance measured by both initial performance (L_1) and learning metric. DR thresholds were log transformed for normality. In examining L_1 performance, linear best fit lines show a positive correlation between performance and threshold across all treatments. However, only nGVS subjects showed a significantly non-zero slope [$F(1,5) = 6.739$, $p = 0.05$, $R^2 = 0.57$]. Neither sham nor high GVS subjects showed significant slopes ($R^2 = 0.04$ and 0.28, respectively) (**Figure 5A**). In examining the relationship between threshold and learning during the manual control task as assessed by the learning metric, no treatments showed a significant slope. However, sham and nGVS subjects showed a best fit line with a positive slope ($R^2 = 0.03$ and 0.12, respectively) while high GVS subjects showed a best fit line with a negative slope ($R^2 = -0.49$) (**Figure 5B**). Due to low sample size, non-parametric analysis of correlation through Spearman Rank was also performed. No significant correlation between DR threshold and L_1 was seen for sham (Spearman $r = 0.36$, $p = 0.44$), nGVS ($r = 0.68$, $p = 0.11$) or high GVS subjects ($r = 0.50$, $p = 0.45$). Similarly, no significant correlation between DR threshold and learning metric was seen for sham ($r = 0.29$, $p = 0.56$), nGVS ($r = 0.29$, $p = 0.56$), or high GVS subjects ($r = -0.50$, $p = 0.45$).

DISCUSSION

How Does Noisy Galvanic Vestibular Stimulation Treatment Affect Sensorimotor Learning in Functional Mobility and Manual Control Tasks?

Analysis of functional mobility data supports that nGVS treatment improved performance across the learning phase, with a statistically significant higher learning metric compared to sham. This improvement in performance was retained even once GVS treatment was removed, as indicated by retention metrics for the nGVS subject group that were not significantly different than zero (retention metric = 0 indicates no change in performance between the end of the learning phase and the aftereffect phase). Analysis of manual control nulling task data showed no significant difference in learning metric between GVS groups, and no significant difference in performance at the end of the learning phase or average performance during aftereffects between sham and nGVS subjects in this task. As an initial investigation of the effect of GVS on learning of sensorimotor tasks, we acknowledge the low sample size in this study. Effort was taken to interpret results with caution and use non-parametric analysis when appropriate.

Learning metric differences between manual control and FMT performance suggests that nGVS provides benefit to learning for tasks like FMT which involve multisensory integration, body coordination, and strategy. Previous studies of GVS support enhancement in functions essential in functional mobility task success, such as gait (Iwasaki et al., 2018), locomotor stability (Mulavara et al., 2015), and dynamic balance (Stefani et al., 2020). A 2019 study showed evidence that nGVS enhances spatial memory, an essential part of obstacle avoidance and overall success in course completion (Hilliard et al., 2019). Further, another study found improved visual perceptual thresholds during nGVS application, suggesting cross-modal stochastic resonance (Voros et al., 2021). In contrast, the manual control task is mostly a vestibular sensation and motor reaction task. It is possible that nGVS is unable to enhance learning of such a sensorimotor task that is predominantly vestibular perception related. The small sample size in this study may have prevented a measurable effect of nGVS on learning of the manual control task, however, the data do not trend toward a positive relationship between this treatment and improved performance.

Alternatively, it is worth noting that in a previous study that found nGVS to improve roll tilt direction recognition thresholds (Galvan-Garza et al., 2018), the investigators used subject-specific optimal stimulation levels identified from either 200, 300, 500, or 700 uA. In this study the group optimal level was 300 uA, which motivated our use of that single level to investigate sensorimotor learning with nGVS. Many previous studies have calculated subject-specific optimal stimulation levels, frequently using postural sway tasks (Fujimoto et al., 2016; Iwasaki et al.,

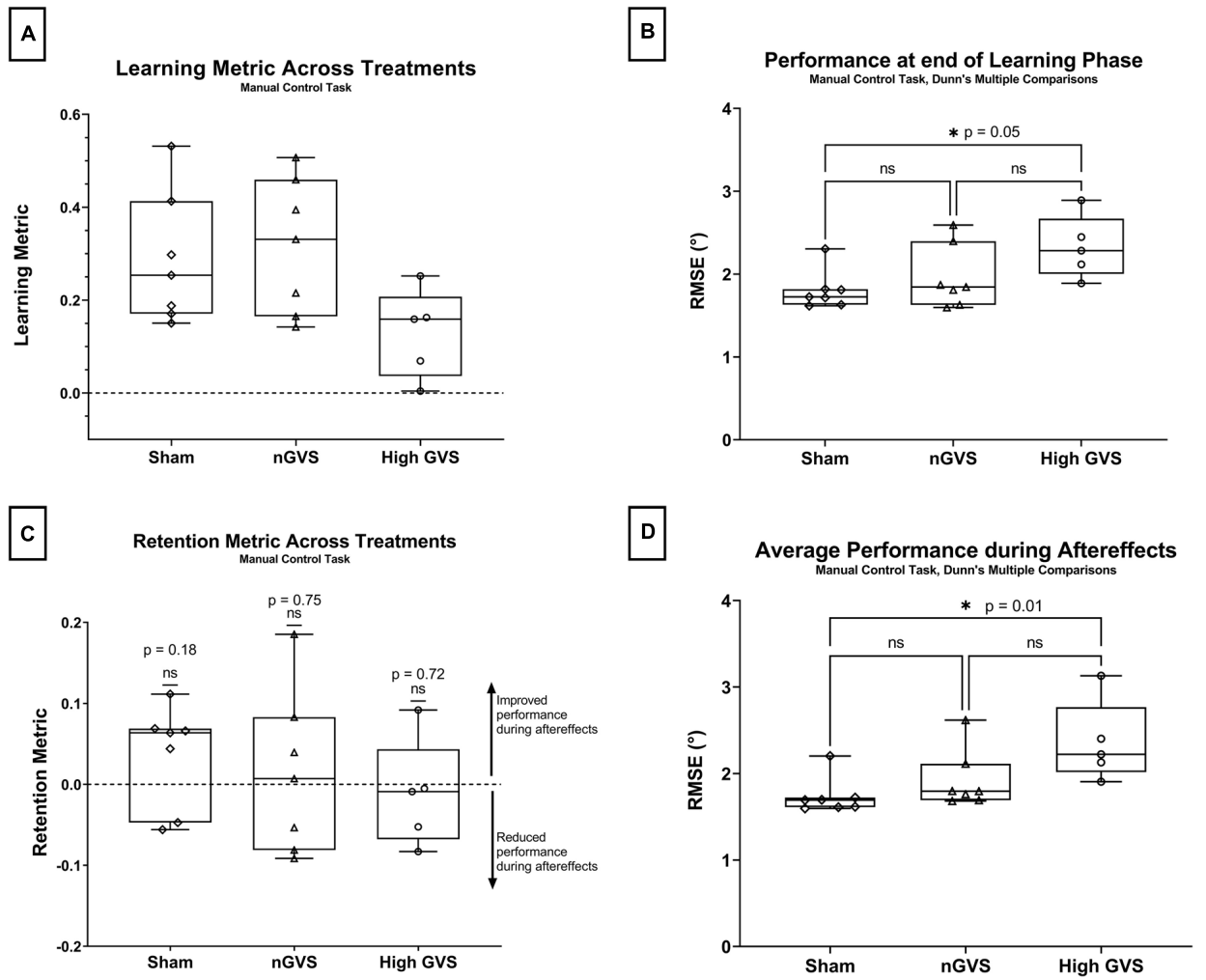

FIGURE 4 | Manual control data analysis. The symbol * denotes significant comparisons at $\alpha = 0.05$. **(A)** Comparison of learning metric across treatments. No global significance between treatments. **(B)** Comparison of nulling performance at the end of the learning phase (L_{end}) across treatments. **(C)** Comparison of retention metric across treatments shows no significant change in performance between end of learning phase and average performance during aftereffects for any treatment. **(D)** Average nulling performance during aftereffects (AE_{avg}) across treatment groups.

2018; Keywan et al., 2018, 2019, 2020). As such, differences in learning metric across nGVS subject could be a result of the use of a single nGVS current level of 300 uA for all nGVS subjects. It may be that 300 uA happened to be optimal or near optimal for some subjects, and less so for other subjects, such that they produced less clear improvements with nGVS in our study, but might have if we were able to optimized the nGVS treatment for them. Since learning was a primary metric in this study, it was not possible to perform repeated measures of performance with varying stimulation levels in order to determine optimal stimulation, as subjects would learn during this time and that learning would not be captured. In order to determine subject-specific optimal levels, we would need to test subjects on a separate task. Doing so assumes that optimal GVS stimulation level for once task, such as a postural sway assessment, is also a subject's optimal for another task, in this case functional mobility or manual control. We did not feel confident making that assumption, thus chose 300 uA as the treatment level for all nGVS subjects. Future work to explore how optimal GVS levels differ across a multitude of tests such as postural sway, gait or dynamic balance, functional mobility, manual control, or direction recognition thresholds may support future selection of subject-specific optimal stimulation levels, particularly for learning of sensorimotor tasks.

Improvement in performance during learning was retained even once GVS treatment had been removed for both tasks. This result suggests that application of nGVS treatment during active task learning could provide sustained operational benefits. Previous studies have found that passive GVS treatment before task completion does not show evidence of aftereffects (Keywan et al., 2020). This difference in results suggests that sustained improvement is only achieved when nGVS is applied *during active learning of a task*. However, retention was only measured for approximately 15 min of aftereffects performance in both

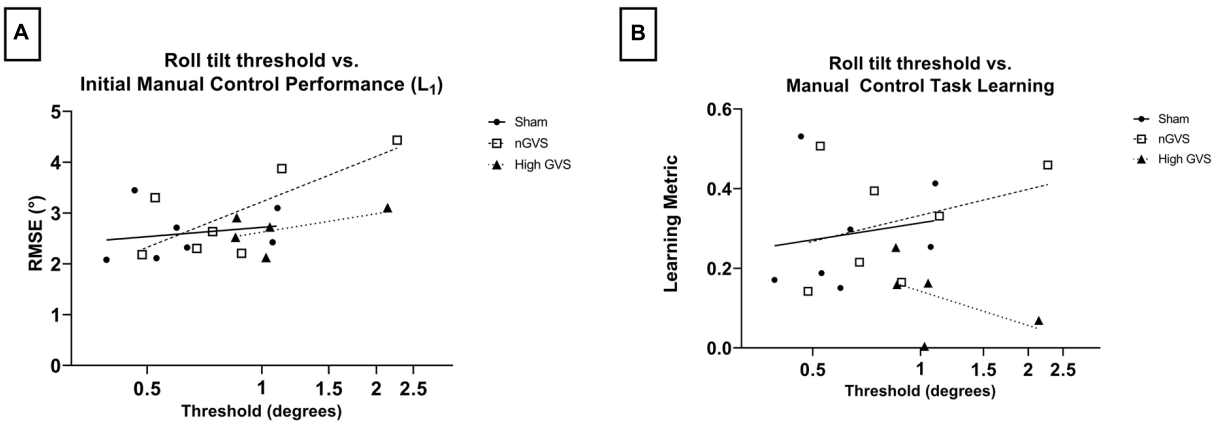

FIGURE 5 | Correlating DR threshold and task performance for manual control subjects. **(A)** While all subjects show a positive correlation between DR threshold and initial task performance, this relationship is only significant for nGVS subjects ($p = 0.05$). **(B)** No significant correlation between DR threshold and manual control task learning across any treatment group.

tasks. Long-duration retention of improvement cannot be concluded from the results of this study. Future work should explore retesting subject performance hours or even days after learning with active stimulation was completed in order to assess long-term sustained retention of improvement. Nonetheless, it is highly promising that the benefits of nGVS in enhancing functional mobility learning, as compared to sham, are sustained even after the nGVS is turned off.

How Does High Galvanic Vestibular Stimulation Treatment Affect Sensorimotor Learning in Functional Mobility and Manual Control Tasks?

High GVS treatment caused some impairment of learning, however, this impairment was not significantly different from sham for either functional mobility or manual control. Removal of treatment in aftereffects did not prompt increased learning for either task, as might be expected when deactivating the destabilizing treatment.

The original intention of the high-level stimulation was to create a disruptive and difficult environment for learning. If learning was conducted in a disruptive environment, it was hypothesized that removal of that disruption and easing of the task environment may prompt improved aftereffects performance. This method of learning can be likened to resistance training, where removing additional weights or resistance that were used in training prompts increased performance in an "easier" environment. Previous studies have used high levels of GVS to create a disruptive balance environment (MacDougall et al., 2006; Sloot et al., 2011).

High GVS treatment as used in this study may not have caused improved learning when removed due to the stimulation profile selected not providing a sufficiently disruptive learning environment. Sloot et al. (2011) used pseudo-random sum of sinusoids with a maximum amplitude of 2.2 mA of current to create balance impairment. MacDougall et al. (2006) used an even higher profile to create disruption, with stimulation up to 5 mA. An additional study which found high level GVS to disrupt body ownership and "self-advantage" also used stimulation levels with a maximum of 5 mA (Hoover and Harris, 2015). However, stimulation in this study was selected to be disruptive while minimizing skin surface sensation in order to keep skin sensitivity blinded through the transition between learning and aftereffects, when all subjects were transitioned to sham. Pilot testing found that current levels about ∼750 uA were frequently detectable via skin surface sensations, thus the High GVS treatment was set at a maximum intensity of 700 uA. High level subjects tested in this study showed no significant improvement in performance during the learning phase for either functional mobility or manual control, demonstrating that the 700 uA stimulation level was indeed somewhat disruptive to learning. However, this level may not have been disruptive enough to instigate aftereffects learning as originally hypothesized.

Are Roll Tilt Direction Recognition Thresholds an Indicator of Inter-Subject Variability of Performance in Manual Control Tasks?

Simple linear regression found a significant and positive correlation between roll tilt direction recognition threshold and initial manual control task performance for nGVS subjects, but not sham or high GVS subjects. No significant correlation was found between an individual's threshold and learning metric for any treatment. However, only high GVS subjects showed a negative correlation between threshold and learning. This result may indicate that task learning is least disrupted by high GVS treatment for subjects with lower baseline roll tilt thresholds. While not significant, the magnitude of impact of GVS treatment on learning trended higher for subjects with a higher baseline threshold, while subjects with lower baseline thresholds experienced learning more similar to sham.

It should be noted that this analysis was performed only for subjects assigned to the manual control task, resulting in a low n and a reduced continuum of possible baseline thresholds for each treatment group. Preliminary trends seen here may pose an area of exploration for a future, higher power study focused on exploring inter-subject variability in sensorimotor task performance. Our study was not powered for or designed to explore sex differences in response to GVS. However, previous research has found no sex differences in sensorimotor adaptations to gravity transitions, nor in vestibular perceptual thresholds (Reschke et al., 2014; Bermúdez Rey et al., 2016). Based on this literature, we did not explore sex as a variable in our analysis.

Operational Context

Based on the results reported here, nGVS may be an effective tool to improve the learning of complex sensorimotor tasks like the functional mobility task. This has strong operational applications, particularly for astronauts. Post-flight sensorimotor impairment poses risks for mission essential task completion in orbit as well as safety when returning to earth (Bloomberg and Mulavara, 2003; Bloomberg, 2015; Bloomberg and Madansingh, 2015; Clark, 2019). Perhaps more importantly, maladaptation in long duration spaceflight poses significant risks to safety and successful task completion for mission essential surface operations in Moon or Mars missions. When astronauts arrive to a new planetary surface, they will have to "relearn" performance of tasks in their new gravity field and environment, especially when adapting to the weight and mobility challenges of a spacesuit. nGVS may be useful to improve task performance through application during active performance of tasks, as demonstrated in this study. It may also enhance learning of tasks as astronauts adapt to their new environment. Even if application is only done for initial periods of task learning and completion, the results of this study demonstrate that improvement in performance is retained even when nGVS treatment has been removed.

Beyond spaceflight applications, nGVS has the potential to benefit any individual for which functional mobility is an essential part of their work, such as firefighters, high performance athletes, or soldiers. While we only studied healthy participants, the benefits of nGVS also have the potential to aid elderly people or patients recovering from a condition that prevented locomotion. The findings of this study supporting the sustained benefit of nGVS when applied during active task learning can be used to implement GVS as a tool for all of these groups. In this preliminary study of sensorimotor learning, we acknowledge small sample size limitations on our findings. We believe that the results of this study justify further exploration into the effect of GVS on sensorimotor learning.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the CU Boulder Institutional Review Board (IRB Protocol Number 20-0097). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EP carried out all subject testing, collected the data, wrote the manuscript with support from TC and RG-G analyzed the data, and interpreted the results with the support of TC. RG-G and TC conceived of the experiment and designed it with support from EP. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnhum.2021.756674/full#supplementary-material

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Academic Publication of Neurodegenerative Diseases From a Bibliographic Perspective: A Comparative Scientometric Analysis

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Background: For measuring the impact in clinical and scientific research, the citation count of the articles is used in the bibliometric analysis, although there is no comprehensive summary of neurodegenerative disease research. This study intends to provide the neuroscientists and investigators with a practical reference guide to appraise the most important and influential articles written on this subject through a macroscopic view of the research activities on neurodegenerative diseases.

Materials and Methods: The Clarivate Analytics Web of Science was searched in July 2020. To ensure the breadth of the search scope, the search terms were confirmed as "multiple sclerosis" (MS) or "amyotrophic lateral sclerosis" (ALS) or "Parkinson's" or "Alzheimer's" or "Huntington's" or "neurodegenerative." After excluding completely unrelated articles, the top-cited articles were collected and evaluated from special characteristics. The data analysis was performed using SPSS 18.0. The articles were characterized by citation number, publication year, topic, study type, authorship, journal, country, and institute of responding author and foundation.

Results: The query identified 593,050 articles. A total of 45% of the top-cited articles were published during 2000–2009, followed by 30 articles from 1990–1999. Diagnosis and pathology were the main research categories ($n = 62$). Alzheimer's disease (AD) was the main study topic ($n = 43$). Meanwhile, the United States confirmed the tremendous impact on the field of neurodegenerative diseases. Notably, 69 of 100 articles were studied in the United States, and the National Institutes of Health sponsored 49 articles. There were only 22 articles that can be divided by evidence level. No article was categorized as level 1 evidence. In the journal list with multiple articles, seven of 15 were general journals. The 58 authors, who contributed to more than one article, have been identified by VOSviewer, and the clusters of authors reveal the evolution of research focus in neurodegenerative diseases.

Conclusions: This study analyzed the bibliometric characteristics and connections of 100 top-cited articles in the field of neurodegenerative diseases in the Web of Science. Their main outcomes were as follows: First, the pathology and diagnostic researches

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took a major role in top-cited articles while the therapy articles are relatively less. Second, the United States confirmed the tremendous impact on the field of neurodegenerative diseases. Third, researchers also submitted their researches to general journals, not just focused on specialty journals.

Keywords: neurodegenerative diseases, aging neuroscience, bibliometric analysis, comparative scientometric analysis,visualization, women in neuroscience

INTRODUCTION

Neurodegenerative diseases, which are characterized by the progressive loss of neurons in the central nervous system, affected more than 50 million people worldwide (Nuic et al., 2018; Ehrenberg et al., 2020). At present, no treatment exists to arrest or slow down the neurodegenerative process (Du et al., 2015). Moreover, due to global demographic changes and life expectancy increases in humans (Kepp, 2019), aging-related neurodegenerative diseases are ever-increasing (Agarwal et al., 2016). Bibliometric sciences offer both a statistical and quantitative analyses of published articles and provide a measurement of their impact in a particular field of research (Baier-Fuentes et al., 2020). This field of science has continuously evolved since 1987 (Garfield, 1987; Ahmad et al., 2014), but the bibliometric analysis in neurodegenerative diseases has not been reported.

As a quantitative study of previously published articles, the bibliometric analysis is used to study various aspects of science (Ellegaard and Wallin, 2015; Du et al., 2020). An analysis of the top 50 or 100 most-cited articles can help researchers review the research history in a specific field and make further contributions based on the classic literature. Thus, we chose the 100 top-cited articles to propose a representative list of intellectual milestones in the field (Du et al., 2020). A few of the top-cited studies in the field of the neurodegenerative disease have been reported. In 2009, a bibliometric analysis related to Alzheimer's disease (AD) has been reported by Sorensen. In 2018, Xue et al. reported "The 100 most-cited articles in Parkinson's disease" (PD) (Xue et al., 2018) for the first time. However, no top-cited bibliometric analysis has been conducted on the whole field of the neurodegenerative disease.

Since the middle of the twentieth century, specialists and researchers are committed to provide hypotheses into neurodegenerative diseases. An increasing number of articles are published annually to show *in vitro* experiments, animal experiments, and clinical applications in this field. Given that, this study aimed at determining a ranking of the 100 top-cited articles regarding all kinds of neurodegenerative diseases. This study can help researchers better determine the direction of their study, meanwhile further analyze for better understanding of the qualities of classical studies and special characteristics of highly cited articles, and highlight the significant contribution of these studies to the field of neurodegenerative diseases.

MATERIALS AND METHODS

Confirmation of Database and Search Scope

To recognize the 100 top-cited articles related to the field of neurodegenerative diseases, the authoritative and professional citation indexing database, i.e., Web of Science, was used on July 22, 2020 (Web of Knowledge, 2020). To ensure the breadth of the search scope, the search terms were confirmed as "multiple sclerosis" (MS) or "amyotrophic lateral sclerosis" (ALS) or "Parkinson's" or "Alzheimer's" or "Huntington's" or "Neurodegenerative." The document type has no restrictions, and the language has been restricted to English only.

Recognition of the 100 Top-Cited Articles

After confirming the search scope, 593,050 articles were expressed in descending numerical order according to the citations. The top 150 articles have been exported, the full text has been read in order by two researchers, respectively. After excluding six articles that (1) were completely irrelevant to the neurodegenerative disease and (2) mentioned the neurodegenerative diseases while focusing on irrelevant topics, the 100 top-cited articles were confirmed (**Supplementary Table 1**).

Extraction and Analysis of the Data

The full record contents were exported from the Web of Science in the format of both plain text (for the analysis in VOSviewer) and Tab-delimited (for the manual check and analysis). The basic information included citation number, publication year, topic, study type, authorship, journal, country, and institute of responding author and foundation. The VOSviewer is a software for creating maps such as coauthorship, co-occurrence, citation, bibliographic coupling, or co-citation links. Other than mapping, all the contents have been checked manually, and the descriptive analysis has been performed using SPSS Statistics for Windows (Armonk, NY: IBM Corp.). The authors of each article were extracted, and the connection between authors was analyzed using VOSviewer (developed by Nees Jan van Eck and Ludo Waltman at Leiden University's Centre for Science and Technology Studies, Leiden, Holland) (version 1.6.15). Items have been grouped into clusters according to the total link strength attribute, which indicates the total strength of the co-authorship links of a given researcher with other researchers. Also, the color of the item is determined by the publication year of their articles. The algorithms of VOSviewer have been explained in the previous study (Van Eck and Waltman, 2017).

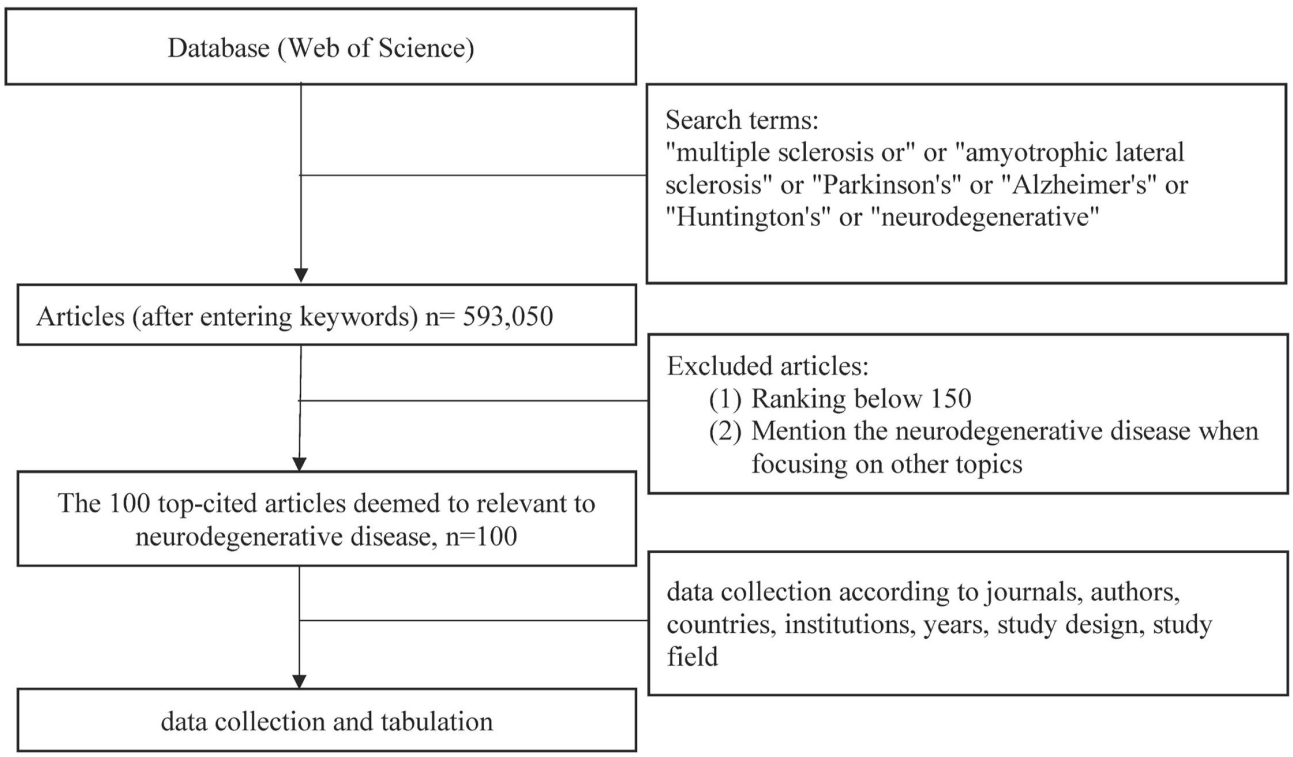

FIGURE 1 | Flowchart of the methodology used in this study.

The evidence levels of the top-100 articles have been determined according to the rating criteria (Guyatt et al., 2011). We read the full text of each article and tried to match each article from level 1 to level 5 according to the description of the rating criteria.

RESULTS

The articles were searched and gathered on July 22, 2020. A total of 593,050 articles were identified from 1950 to 2021 (**Figure 1**). There is an upper trend showed in the number of publications in the field of neurodegenerative diseases. In the last decade (2000–2009), 157,975 articles have been published, and the number doubled (318,864 articles) from 2010 to 2019.

The Basic Characteristics of 100 Top-Cited Articles

The 100 top-cited articles were published from 1963 to 2013 and are listed in **Table 1**. Article citations range from 2,525 to 22,887 in total and 62 to 636 per year. The topic of the top 1 cited article is Alzheimer's diagnosis in 1984 (McKhann et al., 1984), which received 22,887 citations in total and 636 citations per year. The citations in 2019 and 2020 have been independently identified to show the present citation activity of highly cited articles, respectively, ranging from 43 to 1,424 and 24 to 799.

Publication Years of 100 Top-Cited Articles

Notably, 45% of the top-cited articles were published during 2000–2009, followed by 30 articles from 1990 to 1999 (**Figure 2**). After being divided into clinical (which involves clinical trials and other research protocols, and strictly controlled human studies of new therapies), basic research (which were performed in the laboratories using beakers and test tubes, not people. To help people better understand what causes a disease, to analyze how current treatments work, and to develop new potential therapies), and review, it showed that basic research reached a peak at 19 articles during 1990–1999 while clinical research peaked at 15 during 2000–2009. Before 1996, no relevant review is available while there is a great increase during 2000–2009 (i.e., 18 review articles in total). Most of the citations revealed the same trend as the number of publications, but the citation number in the 1980s is larger than that in the 1990's.

Foundation, Country, and Institution of 100 Top-Cited Articles

Considering the origination of these 100 top-cited articles, the respondent authors of the majority of the publications were from the United States ($n = 68$) while nine publications came from the United Kingdom. Japan contributed four articles. Germany, Canada, and France published three articles each (**Table 1**).

Forty-seven articles have been funded by the National Institutes of Health (NIH), which belongs to the United States. Followed by Bayer, contributed 6 top-cited articles. National Multiple Sclerosis Society sponsored five articles while the MS topic only takes up 10% of the 100 top-cited articles. The top seven institutions in terms of the maximum number of articles were situated in the United States. Both Harvard University and NIH were associated with the same numbers of articles (eight of the 100 articles), followed by five articles from the University

TABLE 1 | Country, institute, and foundation with multiple articles in the most-cited list.

Article characteristics			No. of articles	Citation/article
Country		United States	68	4,333
		United Kingdom	9	3,794
		Japan	4	3,278
		Germany	3	5,130
		Canada	3	5,000
		France	3	3,283
Institute		Harvard University	8	4,147
		National Institutes of Health	8	4,947
		University of California	5	3,429
		Johns Hopkins University	4	3,827
		Washington University	4	3,460
Foundation	Governmental organization	NIH	49	3,856
		United States Department of Health & Human Services	4	4,142
		Medical Research Council UK (MRC)	3	3,473
	Companies	Bayer	8	4,157
		Pfizer	4	4,304
		Merck	4	5,119
		Eli Lilly	3	4,303
		Novartis	3	4,592
	Non-governmental organization	National Multiple Sclerosis Society	5	4,432
		Wellcome Trust	5	4,056
		Howard Hughes Medical Institute	3	4,898

of California. Moreover, in the top 10 institutes with multiple articles, six of which are universities.

Journal and Author of 100 Top-Cited Articles

Table 2 shows the top journals contributing maximum articles in the 100 top-cited articles in the field of neurodegenerative diseases. We categorized journals with multiple articles into general journals ($n = 7$) and specialty journals ($n = 9$). As our research focused on the field of neurodegenerative disease, we defined "Specify Journal" in this study as those journals that have been classified into "Neurosciences and Neurology" in Web of Science. Other journals that have been classified into "Science and Technology—Other Topics" have been regarded as general journals in our research. Journal SCIENCE [2020, Impact Factor (IF) = 47.728] contributed 17 articles, and the average citations of 17 articles are 4,058. While NATURE (2020, IF = 49.962) had 12 articles, followed by NEUROLOGY with nine articles. The average citations of the articles published in NEUROLOGY rank first at 6,636. In total, only 15 journals have more than two articles, but the average of citations has no huge difference, ranging from 2,690 from NEW ENGLAND JOURNAL OF MEDICINE to 6,836 from NEUROLOGY.

Figure 3 showed the connections and publication orders between authors. The 58 authors, who contributed to more than one article by VOSviewer, have been identified. To make a clear connection between authors, we hid the names of those authors, which have no connection or one link strength with others ($n = 9$). In cluster A, those authors published articles before 1995 and mainly focused on improving the clinical diagnosis. Cluster B has the largest connection, which included 12 authors. They came up with the amyloid hypothesis of AD around 2000. Cluster C included the author who mainly focused on making the criteria for the MD diagnosis. The latest articles in 100 top-cited articles were mainly published by the authors in cluster D. They contributed to defining the stages of AD, and some of them revealed the stage of the disease called "mild cognitive impairment." To conclude, the clusters of authors reveal the evolution of research focus in neurodegenerative diseases.

Study Topic and Study Type of 100 Top-Cited Articles

Among the 100 research articles, the articles with the topic of AD take the biggest part ($n = 43$), followed by 30 articles related to neurodegenerative diseases in general. Another two topics, PD and MS, respectively, contributed 11 and 10 articles. Only one article focuses on Huntington's disease (HD). However, there is an article about dementia with Lewy bodies shown in the 100 top-cited articles while this disease is not used as the search term. We classified the articles by our researchers according to the topic and the contents. We referred to some published articles to decide our study types (Nichols et al., 2004; Park et al., 2020). Study type in these 100 articles can be categorized into pathology ($n = 39$), etiology ($n = 7$), genetics ($n = 11$), imaging ($n = 4$), diagnosis ($n = 23$), and therapy ($n = 8$) (**Figure 4**); the type "descriptive" encompasses those articles, which in general describes a disease

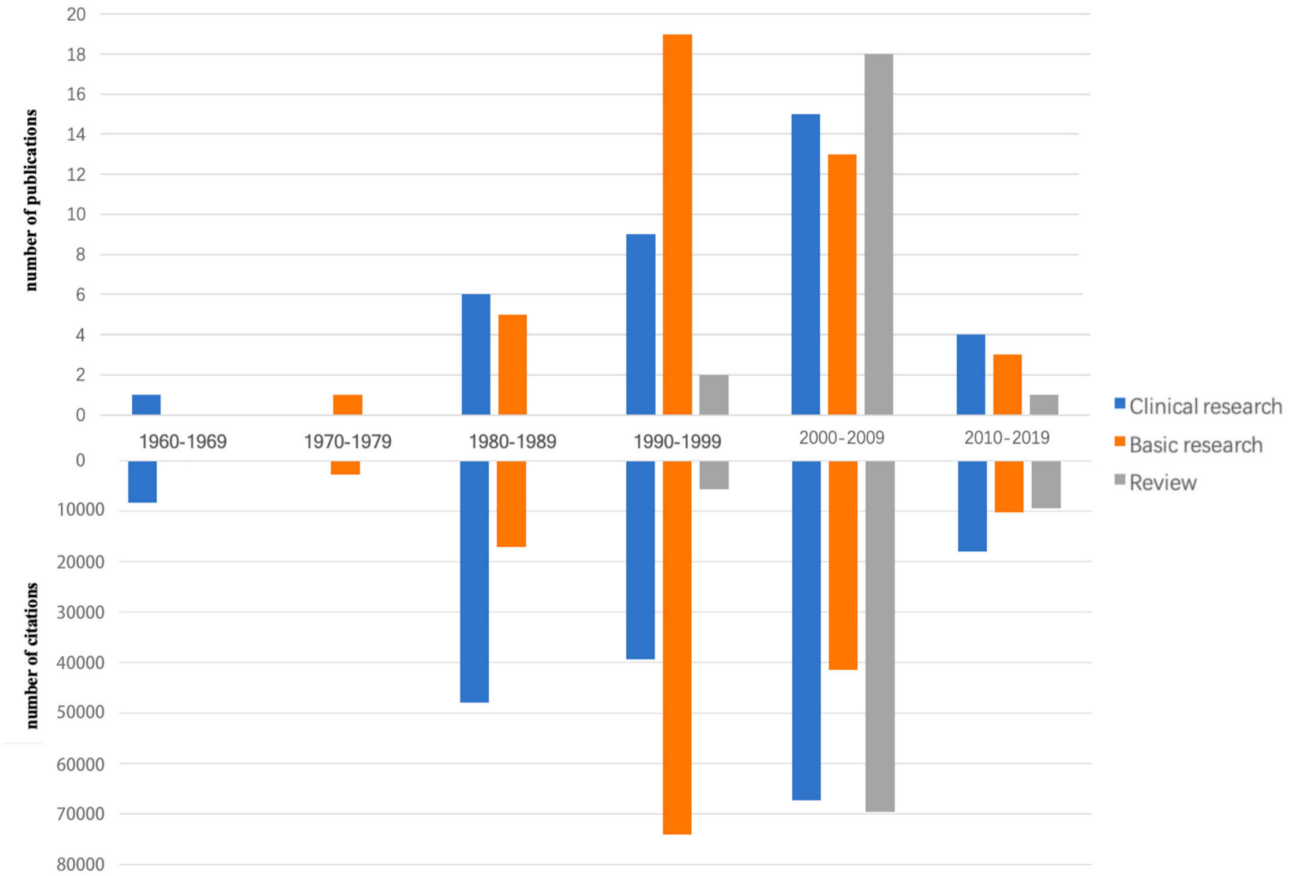

FIGURE 2 | The total number of publications and citations according to a decade in 100 top-cited articles in neurodegenerative diseases.

(e.g., Parkinsonism: onset, progression, and mortality; Mm and Yahr, 1967).

Evidence Level of Clinical Articles ($n = 22$)

As most of the highly cited articles were published at the early stage of the research in the field of neurodegenerative diseases, basic researches take a bigger role in these 100 articles ($n = 45$), in which 22 clinical articles have been identified and categorized. Only one article belongs to evidence level 2 in the field of AD (published in 2004). While level 4 (case series with or without intervention; cross-sectional study) included six articles in total, there are 15 articles in evidence level 5. All the articles were about the diagnosis criteria from the opinions of respected authorities. The research focus is displayed in **Table 3**.

DISCUSSION

Bibliometric analysis can provide enormous information with journals, institutions, authors, and countries, which is available for identifying landmark articles and high-impact journals (Shi et al., 2021). It not only provided a historical prospect in the

TABLE 2 | Journals with multiple articles in the most-cited list.

Category	Journals	No. of articles (%)	Citation/ article
General journals	SCIENCE	17	4,058
	NATURE	12	3,741
	CELL	5	4,271
	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATE OF AMERICA	3	3,644
	LANCET	3	2,961
	NEW ENGLAND JOURNAL OF MEDICINE	2	2,691
Specialty journals	NEUROLOGY	9	6,836
	ANNALS OF NEUROLOGY	6	4,402
	PHYSIOLOGICAL REVIEWS	4	4,308
	ALZHEIMER'S AND DEMENTIA	3	4,304
	ARCHIVES OF NEUROLOGY	3	4,066
	NEURON	3	3,348
	NEUROBIOLOGY OF AGING	2	4,148
	JOURNAL OF INTERNAL MEDICINE	2	3,440
	CLINICAL NEUROPHYSIOLOGY	2	3,404

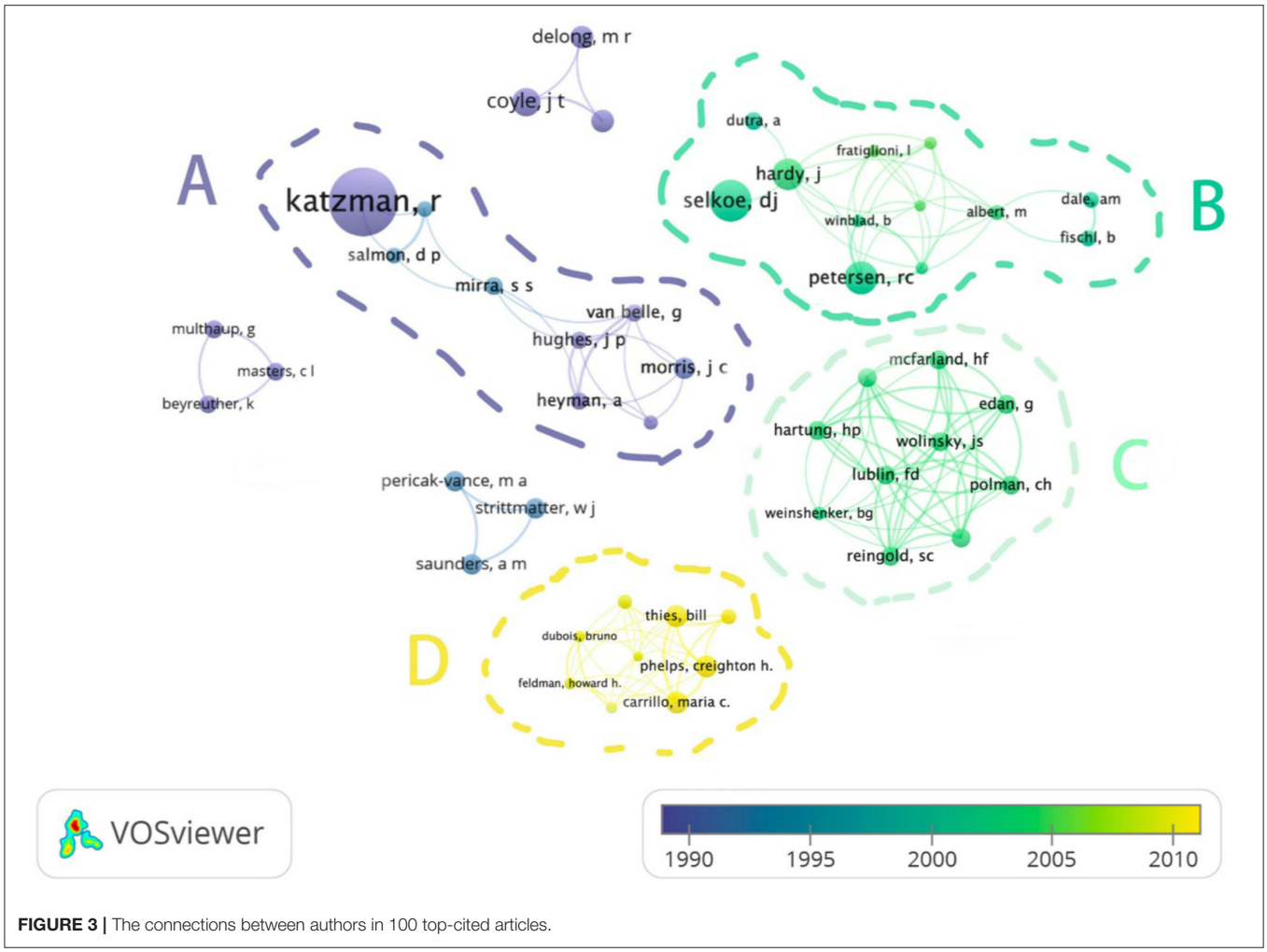

FIGURE 3 | The connections between authors in 100 top-cited articles.

field of neurodegenerative diseases but also revealed the trend of researches.

A few of the top-cited studies in the field of the neurodegenerative disease have been reported. In 2009, a bibliometric analysis related to AD has been reported by Sorensen. In 2018, Xue et al. reported the first time "The 100 most-cited articles in Parkinson's disease." However, no top-cited bibliometric analysis has been conducted on the whole field of the neurodegenerative disease, which encompasses a broad array of conditions that include AD, PD, HD, MS, and ALS, with distinct clinical and pathological features. The articles of several citation analyses have been conducted on a specific disease [Aaron A conducted a citation analysis in the field of AD (Shen et al., 2019)]. However, neurodegenerative diseases share some common features, such as they are all related to progressive functional loss and neuron death within the central nervous system, also some intracellular protein degradation pathways (Laplante and Sabatini, 2012), such as autophagy. In order not to miss the researches on the common features of neurodegenerative diseases, our research encompasses all the neurodegenerative diseases.

The older an article is, the more likely it is cited (Jiang et al., 2016). Thus, articles published after 2010, while ranking top 100, might be the recent research hot spots. In our study, four of 10 articles, which were published after 2010, focused on improving the assessment tools of neurodegenerative diseases. Meanwhile, there is a turning point in the trend of citations while the number of publications increased decade by decade (**Figure 2**). It might mean the large outbreak happened in the 1980's and 2000's, which caused a huge increase in the number of citations.

In this study, the articles on neurodegenerative diseases from 1963 were bibliometrically analyzed. We found that all the articles are still been actively cited nowadays, especially in the diagnostic type. Similarly, the Montreal Cognitive Assessment (MoCA) got 799 citations during 2020, and the NINCDS-ADRDA (Nasreddine et al., 2005; Shen et al., 2019) (clinical diagnosis of AD) got 318 citations. The study type "diagnostic" takes up 23% of the 100 top-cited articles, but most of them are the opinions of respected authorities, and only one research conducted a clinical trial (Sorensen, 2009). Meanwhile, according to the result in VOSviewer (**Figure 3**), it also showed that the topic AD among neurodegenerative diseases attracted most

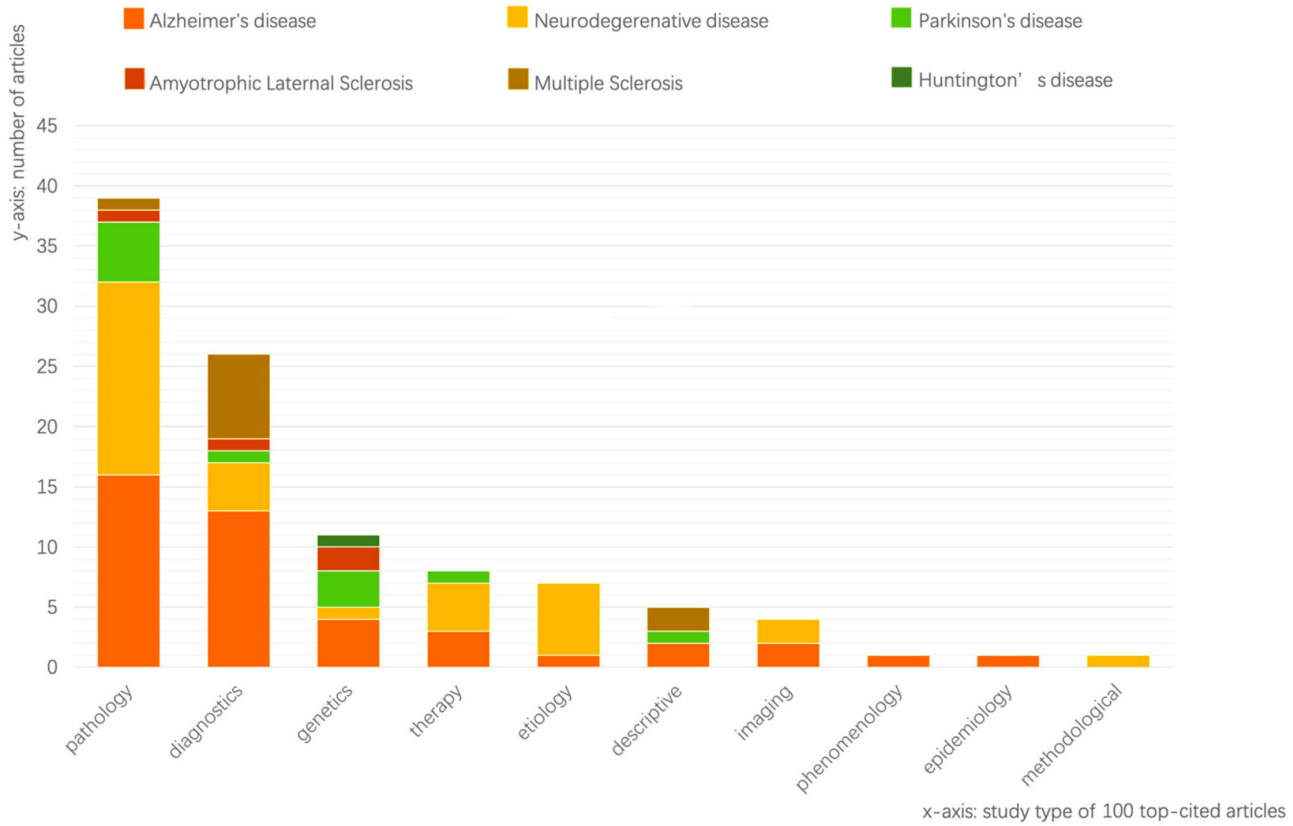

FIGURE 4 | Statistics of the study topic and type of the 100 top-cited articles.

of the researchers and has been studied internationally and collaboratively. Apart from the amyloid hypothesis, the other two major clusters were both focused on the diagnostic of AD. It might indicate that a lot of researches focused on the improvement of the diagnostic assessment tools in the field of neurodegenerative diseases. Moreover, the therapy articles are relatively less, and none of them conducted clinical trials. Thus, more effort should be paid to the studies on the therapy of neurodegenerative diseases other than AD.

We found that the United States confirmed the tremendous impact on the field of neurodegenerative diseases. The top five institutes with multiple articles cited are all located in the United States; meanwhile, the United States governmental organization NIH financially supported 49 types of studies. There is no doubt that the United States is a country with a research atmosphere, but it should also be concerned that most journals are from the United States, and the American reviewers possibly prefer US articles (Klunk et al., 2004). However, recently, other countries, such as Japan and China, have contributed significantly to global Alzheimer's research. Still, strengthening international cooperation could improve the quality and number of publications (Link, 1998).

The result of the journal analysis showed that the top-cited articles were published both in specialty journals (such as ALZHEIMER'S and DEMENTIA and NEUROBIOLOGY OF AGING) and in general journals (such as LANCET and NATURE). In the field of neurodegenerative diseases, specialty journals have attracted more attention to scientists in the past several decades (Dong et al., 2019). However, the journal that published most articles in the top-cited list is SCIENCE ($n = 17$), which is a general journal. This indicates that researchers should be encouraged to submit their articles to general journals that might attract the attention of more researchers from different fields.

A general limitation of our analysis is that bibliometric analysis is not an "exact science" and relies on interpretation and reiteration to achieve a "best fit" data set that will adequately describe the research area while excluding the articles of marginal relevance (Lasjaunias, 1999). We chose Web of Science because it is informally considered as the most accurate bibliographic source in the world, spans over 100 years, and includes several dozens of millions of publication records (Šubelj et al., 2014; Breugelmans et al., 2015). Another limitation is that the 100 top-cited articles are low-level evidence research because most of the highly cited articles were published at the beginning of the field, and some of them were raising important hypothesizes or diagnostic standards, which lack high-quality clinical evidence. In the future, instead

TABLE 3 | Evidence levels of clinical articles in 100 top-cited articles in neurodegenerative diseases.

Level	Rating criteria	No.	Corresponding article	Participants	Measurements
1	Properly powered and conducted randomized clinical trial; systematic review with meta-analysis	0	-	-	-
2	Well-designed controlled trial without randomization; prospective comparative cohort trial	1	76	16 AD patients ranging in age from 51 to 81 years old	Whole-blood samples after injection of 18F-fluorodeoxyglucose(18-FDG)& PIB
3	Case–control studies; retrospective cohort study	0			
4	Case series with or without intervention; a cross-sectional study	6	4	802 patients exhibited some or all of the accepted cardinal signs	263 patients during the 2-year period
			79	Group1-19 men and 8 women with Alzheimer's disease and 26 normal subject group2-10 Alzheimer's subjects and 10 normal subjects	Two raters evaluated every subject to obtain inter-rater reliability of the diagnostic tool. A third interview was conducted 12 months after the initial session.
			6	94 patients meeting with mild cognitive impairment, 93 patients with mild Alzheimer's disease, 90 healthy elderly controls	The MoCA and MMSE of patients
			66	25 patients with chronic progressive MS with Expanded Disability Status Scale scores ranging from 3.0 to 6.5 29 patients with systemic lupus erythematosus 20 normal healthy adults.	28-item fatigue questionnaire
			13	76 consecutively evaluated subjects with mild cognitive impairment 234 healthy control subjects 106 patients with mild AD	Mini-Mental State Examination, Wechsler Adult Intelligence Scale–Revised, Wechsler Memory Scale–Revised, Dementia Rating Scale, Free and Cued Selective Reminding Test, and Auditory Verbal Learning Test were administered to all participants.
			7	100 consecutive cases clinically diagnosed invasive pneumococcal disease	Brains collected between June 1987 and August 1990
5	Opinion of respected authorities; case reports	15	8, 15, 19, 22, 34, 35, 40, 42, 59, 65, 69, 80, 87, 92, 95	-	-

of measuring the validity or the scientific quality of top-cited publications, the impact and the trend of the research might be focused more, and new analysis strategies, such as calculating the number of citations in the recent years and the number of citations per year, which showed the activeness of the articles, should be added to the basic analysis of the bibliometric studies.

CONCLUSIONS

In conclusion, this study provided information on the top-cited articles in neurodegenerative diseases by using specific keywords for reference search in Web of Science. By systematically analyzed the quantity and quality of the articles in neurodegenerative diseases, it reveals that, first, the pathology and diagnostic researches took a major role in 100 top-cited articles while the therapy articles are relatively less. Second, the United States confirmed the tremendous impact on the field of neurodegenerative diseases. Third, researchers also submitted their researches to general journals, not just focused on specialty journals.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JS and ZL did all the revisions, wrote the latest versions, and screened the articles. XK, MY, and ZL extracted the data from each of the articles. MY, WW, and ZL performed the analysis and drafted the manuscript. JS supervised the project. All

authors contributed to the conception and design of the study, commented on the previous versions of the manuscript, and read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2021.722944/full#supplementary-material

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BRIEF RESEARCH REPORT
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Blocking HMGB1/RAGE Signaling by Berberine Alleviates A1 Astrocyte and Attenuates Sepsis-Associated Encephalopathy

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As a life-threatening multiple organ dysfunction attributable to maladjusted host immune responses to infection, sepsis is usually the common pathway to serious prognosis and death for numerous infectious diseases all over the world. Sepsis-associated encephalopathy (SAE) is frequently complicated by septic conditions, and is one of the most important reasons for increased mortality and poor outcomes in septic patients which is still an urgent clinical problem need to be solved. In this research, a conspicuously discovery of treatment-related translational use for berberine was elaborated. The results revealed that berberine treatment significantly restored cognitive impairment in sepsis mice. Reduced expression levels of TNF-α, IL-1α, and C1qA were exhibited in the hippocampus of the berberine treatment group, and attenuated effect of declining neo-neuron, activation of microglia and astrocytes in the hippocampus of mice with sepsis were also found. Moreover, berberine inhibits microglia-stressed A1 astrocytes by inhibiting HMGB1 signaling was revealed, then the molecular mechanism of HMGB1/RAGE signaling inhibition leads to the better outcome of SAE was elucidated. To summarize, this research indicated that berberine targets HMGB1/RAGE signaling to inhibit microglia-stressed A1 astrocyte and neo-neuron decline, which consequently alleviates sepsis-induced cognitive impairment. Collectively, berberine may serve as potential therapeutic drug and HMGB1/RAGE signaling would be a novel target for medicine development for treating SAE.

Keywords: berberine, sepsis-associated encephalopathy, HMGB1/RAGE signaling, cognitive impairment, inflammatory cytokines, neuropharmacology, women in neuroscience

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INTRODUCTION

Sepsis is one of the most important leading causes of mortality and morbidity worldwide. According to the third international consensus definitions for sepsis and septic shock (Sepsis-3) in 2016, sepsis should be defined as life-threatening organ dysfunction caused by a dysregulated host response to infection (Singer et al., 2016). Approximately half of sepsis patients suffer from encephalopathy, which induced most of cognition damage and changed mentality in intensive care units (ICU) (Liddelow et al., 2017).

Sepsis-associated encephalopathy (SAE) is a diffuse brain disturbance that usually occurs following infection in the body with hardly any central nervous system infection. SAE brought greater risks for long periods of cognitive impairments, which contains alteration in visual-spatial abilities, deficits in visual and functional memory with depressive and/or anxiety disorders (Iacobone et al., 2009; Feng et al., 2019). The quality of life of sepsis patients will be harshly affected by all these faultiness. Furthermore, a lot of limitations and difficulties in clinical practical operation strongly impeded the accurate assessment of cognition and sensory functions in sepsis patients. Consequently, SAE has enhanced harmful effects on patients and brought society a heavy and painful burden.

Despite the growing number of studies have focused on the pathophysiology and related molecular mechanisms, however, the precise etiopathogenesis of sepsis as well as SAE still remain obscure. In sepsis, a great amount of pro-inflammatory cytokines and damage associated molecular patterns (DAMPs) released, including tumor necrosis factor-α (TNF-α), interleukin (IL) family and high mobility group box 1 (HMGB1) which lead to further organ dysfunction and multiple cell death (Andersson and Tracey, 2003; Lu et al., 2013; Denning et al., 2019; Feng et al., 2019; Wu et al., 2021). Exposed to all these inflammation related factors usually cause tissue damage in susceptible areas of the brain like hippocampus. Therefore, effective treatment towards related molecular targets and other key mediators in the pathogenesis of sepsis were imperative in avoiding the happening of SAE (Gofton and Young, 2012; Kuperberg and Wadgaonkar, 2017; Zhong et al., 2020).

Recently, with the rapid development in neuropharmacology, plenty of neurotherapeutic natural products were identified, including ginsenoside Rg1, baicalein, curcumin and gastrodin have proved effective in treating SAE through suppression of different signaling pathways (Li et al., 2017; Fu et al., 2018; Alikiaii et al., 2021). Berberine, a quaternary ammonium salt from the protoberberine group of benzylisoquinoline alkaloids found in such plants as Berberis which can be isolated from a variety of plants, has already shown its excellent effect in anti-oxidant, anti-cancer and anti-diabetic treatment (Prajwala et al., 2020; Yuan et al., 2021). In the field of sepsis research, berberine could partly attenuate sepsis-induced multiorgan dysfunction and neutrophil tissue infiltration, it could also prevent intestinal mucosal barrier damage at the early stages of the disease (Li et al., 2015; Pierpaoli et al., 2020). However, whether berberine treatment attenuates SAE and improves cognitive functions after sepsis still remain to be elucidated.

In this study, we aimed to evaluate the effects and the underlying mechanisms of berberine on cognitive deficits induced by caecal ligation and puncture (CLP) in mice, which may facilitate the medicine development for treating SAE.

MATERIALS AND METHODS

Ager$^{-/-}$ (or *Rage*$^{-/-}$, KOCMP-11596-Ager-B6N-VA, Syagen) and WT C57BL/6 male mice (Hunan SJA Laboratory Animal Co., PRC) (8–10 weeks, 22–25 g) were adopted herein. The animals were kept under a special sterilized status in Central South University (CSU) with normal parameters (22–25°C and a 12-h light-dark cycle). The entire research was accepted and coincided with the protocols of CSU (IRB 2021-S076).

For the CLP pattern, the caecum was subjected to exposure posterior to a 1.5 cm longitude middle line incision in mice under the anesthesia via 2% isoflurance with O_2. A polymicrobic septic pattern was completed via the ligation of half of the caecum (Mild-grade type with around 40% expected mortality) and the squeezing out of a little egesta from a complete puncture by 18-gauge needles. The caecum was replaced and the stomach was sealed. NC (37°C; 5 ml per 100 g body weight) was given by subcutaneous injection to awaken the animals from anesthesia. Pseudo operation mice were treated with the identical process apart from the ligating and puncture. Ten days posterior to the CLP treatment, open field test, novel object recognition and Morris water maze test assay were completed.

Open Field Test

The manoeuvrability of the animals was evaluated through Open Field Test before the cognitive assay (Gould et al., 2009). Overall, the animals were placed carefully in a 50 × 50 cm quadrate case, and a 10-min free movement is permitted. The move was recorded, and the overall distance was documented and studied.

Novel Object Recognition

Novel object recognition (NOR) assay was completed as above mentioned (Antunes and Biala, 2012). In short, the same two items were placed symmetrically with the same distance from the central point and the walls. The animals were permitted to finish the training as above mentioned. Afterwards, the time costed for every item was documented. 24 h later, during the test phase, one of the items was substituted with a new one with diverse appearance. The time costed for every item was documented. Their move was documented and studied, while the difference denotes the rate of sniffer time on the new item to the two items.

Morris Water Maze Test

The space learning and retention of the animals were evaluated via Morris water maze (MWM) testing (Vorhees and Williams, 2006). In short, a crystal round terrace was placed 1 cm under the water at one of the quadrants. The animals were permitted to be on the terrace for half a minute to retain the surroundings, and were afterwards placed in the water for training. In every trail, the animals were permitted to discover the terrace in 1 min. If they didn't succeed, they would be led to the terrace and be on it for half a minute. The training was completed 3 times a day for 4 days, where the release quadrant was altered every trial. Then, the terrace was moved away and the animals were placed in the heterolateral quadrant of the terrace. The move was documented in every assay, and the time to the terrace was studied.

Immune Staining

After the perfusion of DPBS (pH 7.4) and 4% PFA, the cerebra were treated with fixation via 4% PFA overnight. The cerebra were subsequently subjected to dehydration via graded saccharose liquor, treated with embedment in OCT and incessantly sliced into 30 μm cristated slices at 20°C. Blocked by 5% BSA and 0.1% TritonX-100 for 60 min at ambient temperature, the sections were

cultivated via the first antibody at 4°C overnight, such as anti-mouse antibody to IBA1 (1:500), GFAP (1:500), DCX (1:500), and NeuN (1: 500, Millipore, America). A cultivation of second antibody (1: 500) for 120 min was subjected completed for the sections. We completed 3 times of cleaning via 0.01 M PBS with 0.1% TritonX-100 between every step. The sections were subjected to image formation via the microscope under the identical illuminous intensity and exposal time.

qRT-PCR

Overall RNA of purified or cultured cells was separated via Trizol (Life Technologies, Gaithersburg, MD) and turned into cDNA through reversal transcription via Reversal Transcription Kit (Cat #k1622, Thermo Fisher Scientific) as per the supplier's specification. Designed primer pair (mouse TNF-α: 5′-CCCTCACACTCAGATCATCTT CT-3′, 5′-GCTACGACGTGGGCTACAG-3′; IL-1α: 5′- CGAAGA CTACAGTTCTGCCATT-3′ 5′-GACGTTTCAGAGGTTCTCAGA G-3′; C1qA: 5′-AAAGGCAATCCAGGCAATATCA-3′ 5′-TGG TTCTGGTATGGACTCTCC-3′; C3: 5′-CCAGCTCCCCATTAG CTCTG-3′; 5′-GCACTTGCCTCTTTAGGAAGTC-3′) and TaqMan Universal PCR master mix (Applied Biosystems, America) were adopted for the amplification of targeted cDNA fragments. Standardization was completed as per the magnification of GAPDH. The expressing levels of the targeted genes were described as the fold variations compared with the control group.

ELISA Quantification of Cytokines

Purchasable kits (88-7324-88 for TNF-α and 88-5019-88 for IL-1α from Invitrogen; WEA747Mu for C1q and SEA399Mu for HMGB1 from Cloud-Clone) were employed to determine the quantity of cytokines in the media or the plasma as per the supplier's specification.

Primary Cell Culture

Primary mouse microglia and astrocytes were separated from P3-4 WT pups. Anti-GLAST MicroBead Kit (Miltenyi Biotec, Germany) was adopted to purify astrocytes. After a challenge of LPS (1 μg/ml, InvivoGen) or recombinant HMGB1 (400 ng/ml, Sino Biological) for 3 h, the medium was harvested and applied to astrocytes for 24 h. The TNF-α quantity in the supernatants were measured via ELISA.

Statistical Analysis

The statistic assay was completed via GraphPad Prism 6.0. The normal distribution of date was assumed based on normality test. The figures were studied via Student's t test to contrast between the two groups. Contrasts among ≥2 groups were finished via ANOVA with post hoc test. A p value <0.05 was deemed as important on statistics. Assays were separately completed ≥3 times while the entire data were expressed as the mean ± SEM.

RESULTS

Berberine Alleviates Cognitive Impairment and Neo-Neuron Decline in Septic Mice

To determine the effects of berberine on SAE, mice were subjected to OFT to assessing the mobility prior to the tests of NOR and MWM. The results documented that no significant difference was found between groups (**Figure 1A**), indicating that mobility was restored and would not interrupt the test of cognitive function. In the training phase of NOR, mice in all groups showed similar discrimination ratio (DR) and the DRs were around at 0.5, which means that mice have equal chance to access the two objects and the setting of NOR is appropriate (**Figure 1B**). When the novel object was set in the testing phase of NOR, as predicted, sham mice administrated with either saline or berberine had a memory of the object with a DR over 0.5, whilst mice with CLP alone lost the memory of the old object and consequently had a DR around 0.5. Administration of berberine significantly restored the decline of cognitive function cause by CLP (**Figure 1C**). In addition, swimming capacity in MWM was not significantly different between groups (**Figure 1D**). In compared with negative setting of sham mice, learning ability as assessed in the training phase of MWM was significantly dampened in the mice with CLP alone, but was reversed by administrating berberine (**Figure 1E**). Similarly, CLP-decreased spatial memory determined in the testing phase of MWM was significantly restored by the treatment of berberine (**Figure 1F**). Collectively, berberine significantly restored cognitive deficits in mice challenged with sepsis.

It is reported that decline of neo-neurons is associated with cognitive impairment in a series of encephalopathy. To test the effects of berberine on sepsis-induced neo-neuron decline, Doublecortin (DCX) of the hippocampus of mice was visualized using immunofluorescence (Reiner et al., 2006; Klempin et al., 2011). The results showed that sepsis significantly decreased neo-neurons, which was reversed by the administration of berberine (**Figure 1G**). Thus, berberine may protect against sepsis-induced cognitive impairment by restoring neo-neuron decline.

Berberine Extinguishes Inflated Microglia and Astrocytes in Septic Mice

It has been shown that the activation of microglia and astrocytes is implicated in sepsis and contributes to impairment of neurogenesis (Liddelow et al., 2017). To determine the underlying mechanism by which berberine protests against SAE, the microglia (Iba1+) and astrocytes (GFAP+) of the hippocampus of mice were determined. Laparotomy alone or with administration of berberine did not have effects on the activation of microglia and astrocytes. By contrast, sepsis robustly activated microglia (**Figure 2A**) and astrocytes (**Figure 2B**) in the hippocampus, which was reversed by the treatment of berberine. Thus, the protection of berberine may attribute to the effects on suppressing activation of microglia and astrocytes in septic mice.

Berberine Alleviates Microglia-Stressed A1 Astrocytes by Inhibiting HMGB1 Signaling

The astrocytes could be classified into A1 and A2 phenotypes, in which A1 astrocytes exudes toxic factors that could kill mature neurons and oligodendrocytes, have been proved to be

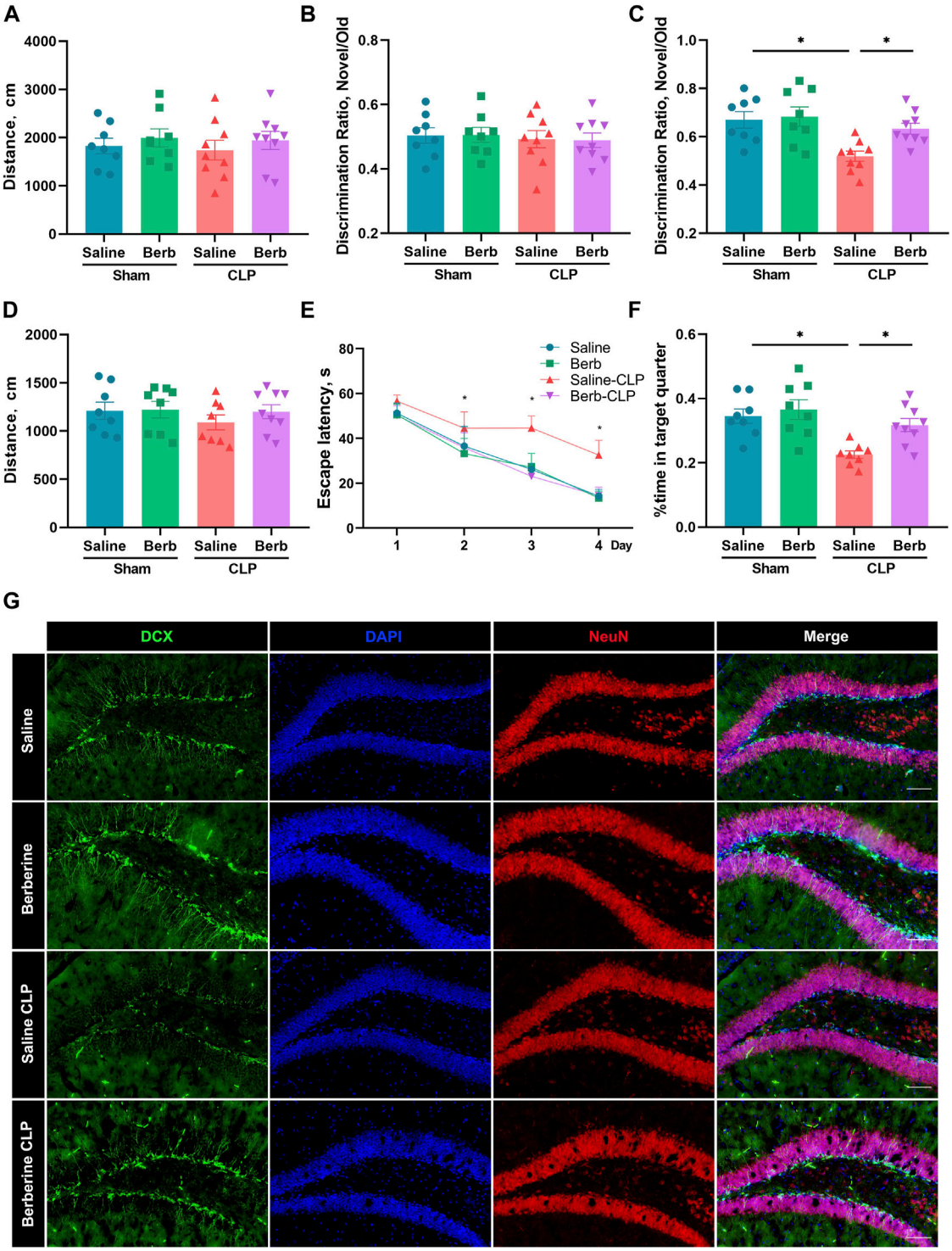

FIGURE 1 | Berberine attenuates cognitive impairment and neo-neuron decline in septic mice. **(A–G)** Mice were administrated with saline or berberine (10 mg/kg) 30 min prior to a challenge of cecal ligation and puncture. Traveling distance in Open Field Test **(A)**, discrimination ratio of training phase **(B)** and test phase **(C)** in Novel Object Recognition (n = 8, 8, 9, 9, respectively), and swimming distance **(D)**, escape latency of training phase **(E)** and time spent in the target quarter of test phase in Morris Water Maze (n = 8, 8, 8, 9, respectively). **(F)** Immunofluorescent staining of DCX (Green, 200×) and NeuN (Red, 200×) in the hippocampus (Scale bar = 100 μm). * indicates $p < 0.05$ in the comparisons of saline-treated CLP group with other groups.

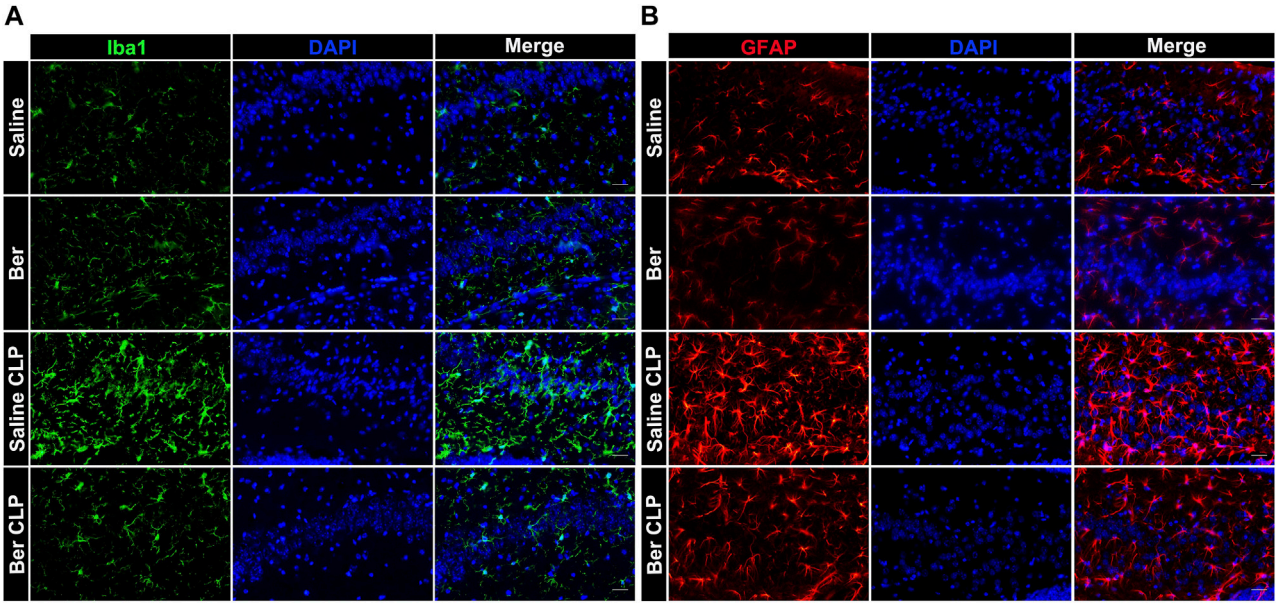

FIGURE 2 | Berberine alleviates activation of microglia and astrocytes in the hippocampus of mice with sepsis. **(A, B)**. Immunofluorescent staining of microglia (Iba1, Green, 400×) and astrocytes (GFAP, Red, 400×) in the hippocampus of mice administrated with saline or berberine (10 mg/kg) 30 min prior to a challenge of cecal ligation and puncture (Scale bar = 100 μm).

involved in a lot of different neurological diseases (Li et al., 2020; Ren et al., 2020) which is a very important factor in SAE. To further assess the mechanisms of berberine-mediated protection, expression levels of TNF-α, IL-1α, and C1qA, essential microglia-secreted cytokines in the formation of A1 astrocytes, were determined in the hippocampus of mice. The results demonstrated that the expression of TNF-α, IL-1α, and C1qA was remarkably increased in the mice challenged with CLP (**Figures 3A–C**). Administration of berberine significantly restored the highly expressed cytokines. LPS was reported to be the major stimulator of microglia-stressed A1 astrocytes. Here, LPS significantly boosted the level of TNF-α in primary microglia, whereas berberine slightly but not significantly decreased the augment of TNF-α (**Figure 3D**), indicating berberine may not inhibit TLR4-mediated TNF-α expression. Given that HMGB1/RAGE signaling is a pivotal upstream of TNF-α and implicated in SAE, the binding of berberine and HMGB1 was assessed using molecular docking. Berberine had a high binding energy with HMGB1 (−7.69, **Figure 3E**). In addition, berberine significantly restored the augment of plasma HMGB1 in CLP-challenged mice (**Figure 3F**). In in-vitro model, treatment of berberine significantly attenuated HMGB1-upgraded TNF-α, IL-1α, and C1q levels in primary microglia (**Figures 3G–I**) and HMGB1-mediated A1 astrocytes (C3, A1 astrocyte-specific, **Figure 3J**) in microglia and astrocyte co-cultured system. Moreover, in *in-vivo* model, berberine had inhibitory effects on C3 expression (**Figure 3K**). Taken together, it can be concluded that berberine reduces pro-inflammatory cytokine release and A1 astrocytes activation by inhibiting HMGB1 signaling.

Berberine Restored Sepsis-Induced Cognitive Impairment and Neo-Neuron Decline by Inhibiting HMGB1/RAGE Signaling

RAGE is the receptor of HMGB1 and mediates HMGB1 downstream signaling (Rauvala and Rouhiainen, 2007; van Zoelen and van der Poll, 2008; Deng et al., 2018). To further confirm the effects of berberine on HMGB1/RAGE signaling in the protection of SAE, WT or *Rage*$^{-/-}$ mice administrated with berberine or not were subjected to CLP or laparotomy alone, followed by cognitive tests. Similar moving distance between groups in OFT confirmed the sufficient mobility restoration for cognition tests (**Figure 4A**). Similar DRs around 0.5 between groups in the training phase indicated appropriate experiment setting of NOR (**Figure 4B**). In the testing phase of NOR, similar to administration of berberine, deficiency of *Rage* significantly restored the memory loss of old objects in septic mice (**Figure 4C**). Administration of berberine in Rage$^{-/-}$ mice did not further improve the cognitive restoration (**Figure 4C**). Moreover, activation of microglia and A1 astrocytes had similar trend (**Figures 4D–F**). Thus, berberine protects against SAE by inhibiting HMGB1/RAGE signaling.

DISCUSSION

In terms of morbidity and mortality among various serious diseases, sepsis imposes a substantial global burden and needs to be diagnosed and treated as soon as possible. The pathogenesis of SAE is inextricably linked to neuroinflammation, with

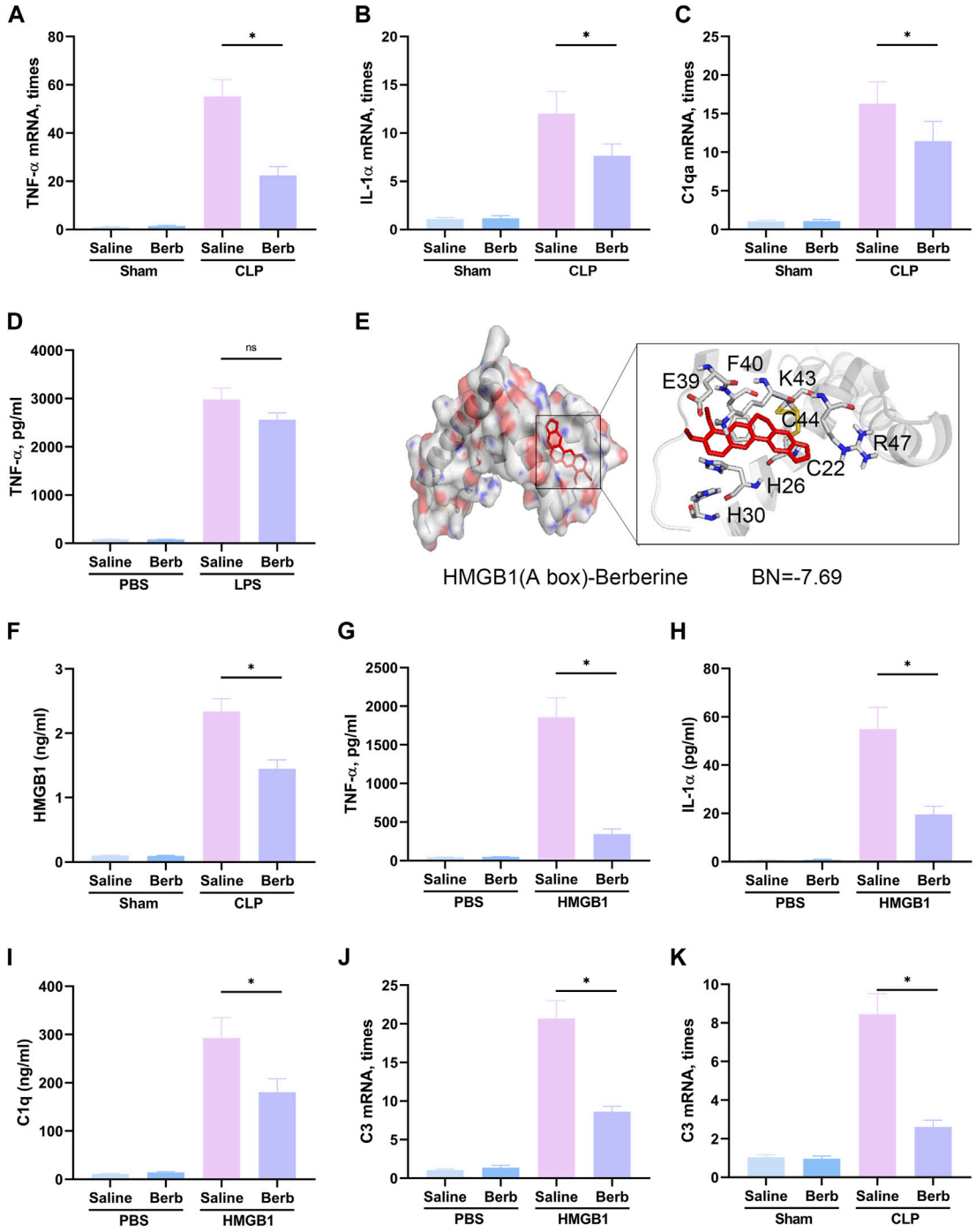

FIGURE 3 | Berberine inhibits microglia-stressed A1 astrocytes by inhibiting HMGB1 signaling. **(A–C)**. Expression of TNF-α **(A)**, IL-1α **(B)**, and C1qA **(C)** in the hippocampus of mice administrated with saline or berberine (10 mg/kg) 30 min prior to a challenge of cecal ligation and puncture. **(D).** Medium level of TNF-α in the microglia treated with beriberine (5 μM) or saline prior to a challenge of LPS (1 μg/ml) or not. **(E)**. Binding of berberine and HMGB1 in the analysis of molecular docking. **(F)**. Plasma levels of HMGB1 in mice administrated with saline or berberine (10 mg/kg) 30 min prior to a challenge of cecal ligation and puncture. **(G–I)**. Medium level of TNF-α **(G)**, IL-1α **(H)**, and C1q **(I)** in the microglia treated with beriberine (5 μM) or saline prior to a challenge of recombinant HMGB1 (400 ng/ml) or not. **(J)**. Expression of C3 (an A1 astrocyte-specific gene) in astrocytes co-cultured with microglia that were treated with beriberine (5 μM) or saline prior to a challenge of recombinant HMGB1 (400 ng/ml) or not. **(K).** Expression of C3 in the hippocampus of mice administrated with saline or berberine (10 mg/kg) 30 min prior to a challenge of cecal ligation and puncture. * indicates $p < 0.05$ in the comparisons of saline-treated with berberine-treated groups.

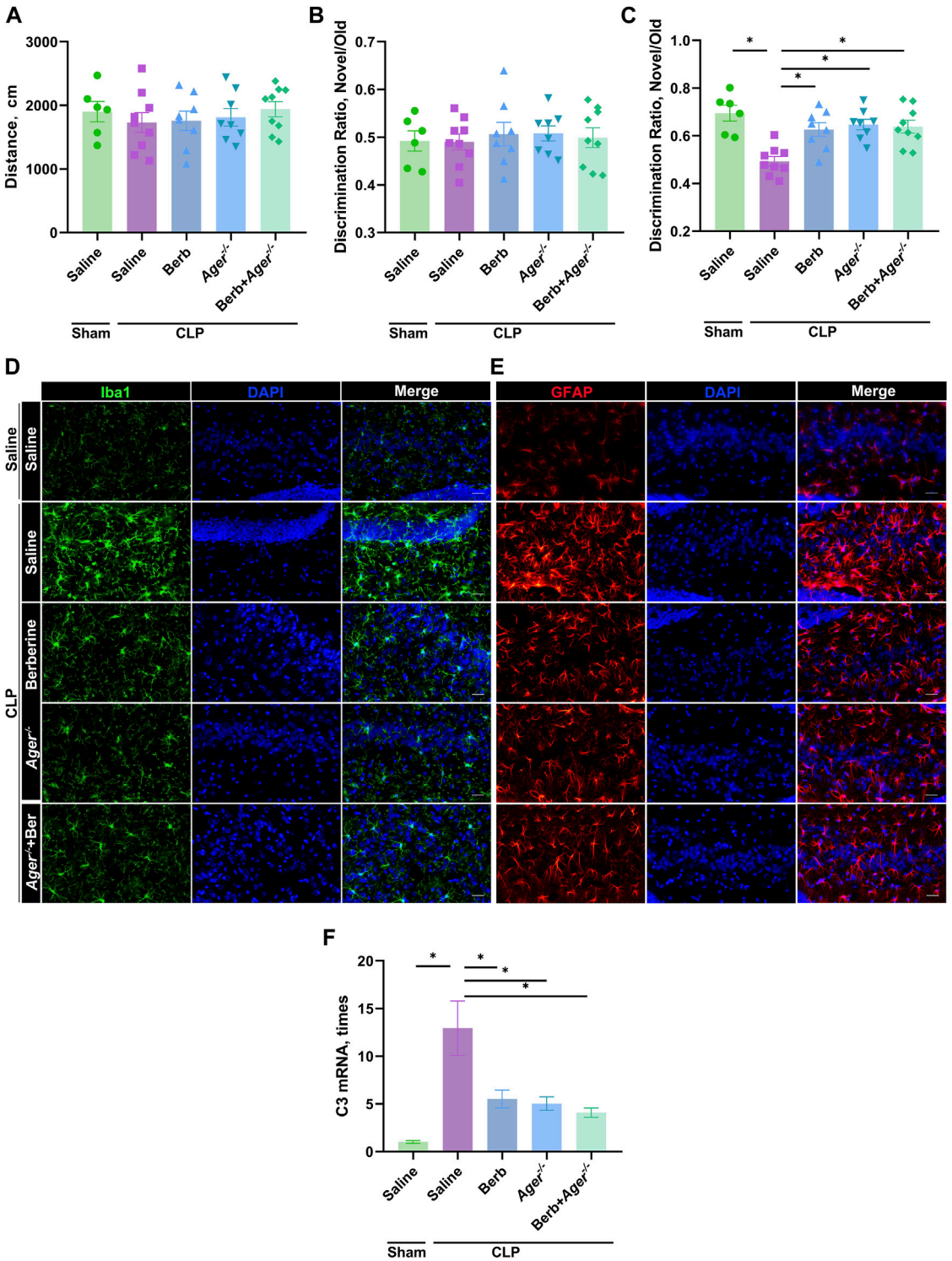

FIGURE 4 | Berberine improve sepsis-associated encephalopathy by inhibiting HMGB1/RAGE signaling. **(A–F).** Ager$^{-/-}$ or WT mice were administrated with saline or berberine (10 mg/kg) 30 min prior to a challenge of cecal ligation and puncture. Traveling distance in Open Field Test **(A)**, discrimination ratio of training phase **(B)** and test phase **(C)** in Novel Object Recognition (n = 6, 9, 8, 8, 9, respectively). **(D)** and **(E)**. Immunofluorescent staining of microglia (Iba1, Green, 400×) and astrocytes (GFAP, Red, 400×) in the hippocampus of mice (Scale bar = 100 μm). **(F)** C3 expression levels in the hippocampus of mice * indicates $p < 0.05$ in the comparisons of saline-treated CLP group with other groups.

uncontrolled inflammation as one of its main characteristics. Neuroinflammation is the major culprit for dysfunction and massive apoptosis of neurons, endothelial cells, and microglial cells (Iacobone et al., 2009; Liddelow et al., 2017). Both local and peripheral inflammation are brought out by the activation of resident brain immune cells, particularly the astrocytes. Previous research have proved that the induction of neuroinflammatory response and worse clinical prognosis are as a consequence of septic complications. And just as importantly, activated microglia can release a number of inflammatory mediators, the most important of which in SAE are TNF-α, IL-1α, and C1qA. These inflammatory mediators will further facilitate the formation of neuron-toxic A1 astrocytes and aggravate neuroinflammation (Xu et al., 2019; Lu et al., 2020). Then sustained damage of endothelial cells caused neuroinflammation leads to cerebral perfusion disorder, which makes SAE a sophisticated and intractable problem. Without any doubt, early diagnosis and treatment of SAE are extremely important.

Previous investigations have demonstrated that berberine and its correlative derivate exhibited extensive anti-inflammation and anti-tumor roles, which could be a new potential therapeutic drug (Kumar et al., 2015; Prajwala et al., 2020). Herein, our study revealed that the utilization of berberine before sepsis could avoid the progress of cognition function disorder in CLP-caused septic mice (Lu et al., 2020). More studies unveiled that such protection effect for cognition damage was closely associated with the decrease of pro-inflammation cell factors and the mitigation A1 astrocyte (Sun et al., 2019; Liu et al., 2020). More detailed mechanisms of those anti-inflammation roles of berberine have not yet been fully elucidated. Numerous signal pathways, such as CCR2 expressing in neutrophilic cells, TLRs, NF-KB, and PPARγ, are targets of berberine *in vitro* or vivo (Xu et al., 2017; Wang et al., 2018). The increasing beneficial berberine effect towards SAE and the precise mechanisms require further investigations.

Current studies have demonstrated that HMGB1, the late mediating factor in sepsis pathogenesis and an essential factor that mediates cognitive impairment in sepsis survivors (Chavan et al., 2012), triggers and sustains the inflammatory response by inducing cytokine release and recruiting leucocytes (Magna and Pisetsky, 2014). These characteristics make extracellular HMGB1 a key molecular target in multiple illnesses. Many approaches have been adopted to suppress HMGB1. Significantly, HMGB1 can activate not only the receptor of advanced glycation endproducts (RAGE) but also other receptors, particularly the Toll-like receptors 2/4 (Lotze and Tracey, 2005). It was also demonstrated that the process of HMGB1 endocytosis requires the RAGE and dynamin-dependent signaling, which in turn influences macrophage pyroptosis during endotoxemia (Xu et al., 2014). And further study has established that cell surface-expressed RAGE could bind to extracellular HMGB1-LPS complexes which will be endocytosed to the endolysosomal compartment (Deng et al., 2018). Moreover, another study has demonstrated that anti-HMGB1 therapeutic modalities caused an extra survival benefit in high-dose endotoxin injected RAGE-deficient mice, which indicating that HMGB1 acts partially through RAGE during septic shock process (Abeyama et al., 2005). These research evidenced that RAGE-mediated internalization is an essential process in Gram-negative sepsis. Additionally, although the HMGB1/RAGE signaling still remains a potential yet promising therapeutic target in SAE, more studies will be required before we know what the functions of RAGE in critical organ disorder implicated in the etiopathogenesis of sepsis and SAE.

Our study results indicated that preprocessing with berberine uplifted the cognition damage by suppressing the quantity of inflammatory events of cell factors, astrocyte activation and neo-neuron decrease in the cerebrum of SAE mice, which revealed that berberine could serve as an underlying medicine for SAE later on. While in clinical and translational studies, berberine is an over-the-counter medicine with verified efficacy to cure enterorrhea and enterogastric dysfunction. Numerous berberine derivates are prepared and assessed for the treatment of multiple illnesses. If berberine and derivate therapy before septic diseases exhibit effectiveness in preventing cognition damage remains elusive and requires more investigations. No matter berberine failed in clinic studies on septic cases or not, it's one of the valid medicines in preventing cognition damage in pathophysiological research in mice and certain studies on the internal diversities between murine sepsis and mankind septic diseases which require investigations in the near future. The main limitations of the study are the lack of physical confirmation of theoretical binding simulation of berberine with HMGB1 and the assumption of this decreasing HMGB1 binding to RAGE. The long-term safety and efficacy of berberine on SAE need to be further validated in controlled clinical trials which is also another limitation and application insufficiency of this study.

CONCLUSION

Overall, our research provides new promising drugs berberine in avoiding the establishment of SAE. Berberine significantly repressed the activation of microglia as well as astrocytes and the decline of natal-neurons and consequently improved cognitive functions in septic mice by blocking HMGB1/RAGE signaling. Hence, berberine marks an underlying HMGB1-targeting medicine for SAE mice, whereas the effectiveness in mankind cases clinically requires more investigations nonetheless.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the IRB of Third Xiangya Hospital, Central South University (2021-S076).

AUTHOR CONTRIBUTIONS

JS and JF contributed to study design. JF, XL, and HX conducted behavioral experiments. JS performed data analysis. MC and JS wrote the article. All authors critically reviewed content and approved final version for publication.

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frontiers in Integrative Neuroscience

PERSPECTIVE
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Integrating Cognitive Developmental Neuroscience in Society: Lessons Learned From a Multidisciplinary Research Project on Education and Social Safety of Youth

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Integrating fundamental science in society, with the goal to translate research findings to daily practice, comes with certain challenges. Successfully integrating research projects into society requires (1) good collaboration between scientists and societal stakeholders, (2) collaboration partners with common expectations and goals, and (3) investment in clear communication. Here we describe an integrative research project conducted by a large Dutch consortium that consisted of neuroscientists, psychologists, sociologists, ethicists, teachers, health care professionals and policy makers, focusing on applying cognitive developmental neuroscience for the benefit of youth in education and social safety. We argue that to effectively integrate cognitive developmental neuroscience in society, (1) it is necessary to invest in a well-functioning, diverse and multidisciplinary team involving societal stakeholders and youth themselves from the start of the project. This aids to build a so-called productive interactive network that increases the chances to realize societal impact in the long-term. Additionally, we propose that to integrate knowledge, (2) a different than standard research approach should be taken. When focusing on integration, the ultimate goal of research is not solely to understand the world better, but also to intervene with real-life situations, such as education or (forensic) youth care. To accomplish this goal, we propose an approach in which integration is not only started after the research has been conducted, but taken into account throughout the entire project. This approach helps

to create common expectations and goals between different stakeholders. Finally, we argue that (3) dedicating sufficient resources to effective communication, both within the consortium and between scientists and society, greatly benefits the integration of cognitive developmental neuroscience in society.

Keywords: cognitive developmental neuroscience, society, integrative method, diversity, team science

INTRODUCTION

In the last few decades, our knowledge on neurocognitive development has grown tremendously. Research on brain development, cognitive behavioral development and the combination of these fields has advanced our understanding of the interplay between brain maturation and behavior (Stuss, 1992; Dahl, 2004; Steinberg, 2008; Blakemore and Robbins, 2012; Crone and Dahl, 2012; Crone et al., 2017; Koenis et al., 2018). In this paper, we outline how knowledge from cognitive developmental neuroscience can be integrated in society. Our examples stem from the NeurolabNL Startimpulse research project on adolescents' academic and social development, conducted by a large Dutch consortium of universities, universities of applied science and societal stakeholders (**Figure 1**).

To illustrate which types of fundamental knowledge on cognitive developmental neuroscience have been previously implemented in practice, we may consider some of the most well known findings in this field (**Box 1**). These findings have informed public health policy and education. For example, the accumulated evidence that the brain is not fully matured at age 18 has raised the age of criminal responsibility in several countries (Cornet et al., 2016; SCAAN, 2017; Matthews et al., 2018; Scott et al., 2018; Schmidt et al., 2020). In addition, findings on adolescents' increased social and emotional sensitivity due to the relatively fast maturing of the limbic area can inform which social-emotional learning activities should be taught in school at what age (Immordino-Yang et al., 2018; Duraiappah et al., 2021), and in general, knowledge about the developing brain can be taken in consideration when designing an optimal learning environment (Trenado et al., 2020). These examples highlight how integrating cognitive developmental neuroscience findings can benefit daily life practices in various domains.

Three Key Challenges

Although the field is advancing, integrating cognitive developmental neuroscience in society remains a challenging endeavor for several reasons outlined below.

Productive Interactive Networks

First, setting up good collaborations between researchers and societal stakeholders (i.e., societal partners) is key. If societal partners are not involved in the project, the fundamental research findings often fail to be picked up and integrated in practice (van Atteveldt et al., 2019; Brouwer, 2021). To enhance the chances of creating long-term societal impact, The Royal Netherlands Academy of Arts and Sciences advises to create so-called 'productive interactive networks' (KNAW, 2018). This requires time and investment of both scientists and societal partners, who don't view this as a primary part of their jobs: scientists focus on gaining fundamental knowledge and publications, while societal partners focus on daily practical use. Still, scientists need to invest in gaining knowledge on daily practice and good communication about scientific results, while societal partners need to invest in understanding scientific practice and results. Additionally, even if societal partners are involved in collaborative research projects, they are often only involved in the last phase of the research. At this stage, the research outcomes are usually not aligned to the needs of daily practice, and integration fails. Therefore, to set up successful integrative research, societal partners should be involved in the project from the start. This makes sure that the research project is tailored to the needs, possibilities and limitations of real-life settings (Cornet, 2019; Nauta-Jansen, 2020; Brouwer, 2021).

To tackle this challenge, it's crucial to have a diverse team of scientists and societal partners. Collaborators with different perspectives and research methods complement each other to create projects that optimally fit with both scientific and societal goals (Cicchetti and Toth, 2006; Ameredes et al., 2015). Today, single disciplinary teams, often with male scientific leaders, still prevail in neuroscience[1]. These teams would benefit from multidisciplinary and diversification. In addition, apart from benefitting fundamental research projects, leadership styles that are more often pursued by women may be particularly suitable for meeting the challenges associated with translating neuroscience to real life, particularly those involving communication and decision-making. A recent report on women leadership showed that female leaders more frequently than male leaders expressed expectations and engaged in participative decision-making (Gipson et al., 2017; McKinsey and Company, 2017). Since integrating cognitive developmental neuroscience in society requires good communication between scientists and societal partners, and setting common goals, we believe that setting up a balanced team of women and men, including more diverse expertise in researchers and societal partners, will benefit integration in practice.

Integrative Method

A second challenge is that for successful integration of cognitive developmental neuroscience in society, all parties involved in the project need to have similar expectations. Often, scientists have different goals and expectations than their societal partners (van Atteveldt et al., 2019). To successfully integrate cognitive

[1]https://www.frontiersin.org/research-topics/19592/women-in-neuroscience#overview

BOX 1 | Well-known findings on adolescent brain development.

Adolescence is marked as the transition period between childhood and adulthood. Its onset is lined up with puberty, which starts around age 9–11 in contemporary society (Dahl, 2004; Crone and Dahl, 2012). Its offset is less well defined. Neurodevelopmental research has shown that cortical brain maturation continues until at least age 21 (Gogtay et al., 2004), white matter maturation reaches it maximum up to age 30 (Yakovlev and Lecours, 1967; Lebel and Deoni, 2018), while cognitive and emotion regulation functions even continue to develop until age 22–25 (Steinberg et al., 2009; Brockmole and Logie, 2013). This is well beyond the age of 18 at which adolescents are commonly agreed upon to enter adulthood in most Western societies. In this paper, we refer to adolescents as youth between the ages of 9 and 21.

One of the most well known findings in cognitive developmental neuroscience is that different brain areas develop at their own speed, and in their own order. Generally, cerebral maturation proceeds from the back to the front of the brain (Stuss, 1992; Gogtay et al., 2004). This means that frontal areas that are involved in the control of behavior are the last to develop. Practically, this suggests that adolescents continue to improve their executive control functions until they are around 21 years of age (Stuss, 1992; Casey et al., 2008; Astle and Scerif, 2009; Crone et al., 2017).

At the same time, the limbic system – a subcortical set of regions that is involved in emotion and reward processing – matures earlier than frontal areas (Casey et al., 2008; Blakemore and Robbins, 2012; Crone and Dahl, 2012), which yields adolescents more susceptible to rewards.

In addition, from puberty onwards, the significance of peer relations increases considerably (Crone and Güroğlu, 2013; Güroğlu, 2021). The combination of peer pressure and the discrepancy in developmental trajectories of the prefrontal versus subcortical brain regions - leading to heightened sensitivity to reward and not yet fully developed behavioral control – leads to dangerous risk-taking and antisocial behavior in some adolescents (Steinberg, 2008; Blakemore and Robbins, 2012). However, for most adolescents, the transition from childhood to adulthood runs smoothly. They take healthy risks that are crucial for development toward independent young adults (such as learning to commute to school with peers instead of parents) and develop mostly prosocial behavior (Dahl, 2004; Crone and Dahl, 2012; Crone and Fuligni, 2020).

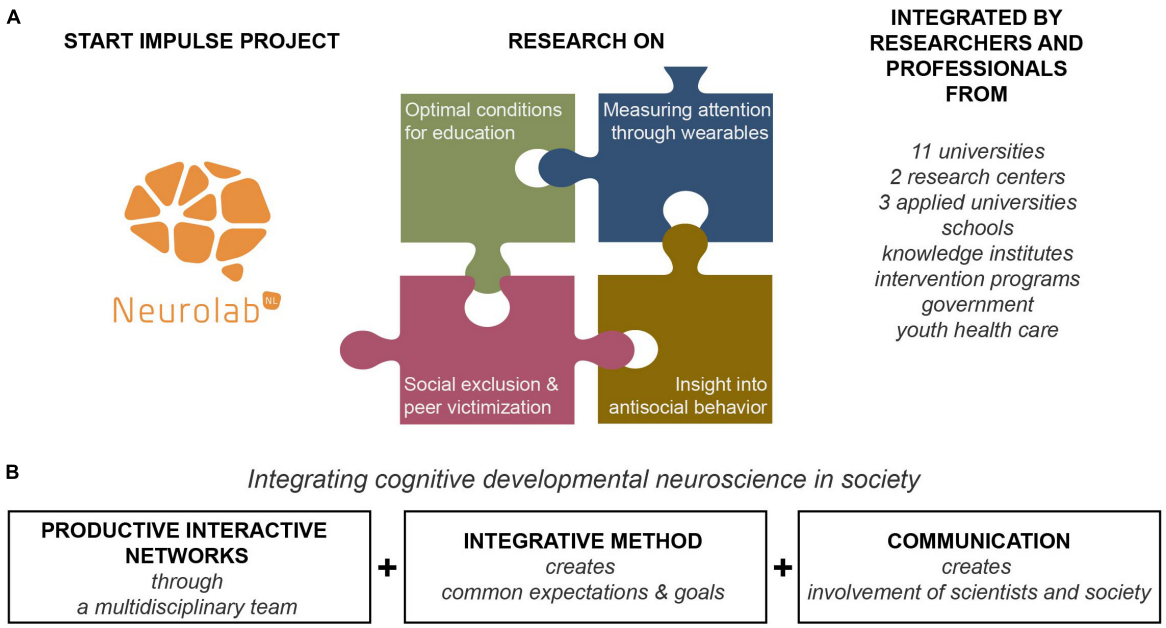

FIGURE 1 | Overview of the NeurolabNL Startimpulse project. **(A)** In four overarching projects, scientists and societal partners worked together to integrate research on optimal conditions of for education (with a focus on motivation and language learning), measuring attention through wearables, insight into antisocial behavior and social exclusion & peer victimization **(B)** The NeurolabNL Startimpulse project integrated cognitive developmental neuroscience in society by forming productive interactive networks, using an integrative method and devoting enough resources to communication.

developmental neuroscience findings, it is therefore important to discuss goals and expectations at the outset of the project.

We argue that integrating cognitive developmental neuroscience in society requires a different than currently standard approach to conducting scientific research. First, when conducting research projects with the goal to integrate the outcomes in practice, societal partners and a diverse research team play a pivotal role. Next, one should think about what the exact goals of the research are and on what level integration can take place (**Figure 2**): consolidation of (existing) knowledge; translation of knowledge in professional education, interventions or tools; implementation of integrated knowledge; monitoring of the use of this knowledge; or evaluation of the integrated knowledge (Nauta-Jansen, 2020). Importantly, this method of doing research is dynamic, meaning that knowledge is constantly updated and information flows both toward, as well as back from society.

When research projects are designed this way, the ultimate goal is not solely to build knowledge and understand the world better, but also to intervene with real world situations. With this approach, fundamental research does not only directly benefit society, but also scientific theory is enhanced through observation and integration of real-life events. By discussing research with various partners, and learning from studies that are conducted in

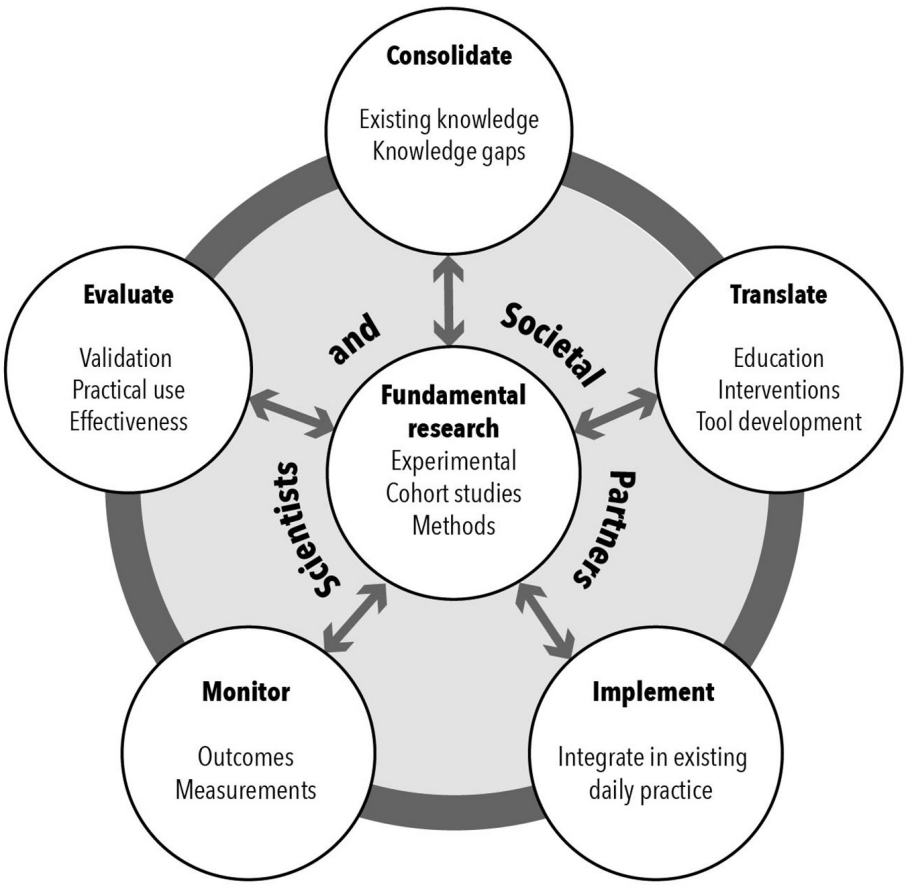

FIGURE 2 | Integrating fundamental research in society at different levels. Fundamental research can have different goals as to how findings may be integrated in society. Depending on the purpose of the project, one can integrate findings by: consolidating knowledge (e.g., exchanging knowledge with stakeholders), translating knowledge (e.g., educational modules), implementing knowledge (e.g., integrate interventions in daily practice), monitoring knowledge (e.g., checking whether scientific knowledge is used properly, and fits with stakeholders' goals), and evaluating knowledge (e.g., evaluating whether scientific knowledge is effectively used). The process is iterative and dynamic: knowledge is integrated from fundamental research to society and back. Importantly, societal partners and a diverse team of researchers should be involved to guarantee successful implementation. This model is based on Nauta-Jansen (2020).

real-life settings, fundamental theory and laboratory experiments can be greatly improved.

Communication

A third challenge relates to communication about research results. For research to be integrated in society, scientific theory and concepts need to be translated such that they are understandable and useful in daily life. This requires skills that scientists are not automatically trained in, since they are used to academic writing.

In addition, it should be clear from the start for both researchers and societal partners what the research may imply on an ethical level: for instance, how will the results change societies' views on adolescent behavior, youth programs and interventions? What are the benefits and possible pitfalls of using the knowledge in practice (Horstkötter et al., 2017; de Kogel, 2019; Brouwer, 2021)? Involving science communication specialists and ethicists to discuss potential (moral) dilemmas can greatly enhance the communication about complex and sensitive topics, thereby facilitating the use of this knowledge in practice.

Outline

In the coming sections, we will outline how the NeurolabNL Startimpulse consortium has conducted a collaborative research project in which cognitive developmental neuroscience findings were integrated in society, to improve education and social safety of youth. We will give examples of the studies that were conducted, and highlight on which level integration of knowledge took place (**Figure 2**). Hereby, we will share our insights into the process and practicalities of integrating cognitive developmental neuroscience in society. When we refer to integration here, we refer to integration of science in society. Integration can of course also take place between different research fields; we see this as one of the success factors for integrating science in society (by setting up productive interactive networks). We will end by making suggestions for best practices to integrate cognitive developmental neuroscience in future research.

THE NEUROLABNL STARTIMPULSE PROJECT: OPTIMAL CONDITIONS FOR EDUCATION AND SOCIAL SAFETY OF YOUTH

In 2016, the Dutch government invited citizens to ask questions to the scientific community about any topic of their interest. This invitation yielded almost 12.000 questions from the general public, about topics ranging from technology to economy and social sciences. From these questions, 140 were selected and clustered into overarching themes, amongst which neuroscience[2]. Based on this "Dutch Research Agenda," a funding scheme was designed that supports research projects in which societal partners are directly involved in conducting research and integrating fundamental knowledge in practice[3]. Within this scheme, the NeurolabNL Startimpulse project was designed to gain fundamental knowledge on brain development and its relation to school performance and social behavior, while at the same time creating optimal conditions for integration of this knowledge in society[4]. Below we outline the current outcomes and their integration within the two major sub-themes of education and social safety.

Education

Education is crucial for adolescents to flourish in contemporary society (Wentzel and Miele, 2016). To create optimal learning conditions, motivation and attention are vital (Wentzel and Miele, 2016). With a team of researchers from universities, research institutes, universities of applied science and teachers in schools, we aimed to further understand academic motivation and attention allocation in groups. Through diverse collaborations, we were able to improve research designs, and develop reflection tools to integrate fundamental knowledge in society.

Motivation

Academic motivation is one of the most important predictors of academic achievement and learning (Deci and Ryan, 1985; Fortier et al., 1995; Vallerand et al., 1997). In their influential self-determination model, Deci and Ryan (1985) determined three needs for motivation: autonomy, competence and relatedness. To help teachers reflect on whether they meet these needs, within the NeurolabNL Startimpulse project, we developed a guideline with questions on how teachers integrate these needs using their current teaching methods[5] (**Figure 2**: *translation*). Additionally, the guideline provides techniques to improve teaching style with regard to these pillars, together with the possibility to experiment with these styles in the classroom.

To further understand individual differences in motivation, we investigated willingness to invest cognitive effort in schools using a behavioral task (Westbrook et al., 2013). We focused on adolescents, as surveys have shown that motivation for school drops during this phase. In a large sample (N = 306), we found that willingness to invest cognitive effort was primarily driven by task-specific ability and to a lesser extent need for cognition (which reflects the degree to which one enjoys thinking). This suggests that adolescents choose strategically how to allocate cognitive resources and do not invest effort when the chance that performance will be successful is small. However, willingness to invest effort was not related to measures of self- and teacher-reported academic motivation. We conclude that in educational practice, increasing the sense of competence and enjoyment may support willingness to invest effort (Kramer et al., 2021).

In the above-described study, we conducted behavioral experiments in schools, and included actual academic achievement measures instead of solely laboratory tasks. Hereby, we were able to link experimental theory to practice. This makes it easier to consolidate these fundamental research findings (*consolidation*). In addition, through this design, we were able to improve our subsequent fMRI studies on the role of brain development in the relationship between cognitive effort discounting and academic motivation (*evaluation*). In addition, in ongoing research we are further investigating dynamic influences on motivation using a diary approach in which adolescents keep track of their motivation for schoolwork (*evaluation*).

Attention

A cognitive ability that is closely related to motivation is attention (Wentzel and Miele, 2016). If motivation is low, a student will inevitably lose attention. Vice versa, if attention is distracted, motivation to learn will decrease.

Stimuli that induce cognitive and affective processing elicit specific physiological responses such as a change in heart rate or skin conductance (Libby et al., 1973; Lang et al., 1993; Bradley and Lang, 2000; Brouwer and Hogervorst, 2014). Such responses enable continuous monitoring of attention based on physiology. In particular, electroencephalographic (EEG) responses can be used to identify to which of several sequentially presented stimuli (e.g., beeps or flashes) an individual is focusing her or his attention on, as demonstrated in Brain–Computer Interfaces (Farwell and Donchin, 1988; Nijboer et al., 2008; Schreuder et al., 2010). While monitoring attention in such a way is possible, it requires training of models using context-specific data. In order to obtain responses in real world settings, it also requires assumptions of what forms 'a stimulus' in the real world, and knowledge of its exact timing. While these approaches are useful in a lab environment, they are difficult to apply outside the lab in for instance a classroom. Using interpersonal physiological synchrony for monitoring attention bypasses these challenges. Interpersonal physiological synchrony refers to the similarity of signals between individuals over time. A high similarity of EEG signals for individuals who watch and/or hear the same stimuli has been shown to correspond to high outward directed attention (Dmochowski et al., 2012; Ki et al., 2016). For this method, no training of models is required, and it can be used to evaluate attention to natural stimuli. The approach of physiological synchrony allows drawing conclusions on group dynamics, which might potentially be useful in classrooms.

[2]www.neurolab.nl/en

[3]https://www.nwo.nl/en/researchprogrammes/dutch-research-agenda-nwa

[4]www.neurolab.nl/en/startimpulse

[5]https://lic.avans.nl/service.lic/publicaties/motivatie-motor

While physiological synchrony in EEG has been demonstrated in the classroom (Dikker et al., 2017), easier, less obtrusive collection and processing of data would make interpersonal physiological synchrony a more suitable measure for attention in the classroom. Therefore, within the NeurolabNL Startimpulse project, we determined whether other physiological measures, namely heart rate and skin conductance, provide similar insights into attentional allocation as EEG (Stuldreher et al., 2020a,b). In this study, participants listened to the same audiobook, mixed with other sounds. Half of the participants were asked to focus their attention on the audiobook; half were asked to focus their attention on the sounds. We showed that indeed, even though EEG is more sensitive, heart rate and skin conductance also contain reliable information on attentional allocation. Classifying a single participant into one of the two instructional groups could be done with 96% accuracy when using synchrony of EEG, and with 73% accuracy when using heart rate and skin conductance. In addition, the reliability of the wearable data that were collected with equipment suitable for real-life settings was similar to the reliability of laboratory equipment (Van Beers et al., 2020), and we observed physiological synchrony in skin conductance and heart rate recorded through wearables in actual classroom settings (Stuldreher et al., 2021). Directly comparing experimental laboratory equipment with equipment suitable for real-life settings form a crucial step in tackling issues around the technical limits to use neurocognitive measures in classroom settings (*evaluation*).

Although scientists often elucidate on possible integration of fundamental findings in practice, it is crucial to engage in a dialog with societal partners themselves to investigate their perspectives on potential benefits and risks of integration. Therefore, in a team of neuroscientists and ethicists, we started qualitative research with focus groups among adolescents to investigate whether using wearable measurements to improve classroom attention and engagement carries their support (*evaluation*). We found that adolescents are in general positive toward using wearables as feedback for teachers, but mainly to be able for them to improve their teaching methods. They are weary of the idea that the devices could be used to monitor individual students. Specifically, students (16/17 years) state that they are old enough to determine whether they pay attention or not: it is their own right to decide. Further research should investigate the sensitivity and added value of using physiological synchrony in realistic cases that potential users – both teachers and adolescents – are interested in.

Social Safety

One of the most important transitions that adolescents go through is exploration that enables them to gain independence from their parent's care and yields them to become independently functioning adults (Crone and Güroğlu, 2013; Crone and Fuligni, 2020; see **Box 1**). While adolescents go through this social-emotional learning process, it is crucial to provide them adequate social safety (Dahl, 2004). By investigating social behavior and brain development with a diverse consortium of researchers from universities, universities of applied science and youth care professionals, we were able to develop educational material and prediction tools to implement knowledge on social safety in practice.

Antisocial Behavior

Many adolescents show more antisocial behavior compared to younger children and adults (see **Box 1**). Antisocial behavior is usually relatively harmless (for example, using a smartphone in class when not allowed), and ceases for most adolescents around age 25 (Liu, 2015; Moffitt, 2018). However, some adolescents remain in life-long trajectories of antisocial behavior that involve behaviors of high severity such as criminal activity. Within the NeurolabNL Startimpulse project, we investigated which neurobiological markers are related to severe antisocial behavior, and how this information can be integrated in practice for diagnostics and treatment to prevent adolescents to become criminally active adults (for an overview of possible implementation in the judicial field, see Cornet et al., 2019).

Neurobiological markers of autonomous nervous system activity, such as heart rate and respiration rate, have been related to antisocial behavior (Zuckerman, 1990; Raine and Liu, 1998; Portnoy et al., 2013; Portnoy and Farrington, 2015; Blankenstein et al., in revision; De Looff et al., submitted). Neuroendocrinological measures such as cortisol and testosterone levels are linked to antisocial and aggressive behavior as well (Alink et al., 2008; Platje et al., 2015; Dekkers, 2018; Blankenstein et al., in revision). However, to study the relation between autonomous nervous system activity and neuroendocrinology with antisocial behavior, often relatively small samples are used. Also, results vary depending on the antisocial behavior that is investigated. Through the extensive collaboration between different universities and youth care facilities, we were able to gain access to and harmonize data from 6 (clinical) samples (Blankenstein et al., 2021). This resulted in a unique dataset of 1,489 participants, displaying none to severe criminal behavior. The findings showed that severe antisocial behavior is linked to neurophysiological measures such as respiration rate, cortisol awakening response and testosterone levels. These findings are of high practical significance as they may be used in addition to psychosocial characteristics to inform diagnostics, risk assessment, psycho-education and eventually (preventive) interventions (Popma and Raine, 2006; Nauta-Jansen, 2020).

Importantly, neurobiological markers cannot be used in their own right, but should be integrated with psychosocial characteristics of individuals. At the moment, an algorithm is being developed to predict antisocial and delinquent behavior from biopsychosocial information within clinical practice (for an example prototype, see[6]; de Ruigh et al., 2021). To implement this algorithm for crime prevention and risk assessment, the current consortium involves partners from Juvenile Justice Institutions and Youth Care to guarantee the feasibility and usability of this tool (*translation*). For example, it is important to consider how therapists choose their intervention programs, such that the knowledge from cognitive developmental neuroscience can add to this. In addition, the assessment of neurobiological

[6]https://architecta.shinyapps.io/PredictingYouthReoffending/

markers should be made as easy as possible for both therapists and adolescents, such that they can be used without scientific supervision in daily practice. By conducting the next phases of this project within Juvenile Justice Institutions, and at the same time designing the tools together with different societal partners, implementation of the tools will be more successful.

Even though much is known about the relationship between brain development and antisocial behavior, this information does not automatically reach professionals working with youth. Within the NeurolabNL Startimpulse project, researchers and teachers from universities and applied universities together developed an education package for youth professionals (*translation*). The education package is based on the fundamental knowledge of cognitive developmental neuroscience – specifically during adolescence – that plays a pivotal role in the development of severe and persistent delinquent behavior. In addition, to determine which knowledge is currently lacking in practice, and which knowledge is most important to professionals, teachers and youth themselves, several focus groups were organized among professionals, professionals in training and delinquent youth. Based on this, educational modules on the neurobiology of antisocial behavior, consisting of knowledge clips and accompanying in-depth study assignments for (future) professionals were designed. The modules will become publicly available through websites of the universities of applied science and Dutch governmental bodies, such as the Dutch Youth Institute[7]. This method secures the use of the to-be-developed tools in practice. By collaboration in such a multidisciplinary consortium, the usability and possibility for implementation in practice are greatly enhanced.

Social Exclusion and Peer Victimization

Across adolescence, peer relationships become increasingly more important (Crone and Güroğlu, 2013; Güroğlu, 2021). Friendships in this period are among the most important relationships. However, peer relationships do not only involve positive interactions with peers. Unfortunately, less pleasant and adverse interactions with peers, such as social exclusion or victimization, regularly take place in peer contexts. To achieve social-emotional well-being and good mental health, adolescents need to learn how to cope with these adverse peer experiences and related social stress. In addition, to support adolescents in this development, we need to better understand whether some adolescents are more sensitive to negative peer experiences and how this affects their development. More recently, neuroscience research combining examination of peer experiences with neural basis of social interactions has contributed significantly to our understanding of social development (Güroğlu and Veenstra, 2021). Within the NeurolabNL Startimpulse project, we investigated the neural and physiological underpinnings of social exclusion and peer victimization across several lines of research.

In one line of research, we collaborate with two large national prospective population based cohort studies (Generation R: Kooijman et al., 2016; ALSPAC: Boyd et al., 2013; Fraser et al., 2013) to investigate the links between adverse peer experiences and structural brain maturation of children aged 7–10 (on average). These datasets include information on participants' social networks and peer relationships as well as assessments of DNA methylation and brain structure using MRI. Our findings showed that being bullied is associated with a change in DNA methylation. The gene that shows less methylation has been previously associated with stress in rats (Mulder et al., 2020). This suggests that being bullied affects the adolescents' stress system. Findings also suggested that white matter microstructure integrity is higher for adolescents who were bullied compared to adolescents who were not bullied. Since white matter integrity increases when the brain develops, we currently interpret these findings to suggest that victimized youth show advanced brain maturation compared to their non-victimized peers (Mulder et al., in prep). Together, these findings add to the growing literature showing that adverse experiences of social stress might have lasting impact on brain development (Teicher et al., 2016; McLaughlin et al., 2019). In the NeurolabNL Startimpulse project, we actively engage societal partners such as teachers and developers of anti-bullying interventions, to pinpoint what these findings might contribute to daily practice (*consolidation*). We do so by organizing regular meetings in which we inform societal partners about our results. Their questions and viewpoints on what these findings imply advance the scientific valorization of our project.

In a second line of research, we investigated the stability of rejection sensitivity, which is a trait that has been associated with peer victimization. Our behavioral study employing longitudinal data on peer victimization and rejection sensitivity has shown that, although victimization and rejection sensitivity are related to each other, they do not enhance each other over time: adolescents who are victimized do not get more sensitive to rejection, and those adolescents that are sensitive to rejection do not get bullied more over time (Kellij et al., in prep). This suggests that certain adolescents are more sensitive to rejection than others, making them more likely to be victimized, but this process does not impact the further development of rejection sensitivity. One crucial open question, however, refers to the impact of chronic experiences of peer victimization over time. To address this question, we have set up a collaboration with an anti-bullying intervention program (KiVa[8]) that is implemented in more than 400 elementary schools nationwide. Schools participating in the program collect data on peer experiences twice across a school year in order to monitor interactions involving bullying and victimization. This unique collaboration between our team of researchers and the anti-bullying program enabled us to access schools with existing longitudinal data on peer victimization, yielding students from these schools a valuable pool of potential participants for our study (*consolidation*). In ongoing research, we are recruiting pupils from these schools for participation in a neuroimaging study that investigates the neural correlates of social cognition in relation to prior victimization experiences.

Our findings, together with previous fundamental knowledge on bullying and social-emotional competence, were used to inform teachers and professionals working with youth through several ways. Together with the Dutch Youth Institute, a publicly available document was constructed to inform teachers and youth

[7] www.nji.nl/english

[8] https://www.kivaschool.nl/

professionals about essential scientific knowledge on bullying and adolescents' social-emotional development[9] (*consolidation*). In addition, we used this fundamental knowledge to further develop a taxonomy on social cognition (de Mooij et al., 2020). This taxonomy served as a basis to evaluate anti-bullying interventions in schools through a content analysis. For this analysis, programs were scored based on inclusion of markers that should be present based on this literature. Different researchers scored the programs, and compared their scores to reach consensus on the evaluation. Specifically, the evaluation revealed that programs should focus more on the role of social information processing, and the development of emotion regulation strategies and negotiating skills in bullying (van den Bedem et al., in prep). Because of the direct involvement of youth professionals working on anti-bullying intervention programs, the organizations of three widely used intervention programs in the Netherlands cooperated and improved their intervention schemes (*evaluation*). If the intervention program developers had not been involved from the start of the project, this would have been a more difficult trajectory.

BEST PRACTICES

In this paper, we reviewed outcomes of the NeurolabNL Startimpulse project; a national Dutch research program on optimal conditions for education and social safety of youth. The focus of this program was to integrate cognitive developmental neuroscience in society, specifically in education and (forensic) youth care. We identified three main challenges that are often encountered when integrating cognitive developmental neuroscience in society: setting up good collaboration between scientists and societal partners, creating common goals and expectations, and good communication – both within the consortium and between scientists and society. We argue that three factors contribute to overcome these challenges (**Figure 1B**): (1) forming a well functioning, diverse and multidisciplinary consortium to create productive interactions; (2) using an integrative model to design the research project; and (3) dedicating resources to professional communication. In addition, we highlight the importance of ethics in such as large-scale project, and discuss its translatability to other countries. Below, we outline our lessons learned.

Forming Productive Interactive Networks

To integrate cognitive developmental neuroscience in society, one of the main challenges is to create productive interactive networks. The necessary basis of such networks is a well-functioning, multidisciplinary team. To tackle this challenge, we have five general recommendations that are elaborated upon below.

Early Involvement of Societal Partners

Different stakeholders often have different goals for a project (van Atteveldt et al., 2019). If only one of the stakeholders, often the scientific partner, starts the project, and later on involves other partners, their goals will not be aligned. This will render integration of fundamental knowledge in practice difficult (Cornet, 2019; Brouwer, 2021; Nauta-Jansen, 2020). Throughout the NeurolabNL Startimpulse project, we consulted with different societal partners on the questions they had regarding fundamental research. For example, the fMRI study on chronic peer victimization that is currently being conducted was designed together with input from professionals working with anti-bullying interventions (*consolidation*). For integration of practical tools such as guidelines or algorithms (*translation*), early collaboration is even more important. In our project, for the development of the algorithm that can help predict antisocial and delinquent behavior using neurobiological markers, the involvement of societal partners in juvenile justice settings is crucial for successful integration in practice.

Merge Fields – Different Expertise, Ethicists and Diverse Leadership Styles

The success of our integration projects did not only depend on the involvement of societal partners, but also on the diversity of the researchers involved in the project (Cicchetti and Toth, 2006; Ameredes et al., 2015). The topics that were investigated were relatively diverse to start with: motivation, attention, social stress and antisocial behavior. Thereby, the consortium included scientists and applied scientists with various backgrounds, such as in psychology, neuroscience, sociology, criminology and ethics. By regularly meeting and discussing research outcomes, the projects became more integrated with different societal issues, and new collaborations between different fields emerged. For example, wearables were used in a project on social stress at schools, thereby providing a pilot in practice for the wearables on the one hand, and physiological data on social stress on the other hand (*evaluation*).

In addition, we argue that leadership styles that are more often pursued by women, such as expressing expectations and engaging in participative decision-making (Gipson et al., 2017; McKinsey and Company, 2017), greatly contribute to creating a well-functioning team. These leadership styles facilitate setting common goals and expectations between scientists and societal partners, and make sure that the project is tailored to the possibilities and limitations of science and daily practice. Therefore, we believe that setting up a balanced team of women and men, including more diverse expertise in researchers and societal partners, will benefit integration in practice.

Involve Youth

Along with creating a diverse research team consisting of adults, youth themselves can greatly enhance successful integration of cognitive developmental neuroscience in society (*consolidation and translation*). Indeed, the knowledge that is integrated is about them, and any intervention that stems from this knowledge should fit with their goals and needs (Gibbs et al., 2018; Grootens-Wiegers et al., 2020; Nauta-Jansen, 2020). Even if the integrated knowledge is perfectly aligned with their environment, if they themselves do not support the intervention that uses this knowledge, the intervention will fail. For example, to reflect on

[9] www.nji.nl/nl/Kennis/Dossier/Pesten

the use of wearables in classes and the use of knowledge on social safety, we conducted several youth focus groups. The input from these focus groups is used to further design follow-up integrative research projects.

Knowledge-Brokers

Another successful factor for adequate knowledge communication and integration of fundamental knowledge in practice is to have a person dedicated to this task[10]. Both a researcher's and a youth professional's job are not centered on collaborating with each other. A so-called "knowledge-broker" greatly increases the time that can be invested in the collaboration and integration process, thereby enhancing the success of the implementation in practice. For example, in the current project, two knowledge-brokers organized youth panels (*translation, consolidation, and evaluation*), evaluated current interventions based on new scientific knowledge (*evaluation*), and organized networking events (diverse team building). We therefore highly recommend the inclusion of budget for such a position in future grants. Alternatively, if this budget is not available, we recommend having a post-doctoral researcher dedicate some of her time to this task, as it will greatly enhance the collaboration between scientists and societal partners, and between scientists with diverse backgrounds.

Networking – Old and New

Finally, the ability – time and moneywise – to network with different stakeholders is crucial for the field to progress. To start with, the research of the teams within the NeurolabNL Startimpulse consortium that had already established connections took off fastest. They had the advantage of already knowing each other's way of working, and more easily reached out to each other. Therefore, we recommend creating a consortium with at least some relations that are already established. Relatedly, arranging time for the team to get to know each other better, and for the network to grow through match making sessions allows for better integration of different fields and viewpoints. Two of our sub teams tremendously grew and gained more success by active match-making with new partners. By creating a denser and more consolidated network, knowledge can be integrated faster and with more success (*all levels of integration*).

The Integrative Model

By setting up different types of integration projects (**Figure 2**), we learned which integrative methods are valued by society and work efficiently. The focus of the NeurolabNL Startimpulse project was to integrate knowledge mainly on the level of consolidation, translation and evaluation. In follow-up projects, we will continue to integrate this knowledge on the level of implementation and monitoring as well. Below, we elaborate on four specific recommendations regarding integrative methods.

[10]https://www.nro.nl/nieuws/onderzoek-en-onderwijspraktijk-verbinden-vier-randvoorwaarden-voor-een-succesvolle?utm_campaign=nieuwsbriefjan20&utm_medium=nieuwsbrief&utm_source=nieuwsbriefjan20

Educational Material

First of all, many different stakeholders, such as adolescents themselves and youth professionals, mention that transferring knowledge through education is very effective (Cornet, 2019; Nauta-Jansen, 2020; *translation*). This may seem like an obvious path to take, but much of the (to scientists) "basic" knowledge on neurocognitive development has not reached professionals and youth education yet. By developing educational packages that adhere to this basic knowledge – for example on neurobiological markers of antisocial behavior for youth professionals working with criminal youth -, we can reach many people with information that supports professional and personal growth.

Research by Practitioners

Throughout the project, we discovered that integration of knowledge also takes place by allowing youth professionals to perform research. In one of our subprojects, a team of teachers in training and scientists conducted studies on motivation for language learning. As part of their teacher training, teachers carried out the experiment at schools, thereby providing the researchers with valuable input on feasibility of the learning method in practice. At the same time, the teachers get acquainted with neuroscience, thereby enhancing their knowledge on brain functioning. By co-creating these projects, researchers can take real-life environmental factors into account, while it helps teachers to develop more efficient teaching environments (Trenado et al., 2020; *integration at all levels*).

Evaluate Tools in Practice

To develop tools that can be used in practice, experimental tools almost always need to be adapted (Brouwer, 2021). Often, laboratory experiments are conducted with highly reliable equipment that is not suited for use in daily-life. When developing a tool for practice, stakeholders should be involved from the start to help improve feasibility of the tool. In addition, the tools should be tested to confirm that they are reliable to be used in practice. In the current project, for example, we were able to test wearable devices that measured heart rate and skin conductance in school settings (Thammasan et al., 2020; Stuldreher et al., 2021). This allowed us to test the reliability of these measurements compared to laboratory settings and equipment (*evaluation*). Importantly, without the collaboration within this diverse consortium, this study would not have taken place this easily.

Cross-Over From Real-Life Experiments to Laboratory Research

Not only should tools be tested in practice, if knowledge is to be transferred from the laboratory to real-life, the experiments that are performed in the lab should adhere to real-life behavior. By piloting laboratory experiments in real-life settings, one can gain insight into the validity of the constructs that are tested, and whether conclusions from the controlled setting can be extended to daily life (Brouwer, 2021; *evaluation and consolidation*). For example, by conducting behavioral experiments on motivation in school settings using actual academic achievement and motivation diaries, our current fMRI experiments have improved validity.

Communication

Throughout the project, we focused on clear and effective communication between consortium partners, to interested stakeholders, and to the general public. Three insights aided us in this communication.

Include Communication Professionals

In addition to strengthening the interactive productive networks, our knowledge-brokers took on communication within the consortium and the dissemination of research results. To do so, they organized several activities for the consortium to get together, to disseminate knowledge to a broad audience (*consolidation*) and served as editor for educational material (*translation*). Importantly, these knowledge-brokers were skilled at translating scientific findings to a broad audience, because of their own scientific background and direct collaborations with societal partners.

In addition, by including ethicists on different research projects, moral dilemmas and potential pitfalls of communication were discussed. For example, the implications of education on neurobiological markers of antisocial behavior, and the use of wearables to track attention were discussed (Horstkötter et al., 2017; de Kogel, 2019; Brouwer, 2021). This enhances the success of translating these results to society (*translation*).

Use Existing Platforms

Translating knowledge by updating or adding information to already existing communication platforms is more effective than setting up new communication pathways. For example, at the outset of the NeurolabNL Startimpulse project, we planned to design a new website for youth professionals to share information about brain development and bullying. Instead, throughout the project, we found that the Dutch Youth Institute already had quite extensive communication channels and were interested to update their information regarding bullying and social emotional capacities. By integrating our knowledge, we were able to communicate to youth professionals in a faster and more efficient manner (*consolidation and translation*). This example shows the benefits of a well-functioning interactive productive network, illustrating that long-term societal impact might be realized through parties that were not part of the initial research consortium.

Target Specific Stakeholders

Although time-consuming, one of the most effective ways to consolidate knowledge is to organize workshops or lectures for stakeholders that directly benefit from the scientific knowledge. In the NeurolabNL Startimpulse project, for example, researchers gave lectures at schools and organized workshops for educational modules for teachers. According to our collaborating teachers and education coaches, this way, knowledge best reaches the interested stakeholders, and makes them more likely to use the information in practice. However, since scientists in general don't have much time to spend on giving lectures for societal partners, this suggestion feeds back into the recommendation of including communication professionals in integrative research projects.

Ethics and Data Privacy

Through our extensive collaboration with different partners, datasets were shared between scientists, as well as between societal partners and scientists. This made data privacy a very important topic in many of our studies. To make sure that privacy regulations were upheld, we consulted with ethical, juridical and data science experts, who advised on reusing data. For example, before sharing data, agreements regarding who had access to data and where it was stored were signed. In addition, as is common in experimental developmental cognitive neuroscience research, we only used data for which participants signed informed consent, stating that their data was made anonymous and only used for research purposes.

Translatability to Other Countries

Since the current project was conducted in the Netherlands, it had a favorable start with respect to the possibility to integrate science in society: in the Netherlands, there is already a well-established connection between science and society, and a government that supports the integration of science in society. This may have led to an emphasis in the current project on setting up good collaborations and the integrative method. We acknowledge that in other countries, this might be different, and starting integration at the level that we describe here is not possible. If the logistics and culture are not supportive of science, or the integration of science in society, communication between scientists and society is even more important, and forms the starting point for future collaborations. Still, if there is a wish to integrate science in society, we argue that the three basic needs for integration – early collaboration, choosing integrative methods, and communication – are similar for all countries.

CONCLUSION

In the last decades, our knowledge on cognitive developmental neuroscience has advanced such that it becomes increasingly more possible to integrate this knowledge in society. However, successfully integrating research findings remains a challenge: fundamental theories and experimental methods cannot be translated to practice one-on-one. Based on the research within the NeurolabNL Startimpulse project, we learned that (1) good collaboration between scientists and societal stakeholders, (2) common expectations and goals between partners, and (3) investment in clear communication are key in successfully integrating cognitive developmental neuroscience in society (**Figure 1B**). We propose to (1) conduct research projects in consortia in which researchers from different backgrounds and societal partners work together in diverse teams, creating interactive productive networks; to do so, it is important to involve societal partners at the start of the project, such that goals and expectations of scientists and societal partners are aligned. Next, the team should consist of researchers and societal partners from different institutional types with different expertise and research methods. Relatedly, we advocate having a diverse team to promote the integration of different leadership styles and communication methods. Related to the topic of this special issue 'Women in Neuroscience,' we believe that leadership styles

that are more often pursued by women, such as expressing expectations and participative decision-making, contribute to optimal transfer of knowledge, both within the consortium, and between science and society. Next, we propose to (2) use an integrative approach in conducting research (**Figure 2**). To effectively do so, the research goals and level of integration should be clearly defined at the start of the project. Last, we propose to (3) dedicate sufficient resources to communication, both within the consortium, and to interested stakeholders and the general public. By adhering to these three recommendations, we believe that fundamental knowledge can most effectively be integrated into practice, while at the same time, scientific theory is strengthened by knowledge embedded within society.

AUTHOR CONTRIBUTIONS

All authors wrote the manuscript together. A-MB initiated the submission. AV coordinated the process and was the main writer. All authors contributed to the article and approved the submitted version.

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NAIAD-2020: Characteristics of Motor Evoked Potentials After 3-Day Exposure to Dry Immersion in Women

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As female astronauts participate in space flight more and more frequently, there is a demand for research on how the female body adapts to the microgravity environment. In particular, there is very little research on how the neuromuscular system reacts to gravitational unloading in women. We aimed to estimate changes in motor evoked potentials (MEPs) in the lower leg muscles in women after 3-day exposure to Dry Immersion (DI), which is one of the most widely used ground models of microgravity. Six healthy female volunteers (mean age 30.17 ± 5.5 years) with a natural menstrual cycle participated in this experiment. MEPs were recorded from the gastrocnemius and soleus muscles twice before DI, on the day of DI completion, and 3 days after DI, during the recovery period. To evoke motor responses, transcranial and trans-spinal magnetic stimulation was applied. We showed that changes in MEP characteristics after DI exposure were different depending on the stimulation site, but were similar for both muscles. For trans-spinal stimulation, MEP thresholds decreased compared to baseline values, and amplitudes, on the contrary, increased, resembling the phenomenon of hypogravitational hyperreflexia. This finding is in line with data observed in other experiments on both male and female participants. MEPs to transcranial stimulation had an opposing dynamic, which may have resulted from the small group size and large inter-subject variability, or from hormonal fluctuations during the menstrual cycle. Central motor conduction time remained unchanged, suggesting that pyramidal tract conductibility was not affected by DI exposure. More research is needed to explore the underlying mechanisms.

Keywords: Dry Immersion, NAIAD-2020, microgravity, TMS, support unloading, trans-spinal magnetic stimulation

INTRODUCTION

Nowadays women participate in space flights (SF) alongside men, and the differences between the sexes should be considered when training crewmembers for space missions. For many years space physiology studies have been conducted with male volunteers (Kozlovskaya and Kirenskaya, 2004; Koppelmans et al., 2015, 2017; De Abreu et al., 2017; Amirova et al., 2020), which was the "gold

standard" of research, and was reasonable at the dawn of manned space exploration. However, physiological changes triggered in the female body by microgravity have been insufficiently investigated, and this may lead to both employment and casual discrimination of female astronauts.

One of the important lines of research in space physiology is studying how weightlessness affects motor system function. Preventing and predicting motor impairments caused by weightlessness is especially significant for increasing SF duration and expanding the scope of motor tasks performed during space missions. The complex of changes occurring in human motor function under the conditions of real or simulated microgravity is called hypogravitational motor syndrome (Kozlovskaya et al., 1988), and it is defined by a deficit in vestibular, proprioception, and support afferent activity (Pechenkova et al., 2019), and substantial alterations in the functional (e.g., atony, a decline in speed-force qualities) and structural (e.g., atrophy and a phenotype deterioration) characteristics of skeletal muscles (Kozlovskaya et al., 1988; Kozlovskaya and Kirenskaya, 2004; Koppelmans et al., 2017; Amirova et al., 2020). In experiments with animal models it was also shown that a hindlimb suspension in rats results in nerve fiber demyelinization, which in turn may play a role in the development of hypogravitational motor syndrome (Islamov et al., 2013).

Because invasive techniques to study the brain and the spinal cord under the conditions of support withdrawal cannot be used, a different method is required. Recently transcranial magnetic stimulation (TMS) began to be utilized in the field of space medicine and biology (Davey et al., 2004; Roberts et al., 2007, 2010; Badran et al., 2020; Romanella et al., 2020; Nosikova et al., 2021). This method is widely used in studying cognition, brain-behavior relationships and the pathophysiology of neurological and psychiatric disorders; in particular, TMS of the motor cortex has a well-established role in clinical neurophysiology. Stimulation is achieved by applying electromagnetic induction to generate suprathreshold current in the brain, and different shaped TMS coils allow stimulation of both deep structures and selected small regions of the cortex. TMS variables that are typically analyzed in clinical and research studies include motor thresholds, motor evoked potential (MEP) amplitudes, and MEP latencies among others. Motor threshold is the minimal intensity of stimulation required to elicit a reliable MEP of minimal amplitude in the target muscle. Thresholds are measured to estimate cortical and spinal neurons excitability and they depend on a number of factors such as coil position and orientation, the individual arousal level, and environmental noise. MEP amplitudes, which reflect muscle contraction magnitude to a select stimulation intensity, are also widely used to study corticospinal excitability. Lastly, MEP latency is the time interval from the stimulus onset to the muscle response. The difference between latencies to stimulation of the motor cortex and spinal roots, called central motor conduction time (CMCT), is calculated to estimate corticospinal conductibility (Rossini et al., 2015). It was reported that MEPs to TMS depend on physical individual features. Specifically, MEP latency increases with age and positively correlates with height. Moreover, females show smaller latencies in upper limbs to both cortical and spinal stimulation when compared to males (Cantone et al., 2019).

Earlier studies were more focused on the female cardiovascular system's reaction to microgravity (Demiot et al., 2007; Hodges et al., 2010; Arbeille et al., 2012; Edgell et al., 2012), and it was shown that female astronauts are more susceptible to orthostatic intolerance after SF than male astronauts (Platts et al., 2014). A few research papers report postural performance impairments (Viguier et al., 2009) and muscle atrophy (Shenkman et al., 2000) after head-down bed rest in women, but the authors do not compare their results to male groups. It was also reported that women are more likely to suffer from space motion sickness and vestibular instability after SF than men (Reschke et al., 2014). As for neuromuscular changes, long-term gravitational unloading leads to less deterioration of muscle force characteristics in females compared to males, but on the other hand, males are better at integrating different sensory inputs when performing explosive motor tasks after unloading (Koryak, 2009). However, there is still very little research on the state of the female neuromuscular system after exposure to real SF or ground-based models, which makes it difficult to select more effective countermeasures for female groups (Holt et al., 2016).

We hypothesized that, as there appear to be some differences in neuromuscular adaptation to support withdrawal between men and women, the motor responses evoked by magnetic stimulation (MS) will possess different characteristics after exposure to simulated microgravity in women compared to men. Thus, we decided on Dry Immersion (DI) as one of the most widely used ground models of microgravity and carried out a 3-day experiment on a group of female volunteers to estimate how their MEP characteristics changed.

MATERIALS AND METHODS

Participants

Six healthy female volunteers (mean age 30.17 $\pm$ 5.5 years) of reproductive age participated in this study. The participants had similar bodily constitution, height (166.6 $\pm$ 3.3 cm) and body weight (62.0 $\pm$ 3.4 kg). All subjects had a natural menstrual cycle and no history of motor impairments or neurological diseases. Each participant signed an informed consent after the experimental procedures and possible consequential effects and risks were explained to them.

The present study was approved by the Bioethical Commission of the Institute of Biomedical Problems of Russian Academy of Science (Protocol No. 544 of July 16, 2020) and fully complied with the principles of the Declaration of Helsinki.

Experimental Design

The study was conducted at the DI facility of the Institute of Biomedical Problems, Russian Academy of Science (Tomilovskaya et al., 2019, 2021). For 3 days, participants lay in the immersion bath without any physical activities and

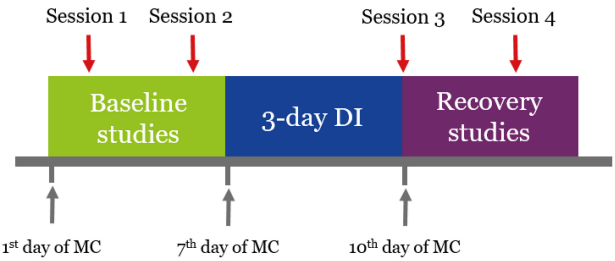

FIGURE 1 | Schedule of the experiment. Sessions 1 and 2 were conducted 5 and 3 days before the start of DI. Session 3 was conducted on the day of DI completion. Session 4 was conducted 3 days after DI. Every experimental stage started on a specific day of the MC.

with moderate movement restriction; among other factors, lower limb activation was limited. The water temperature in the bath was maintained at 32.5 ± 2°C. Every evening the subjects were lifted out of the bath for 15–20 min for hygiene procedures, the majority of which were performed in the supine position. The subjects were also raised from the bath during the day for certain experimental examinations that were carried out in the supine position. The average time spent outside the immersion bath did not exceeded 30 min per day. The crew, consisting of a doctor, an assistant and a technician, provided 24-h monitoring of the participants' health and the working condition of technical equipment. In their free time, subjects were allowed to read, work on a laptop, watch TV, talk on the phone, etc.

Magnetic Stimulation Procedure

Experimental sessions were conducted according to the schedule (**Figure 1**). There were four sessions in total: two before DI (5 and 3 days before the start of DI, baseline studies), one right after DI (on the day of DI completion, referred to as R + 0), and one during the recovery period (on the third day after DI, referred to as R + 3). The start of every experimental stage was matched with a specific day of the menstrual cycle (MC).

Participants were instructed to abstain from alcoholic and tonic drinks the day before each procedure. During the procedure, the subjects lay prone, relaxed and with their eyes open. A support was placed under the ankles for better relaxation. Motor responses were obtained using transcranial and trans-spinal MS, and MEPs were recorded from the soleus and gastrocnemius muscles of the right leg.

Transcranial MS was delivered with the 8-shaped coil (DB-80 Butterfly) of the MagPro X100 magnetic stimulator (Medtronic, Denmark) to the area of cortical motor projections of the right lower leg muscles. The coil was placed 1–2 cm to the left from the intersection of the vertex and the line connecting the pre-auricular points and then was gradually moved to the position at which stimulation led to MEPs with the greatest amplitude and a constant shape. Trans-spinal MS was delivered using a flat round coil with an outer diameter of 114 mm, which was placed at the level of L5–S1 segments of the lumbar spine. If the stimulation area was picked correctly, MEP amplitudes were generally stable, which means MEPs had a constant shape and their amplitudes were similar. Motor responses of soleus and gastrocnemius muscles were recorded with bipolar surface silver-chloride electrodes that were placed in the center of the muscle belly projections with a 20 mm interelectrode distance. Electromyographic signals were recorded using a Viking Quest 4-channel myograph (Viasys, United States) with a 2 Hz to 10 kHz passband. The sensitivity band was 0.1 μV to 10 mV; the input noise did not exceeded 40 μV.

After obtaining the coil positioning which ensured stable motor responses, we first retrieved MEP thresholds by decreasing the stimulation magnitude in steps of 2–5% of maximal output and stimulating the target area with an interval of more than 3 s. The magnitude that evoked responses of 20–50 μV amplitude with a 50 or more percent probability was taken as a threshold (Nikitin and Kurenkov, 2003). The muscle relaxation during thresholds evaluation was monitored *via* real-time electromyogram (EMG). We then increased stimulation magnitude in steps of 5–10% of maximal output until reaching maximal MEP amplitudes or 100% of maximal output. At each step, we recorded at least three MEPs. MEPs to transcranial and trans-spinal MS are referred to as "cortical MEPs" and "spinal MEPs," respectively.

Data Processing and Statistical Analysis

Motor evoked potential data were extracted from muscle curves using Viking Quest 11.1 software, raw latency and amplitude values for each single stimulation were obtained. For each participant we evaluated MEP thresholds and mean maximal peak-to-peak amplitudes at three registration points: baseline (average of two baseline points), R + 0, and R + 3.

For demonstration purposes, threshold and amplitude values were presented as mean ± SEM of percent changes from baseline, which was taken as zero. Statistical analysis was performed with GraphPad Prism 8 software. Data normality was assessed using the Kolmogorov–Smirnov test. Because data were generally not normally distributed, threshold and amplitude mean values were compared using the Friedman test with *post hoc* Dunn's multiple comparisons test. Data were assumed statistically significant at $p < 0.05$.

We also evaluated CMCT, which is calculated with the following formula: CMCT = cortical MEP latency − spinal MEP latency. Latency was measured as the time interval between the MS artifact and the first deflection of the muscular response from EMG baseline. From a series of responses with maximal amplitudes, the MEP with the shortest latency was considered for CMCT calculation.

RESULTS

Both thresholds and amplitudes of MEPs showed differences in range and direction of changes between subjects after DI and during the recovery period (**Supplementary Table 1**). Due to this inter-subject variance, value shifts on R + 0 and R + 3 were mostly not statistically significant compared with baseline. Nevertheless, there were general tendencies present in the majority of the group.

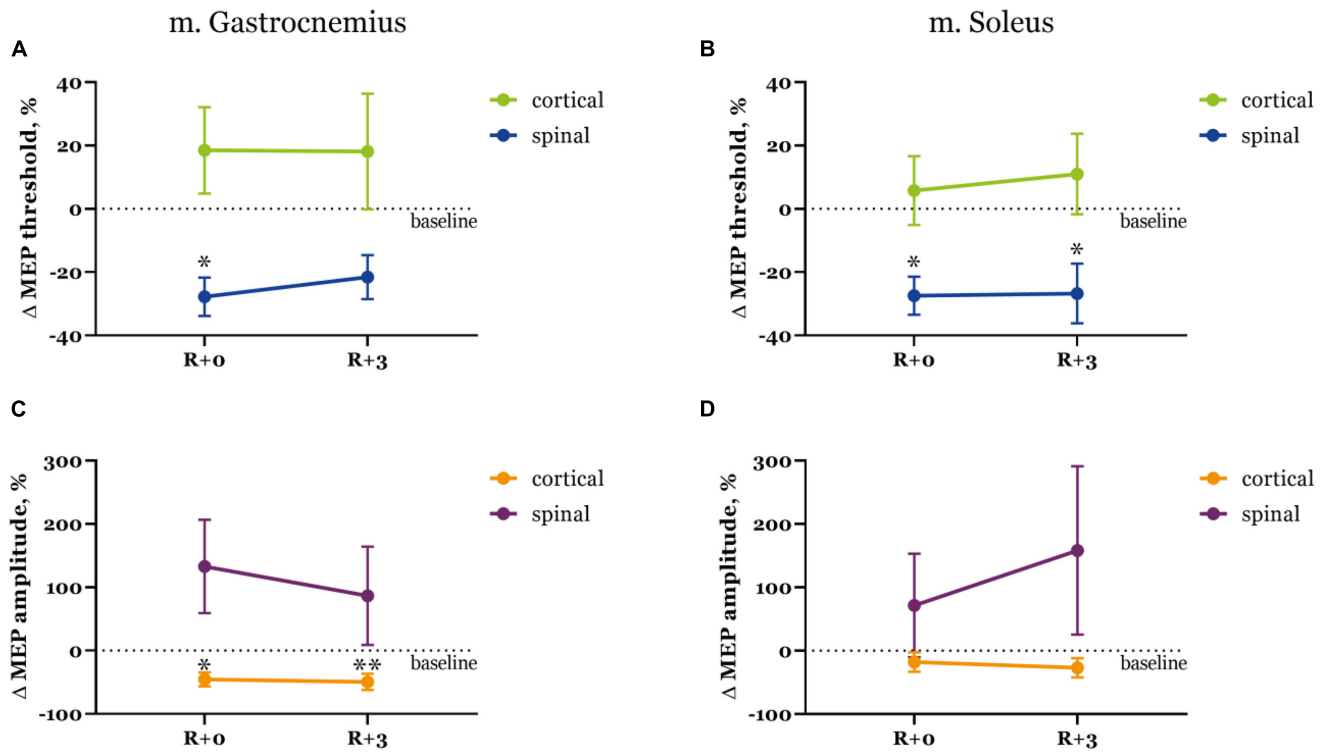

FIGURE 2 | Motor evoked potential thresholds **(A,B)** and MEP amplitudes **(C,D)** in the gastrocnemius **(A,C)** and soleus **(B,D)** muscles. Data is presented as mean ± SEM of percent changes from baseline. Baseline values are taken as zero. R + 0 – day of DI completion, R + 3 – third day after DI. *$p < 0.05$ vs. baseline, **$p < 0.01$ vs. baseline.

Threshold values changed in a similar manner for both recorded muscles, but depended on the stimulation site (**Figures 2A,B**). Specifically, spinal MEP thresholds lowered right after DI and then exhibited a slight recovery, more so in the gastrocnemius muscle. Cortical MEP thresholds conversely were higher right after DI and in the soleus muscle they even showed a tendency to increase during the recovery period. The decrease in thresholds to trans-spinal MS was more prominent (−27.7 and −27.5% from baseline in the gastrocnemius and soleus muscles respectively at R + 0) than the increase in thresholds to transcranial MS (18.5 and 5.8% from baseline in the gastrocnemius and soleus muscles, respectively at R + 0). Only spinal MEP thresholds significantly reduced from baseline ($p < 0.05$) after DI.

Interestingly, changes in MEP amplitudes were opposite for both types of stimulation (**Figures 2C,D**). Cortical MEPs had smaller amplitudes right after DI compared with baseline (−45.4 and −18.0% decrease in the gastrocnemius and soleus muscles, respectively), and their amplitudes decreased even further by the third day of recovery (−49.4 and −27.0% from baseline in gastrocnemius and soleus muscles, respectively). Spinal MEPs were characterized by substantially larger amplitudes on R + 0 (132.8 and 71.6% increase from baseline in gastrocnemius and soleus muscles, respectively) which then decreased to 86.5% in the gastrocnemius muscle and increased to 158.3% in the soleus muscle during the recovery period. Again, as was described for the threshold values, changes in MEP amplitudes were more drastic for trans-spinal MS.

We also calculated CMCT (**Supplementary Table 2**) to estimate possible changes in pyramidal tract conductibility. As shown on **Figure 3**, CMCT did not change significantly under the condition of DI, although it slightly decreased right after DI and then increased beyond baseline during the recovery period. Cortical MEP latencies had the biggest contribution to these CMCT fluctuations.

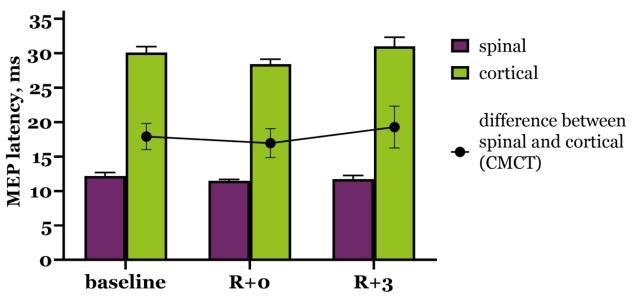

FIGURE 3 | Motor evoked potential latency to trans-spinal (purple bars) and transcranial (green bars) MS. Black dots represent CMCT values. R + 0 – day of DI completion, R + 3 – third day after DI.

DISCUSSION

Evoked responses generally changed in the same direction after DI regardless of the muscle being mostly phasic (gastrocnemius) or tonic (soleus). On the other hand, there were completely different dynamics of changes dependent on the stimulation site, i.e., cranial or spinal placement of the coil.

Motor evoked potential characteristics for trans-spinal MS after DI exposure (i.e., decreased thresholds and increased amplitudes) resemble the phenomenon of hypogravitational hyperreflexia – muscle hyperreflexia developing in the microgravity environment (Kozlovskaya et al., 1988). Previously we carried out a similar experiment on a group of male subjects (Nosikova et al., 2021) and described the same spinal MEPs changes. An increase in spinal excitability has also been shown in unilateral lower limb suspension, the other ground-based model of microgravity. The experiments were carried out on mixed groups, included men and women, and after 4 weeks of unloading an increase in the soleus H-reflex was observed (Clark et al., 2006, 2007). This evidence shows that hypogravitational hyperreflexia probably has a spinal origin and that it develops regardless of the subject's sex.

Cortical MEPs in women had the opposite dynamic: for most subjects their thresholds increased and amplitudes became smaller after DI. However, in men cortical MEPs changed with the same dynamic as spinal MEPs throughout the experiment, although shifts to transcranial MS were not as severe (Nosikova et al., 2021). TMS studies by other authors in this field are scarce, and their results appear to be inconsistent. For instance, in a parabolic flight induced weightlessness a facilitation of MEP responses was reported, suggesting an increase in corticospinal excitability during 0 G (Davey et al., 2004). In a similar study on a small male group zero gravity also led to a decrease in MEP thresholds, and the authors provided a few possible explanations for the observed phenomenon, including corticospinal excitability increase (Badran et al., 2020). Such changes in excitability were also observed after 10 days of lower leg immobilization (Roberts et al., 2007), although the authors hypothesize that excitability increases because of motor recovery and re-learning. Additionally, there were no significant differences in resting motor thresholds across experimental sessions. By contrast, in a long-term bed rest study corticospinal excitability decreased in the immediate post-bed rest period (Roberts et al., 2010). It is important to note that both studies by Roberts et al. (2010) were conducted on mixed groups, but no comparison between male and female participants was made.

Considering the literature, it is hard to tell whether the difference between male and female groups in two our experiments was sex dependent. The general decrease in amplitudes and increase in thresholds of cortical MEPs in women suggests a decrease in corticospinal excitability, but since such changes were not present in men (Nosikova et al., 2021), we cannot confidently conclude that corticospinal excitability reduces as a function of DI or support withdrawal. There is also an inconsistency in the dynamics of MEP characteristics in different models of microgravity (Davey et al., 2004; Roberts et al., 2007, 2010; Badran et al., 2020). It is possible that the small group size and large inter-subject variability affected our results.

We can also take into account hormonal fluctuations during the MC, which may affect nervous control of muscle activity. For example, estrogen has an excitatory impact on the nervous system, and progesterone induces inhibition, resulting in shifts of neuromuscular function throughout the MC (Ansdell et al., 2019). We suppose that changes in the endocrine profile do not greatly affect spinal conductibility, as spinal MEPs appear to be similar in men and women, although MEP amplitudes in women varied substantially across subjects (**Figures 2C,D**). Spinal MEP latencies also were unchanged after DI (**Figure 3**), which proves that spinal conductibility was not affected. Cortical MEPs pose a more challenging question about the origin of their changes that could be influenced by the MC. Specifically, varying dynamics of MEP characteristics between subjects could signify diverging adaptive reactions of neurons, therefore, one should judge the described changes carefully. More research is needed to explore and better understand the underlying mechanisms.

Limitations

The main limitation of the study is the sample size of 6 as it is too low to provide reliable outcomes measures. However, this is a novel study, and we believe that the data obtained in the first female DI experiment might be helpful for the future research in this field.

CONCLUSION

The results of our study show that 3-day support withdrawal in women leads to an increase in spinal excitability, which manifests as a threshold decline and amplitude increase of trans-spinal MS evoked motor responses in the lower leg muscles. These data are in line with our previous research conducted on a male group, as well as with studies carried out with the participation of both men and women. The changes in corticospinal excitability were ambiguous and could possibly be affected by a number of factors such as large inter-subject variability or the sex hormones profile. As this finding is not fully supported by the literature, it demands further and more careful research.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Bioethical Commission of the Institute of Biomedical Problems of Russian Academy of Science. The

patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IN and AR collected the data and wrote the draft of the manuscript. LA contributed to the data analysis and design of the figures. VK contributed with the technical support. ET made a revision of the manuscript and was a supervisor of the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnhum.2021.753259/full#supplementary-material

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frontiers in Aging Neuroscience

ORIGINAL RESEARCH
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Subcortical Volume Changes in Early Menopausal Women and Correlation With Neuropsychological Tests

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Background: The aging process and declining estradiol levels are two important factors that cause structural brain alterations. Many prior studies have investigated these two elements and revealed controversial results in menopausal women. Here, a cross-sectional study was designed to individually evaluate estradiol-related structural changes in the brain.

Methods: A total of 45 early menopausal women and 54 age-matched premenopausal controls were enrolled and subjected to magnetic resonance imaging (MRI) scans, blood biochemistry tests, and neuropsychological tests. MRI structural images were analyzed using FreeSurfer to detect changes in subcortical and cortical volumes as well as cortical thickness. Finally, structural brain data as well as clinical and neuropsychological data were used for Pearson's correlation analyses to individually determine estradiol-related structural and functional changes in the brains of early menopausal women.

Results: Compared with the premenopausal controls, the early menopausal women showed significant subcortical volumetric loss in the left amygdala and right amygdala, higher serum follicle-stimulating hormone (FSH) levels, more recognizable climacteric and depressive symptoms, decreased quality of sleep, and decreased working memory and executive functions. Simultaneously, FSH levels were related to lower working memory accuracy and longer working memory reaction time. Decreased subcortical volume in the bilateral amygdala was also related to lower working memory accuracy and longer executive reaction time in early menopausal women.

Conclusion: The data suggest that estradiol deficiency in early menopausal women can lead to subcortical volume and functional brain changes, which may contribute to further understanding the neurobiological role of declined estradiol levels in early menopausal women.

Keywords: early menopausal women, FreeSurfer, subcortical and cortical volume, amygdala, cognition, emotion

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INTRODUCTION

With the aging process and a progressive reduction in naturally circulating levels of sex hormones, menopausal women experience a range of menopausal symptoms, including declined cognition and structural alterations in the brain (Engler-Chiurazzi et al., 2016; Koothirezhi and Ranganathan, 2021; Peacock and Ketvertis, 2021). To illuminate the neuroprotective affection of estradiol, magnetic resonance imaging (MRI) studies have been performed, but paradoxical outcomes have emerged.

Some studies found that postmenopausal women who received hormone therapy (HT) displayed larger hippocampal volumes and better cognition than those who used placebo (Boccardi et al., 2006; Genazzani et al., 2007; Erickson et al., 2010a). In contrast, other studies demonstrated no differences in hippocampal volume and cognition before and after HT (Eberling et al., 2004; Raz et al., 2004). In addition, a handful of studies revealed that hippocampal and prefrontal cortex volumes were smaller in women who were subject to HT than in controls (Coker et al., 2009). It should be noted that these studies combined the two factors of sex hormone deficiency and age, which worked together to cause the neurotoxicity effects (Jose et al., 2013; Grimm and Eckert, 2017). There have been very few neuroimaging studies that individually evaluated hormone-related changes in the brain among perimenopausal women.

Previous research found obvious estradiol-related alterations in cerebral activity in the left amygdala and bilateral middle occipital gyrus in early menopausal women. However, no significant voxel-based morphometry structure changes were found (Zhang et al., 2018). It is noted that these findings differ from a common perspective that postmenopausal women who receive HT, who exhibit higher serum estradiol levels, show larger hippocampal/parahippocampal volumes than those receiving placebos (Morrison et al., 2006; Lord et al., 2008; Girard et al., 2017). It is suspected that the possible reason for these results was that the experimental subjects were in a state of sex hormone deficiency, which contrasts to subjects in prior research.

Current mainstream opinions in neuroscience consider the view that the hippocampus/parahippocampus-related brain function changes in menopausal women compared to healthy controls (Lord et al., 2008; Rebekah and Christian, 2013; Miller and Harman, 2017). Thus, it was proposed that there may be volumetric changes in the substructures that make up the hippocampus/parahippocampus instead of the hippocampus/parahippocampus in early menopausal women. However, there is no relevant research to support this hypothesis. In addition, to the best of our knowledge, cognitive function is related to cortical thickness and cortical volume, which has also been demonstrated by MRI studies of healthy and brain-lesioned subjects (Watson et al., 2015). Thus, novel techniques should be sensitive enough for segmentation of the subcortical structure of the hippocampus or parahippocampus, which may reveal differences between early menopausal women and controls (Albert et al., 2017).

The FreeSurfer automated segmentation procedure is a novel and reliable method to measure hippocampal subregional volumes (Schmidt et al., 2018; Chiappiniello et al., 2021; Figueiredo et al., 2021). It has been authenticated in many studies, including those investigating Alzheimer's disease (Novak and Einstein, 2013), schizophrenia (Tost et al., 2013), and fetal alcohol spectrum disorder (Guio et al., 2016). In the present investigation (Zhang et al., 2018), 45 early menopausal women with naturally low endogenous estradiol levels were enrolled. A total of 54 age-matched premenopausal women with naturally low endogenous estradiol levels were also enrolled. A cross-sectional survey that aimed to eliminate the influences of age-related structural alterations in the brain was designed to investigate the modulatory effects of hormones on subcortical brain regions using a neuroimaging approach. Subsequently, subcortical volumes as well as clinical and neuropsychological data were used for Pearson's correlation analyses to individually determine the early menopausal women's hormone-related structural and functional brain changes. The goal was to illuminate subcortical structural and volume changes in the brains of early menopausal women and their relation to neurocognitive function.

MATERIALS AND METHODS

Participants

The local Medical Research Ethics Committee of Xinqiao Hospital (Chongqing, China) approved this study protocol, and all subjects provided written, informed consent. A portion of the participants were recruited through advertisements in the community, and others were recommended by gynecology clinics. A total of 45 right-handed early menopausal women (age range: 45–51 years, mean age: 47.38 $\pm$ 1.65 years) who fulfilled the following criteria were enrolled (Chen et al., 2017): (1) irregular menstrual cycle length (i.e., menstrual disorders); and (2) 2 or more of the last 10 menstrual cycles showed an adjacent menstrual cycle length change of at least 7 days (Menopause Subgroup et al., 2018). A total of 54 right-handed and education-matched premenopausal control women (age range: 45–49 years, mean age: 46.89 $\pm$ 1.69 years) with regular menstrual cycles were also enrolled.

All participants were excluded before enrollment for the following conditions: estradiol or progestational hormone use in the past 6 months, bilateral oophorectomy, Schizophrenic disorder, mood (emotion) disorder, personality and behavior disorders, mental developmental disorders, no specific mental disorders, drug or alcohol abuse, history of traumatic brain injury, neuroanatomical abnormalities in the brain, and chronic diseases (i.e., diabetes, hypertension) that may affect brain structure (Liu et al., 2017). Finally, participants with MRI contraindications and poor image quality were excluded (Bixo et al., 2018).

Experimental Design

To eliminate possible influences caused by the aging process, our team strictly selected women in the age range of 45–50 years old so that the specific effects of estradiol on brain structure could be determined. All members tested participants' serum follicle-stimulating hormone (FSH) and estradiol (E2) levels, clinical symptoms as measured by the specialized Kupperman Index (KI,

which describes common symptoms and weighs these factors to evaluate the severity of climacteric symptoms), and scores on the Pittsburgh Sleep Quality Index (PSQI). Other measures that were taken included a neuropsychological assessment using the Beck Depression Inventory II (BDI- II, self-reported depression levels), SAS (self-rating anxiety scale) and MRI scans. Finally, cognitive tests were carried out by the E-prime program on the computer, and executive function and working memory were the main focus of the cognitive domains. Executive function was evaluated by the Stroop Test. The Stroop test were conducted as follows: firstly, the task had four types of stimuli, namely four types of color and words with "red," "yellow," "green," and "blue"; secondly, the subjects ignored the word meaning and only responded to the color; at last, we calculate the reaction time under inconsistent conditions and the reaction time under consistent conditions. The ability of memory was evaluated by the Two-back working memory task. While undergoing all of these assessments, the premenopausal controls were required to be in the follicular phase of the menstrual cycle.

Image Acquisition

Participants underwent MRI scans using a 3.0 T GE MRI system. Acquisition included high-resolution T1-weighted images using a three-dimensional, fast spoiled, gradient-echo (3DSPGR) sequence with a standard eight-channel phased-array head coil and approximately matched parameters (31.25 Hz/pixel bandwidth, TE = 2.8 ms, TR = 450 ms, flip angle = 15°). All scans were 3D sagittal acquisitions with 124 contiguous slices (slice thickness = 1.6 mm, slice gap = 0 mm; FOV = 240 mm × 240 mm, matrix = 256 × 256, isotropic voxel size = 1.6 mm × 1.6 mm × 1.6 mm). Acquisition time was 4 min plus 19 s. Subjects were required to make no movements and close their eyes during the scanning course (Liu et al., 2020).

Standard FreeSurfer Processing Pipeline

For analysis, the T1-weighted images with indistinct gray-white demarcation and obvious motion artifacts were excluded first. The cerebrum cortical reconstruction and volumetric segmentation were conducted by the FreeSurfer image analysis suite (version 6.0), which has good test-retest reliability on scanners of different manufacturers (Chiappiniello et al., 2021). Field strengths were freely obtained online,[1] and technical details of these procedures are described in prior publications.

The processing steps were as follows: (1) motion correction; (2) separation of the brain and non-brain tissue using a hybrid watershed or surface deformation procedure; (3) automated Talairach transformation; (4) segmentation of subcortical white matter and deep gray matter brain structures (i.e., hippocampus, amygdala, thalamus, caudate, putamen, pallidum, and ventricles); (5) intensity normalization; (6) tessellation of the boundaries of the gray and white matter, (7) topology correction, (8) surface deformation; and (9) use of the intensity gradients to properly divide the gray/white and gray/cerebrospinal fluid boundary, which defined the greatest shift in intensity in the other tissue (Brown et al., 2020).

[1] http://surfer.nmr.mgh.harvard.edu

Once the standard processing stream was completed, the data was inspected, and accuracy of the boundaries between gray/white and gray/cerebrospinal fluid were confirmed. Some subjects needed minimal edits, in which case images were run through the second reconstruction, beginning at the point where the edits were applied.

Statistical Analysis

Two-sample *t*-tests were conducted with the statistical software program SPSS 22.0 to detect group differences in subcortical volume, demographic characteristics, clinical data, neuropsychological results, and accuracy and reaction times of the Stroop task and Two-back task. Statistical inspection level was defined as $p = 0.05$. Simultaneously, differences in subcortical volume data were controlled for multiple comparisons using the standard false discovery rate (FDR) approach with a false-positive rate of 5% ($p = 0.05$).

To investigate putative diagnostic-specific relationships and acquire partial correlation coefficients for further group comparisons, hierarchical regression analyses were performed for subcortical volume, clinical data, and neurocognitive function variables. Age and educational degrees were regarded as the covariates. Next, Pearson's correlation global analyses were conducted with SPSS software, and the statistical significance threshold was set as $p < 0.05$.

RESULTS

Demographic, Clinical, and Neuropsychological Results

Demographic, clinical, and neuropsychological group comparisons are exhibited in **Table 1**. There were no statistically significant differences in age ($p = 0.151$), educational level ($p = 0.757$), SAS ($p = 0.936$) and Stroop accuracy rating ($p = 0.051$). Compared with premenopausal controls, early menopausal women showed prominently higher scores on the KI ($p = 0.031$), BDI- II ($p < 0.001$), and PSQI ($p = 0.003$). Neuropsychological results demonstrated that early menopausal women exhibited longer Stroop test reaction time ($p < 0.001$) and Two-back working memory reaction time ($p = 0.002$) but lower accuracy rate in the Two-back working memory test ($p < 0.001$). Early menopausal women group had obviously higher serum FSH levels ($p < 0.001$) and lower estradiol levels than control group. Hormone levels were too low to be measured accurately in part of the early menopausal women; hence, there were no statistical results for this cohort.

Group Differences in Subcortical Volume

A total of 41 selected brain regions were, respectively, segmented. Differences were calculated across the groups, which exhibited that early menopausal women had significant smaller subcortical volumes in the left amygdala ($p < 0.001$), right hippocampus ($p = 0.045$), and right amygdala ($p = 0.001$) (**Table 2**). After the FDR correction, only the left amygdala and right amygdala reflected these differences. Subsequently, subcortical volume

TABLE 1 | Demographic, clinical, and neuropsychological data.

Characteristics	Early menopausal women	Premenopausal controls	*p*-value
n	45	54	
Age at scan (years)	47.38 ± 1.65	46.89 ± 1.69	0.151 (N.S.)
Years of education	12.93 ± 2.76	13.11 ± 2.90	0.757 (N.S.)
FSH (mIU/ml)	46.69 ± 25.65	13.07 ± 10.18	0.000
Kupperman Index	11.73 ± 7.15	8.80 ± 6.21	0.031
BDI-II	10.91 ± 7.62	5.76 ± 5.39	0.000
SAS	41.53 ± 9.81	41.67 ± 6.44	0.936
PSQI	7.00 ± 3.23	4.91 ± 3.49	0.003
Two-back			
ACC	0.9429 ± 0.0277	0.9646 ± 0.0206	0.000
RT (ms)	1113.3818 ± 276.8734	964.3576 ± 192.2480	0.002
Stroop test			
ACC	0.8931 ± 0.1037	0.9320 ± 0.0919	0.051 (N.S.)
RT (ms)	831.2380 ± 125.3691	754.6167 ± 84.2718	0.000

FSH, follicle-stimulating hormone; BDI-II, Beck Depression Inventory II; SAS, self-rating anxiety scale; PSQI, Pittsburgh Sleep Quality Index; ACC, accuracy rating; RT, reaction time; N.S., not significant.

of the two groups in the left amygdala and right amygdala were conducted with hierarchical regression analysis for further Pearson's correlation and global analysis.

Correlations Among Subcortical Volume, Clinical Data, and Neuropsychological Results

After correcting for age and educational level, Pearson's correlation analyses were performed for all experimental data. Results showed that the increased serum FSH levels were negatively correlated with working memory accuracy ($p = 0.001$, $r = -0.315$) and positively correlated with working memory RT ($p = 0.032$, $r = 0.216$). The Two-back working memory accuracy rate was positively correlated with left amygdala ($p = 0.011$, $r = 0.256$) and right amygdala ($p = 0.001$, $r = 0.328$). Executive RT was negatively correlated with left amygdala ($p = 0.046$, $r = -0.201$), and right amygdala ($p = 0.024$, $r = -0.227$). The abovementioned outcomes are shown in **Figure 1**.

DISCUSSION

This research intended to explore the relationships between subcortical brain volumes and sex hormones; therefore, matching for age and education in both groups was conducted. Prior study results revealed estradiol-related aberrant cerebral activity in the left amygdala and bilateral middle occipital gyrus but no obvious voxel-based morphometry structure alterations in early menopausal women (Zhang et al., 2018). Our team expanded the subject sample size and used a novel and reliable method to detect hippocampal subregional volume.

When comparing between the selected subcortical and cortical brain structures (estimated from FreeSurfer Version 6.0) across the groups, the most obvious alterations in brain structure were shrinkage of the left amygdala and right amygdala in the early menopausal women in contrast to the premenopausal controls. Simultaneously, early menopausal women displayed higher serum FSH levels, more severe climacteric and depressive symptoms, declined quality of sleep, and decreased memory and

TABLE 2 | Group differences in subcortical volume.

Brain structure (mm^3)	Early menopausal women	Premenopausal controls	*p*-value
Left lateral ventricle	6252.21 ± 3503.31	5520.95 ± 2401.37	0.223(N.S.)
Left inf lateral ventricle	163.66 ± 110.36	153.91 ± 129.07	0.691(N.S.)
Left cerebellum WM	15288.99 ± 3624.12	16296.78 ± 3154.71	0.142(N.S.)
Left cerebellum cortex	44147.05 ± 3594.56	44710.40 ± 4449.10	0.496(N.S.)
Left thalamus proper	7463.40 ± 884.33	7564.60 ± 752.37	0.540(N.S.)
Left caudate	3223.36 ± 360.89	3267.29 ± 423.03	0.584(N.S.)
Left putamen	4774.27 ± 619.40	4921.89 ± 654.90	0.255(N.S.)
Left pallidum	1166.98 ± 192.86	1159.55 ± 244.04	0.869(N.S.)
3rd Ventricle	899.43 ± 239.45	866.91 ± 213.48	0.477(N.S.)
4th Ventricle	1506.30 ± 327.59	1578.42 ± 410.91	0.344(N.S.)
Brainstem	18776.61 ± 1463.83	18770.68 ± 1786.76	0.986(N.S.)
Left hippocampus	4264.5 ± 378.73	4388.35 ± 325.86	0.083(N.S.)
Left amygdala	1456.82 ± 161.14	1659.83 ± 184.13	0.000
CSF	1064.46 ± 171.35	1053.93 ± 186.33	0.772(N.S.)
Left accumbens area	506.14 ± 86.70	509.22 ± 87.17	0.861(N.S.)
Left ventral DC	3918.08 ± 429.21	4005.81 ± 498.07	0.355(N.S.)
Left vessel	76.63 ± 27.61	87.97 ± 34.66	0.079(N.S.)
Left choroid plexus	1046.57 ± 251.98	1071.09 ± 188.52	0.581(N.S.)
Right lateral ventricle	5294.26 ± 2777.51	5089.72 ± 2360.91	0.693(N.S.)
Right inf lateral ventricle	176.00 ± 135.55	134.56 ± 88.30	0.070(N.S.)
Right cerebellum WM	16407.57 ± 4514.47	16265.99 ± 4161.08	0.871(N.S.)
Right cerebellum cortex	45685.92 ± 4384.58	45987.90 ± 4765.02	0.746(N.S.)
Right thalamus proper	6581.79 ± 535.33	6735.46 ± 564.35	0.171(N.S.)
Right caudate	3248.55 ± 334.75	3219.34 ± 344.27	0.671(N.S.)
Right putamen	4443.09 ± 527.18	4600.41 ± 541.89	0.149(N.S.)
Right pallidum	1184.70 ± 239.19	1204.79 ± 209.65	0.657(N.S.)
Right hippocampus	4317.52 ± 283.55	4449.22 ± 349.49	0.051(N.S.)
Right amygdala	1556.95 ± 135.78	1671.55 ± 182.06	0.001
Right accumbens area	471.36 ± 78.36	495.97 ± 80.29	0.128(N.S.)
Right ventral DC	4054.47 ± 461.67	4142.84 ± 485.22	0.359(N.S.)
Right vessel	83.15 ± 31.99	88.62 ± 47.44	0.512(N.S.)
Right choroid plexus	1135.38 ± 357.74	1198.40 ± 220.87	0.286(N.S.)
Optic chiasm	288.21 ± 59.41	295.87 ± 57.85	0.518(N.S.)
CC_posterior	906.81 ± 153.71	915.53 ± 114.45	0.747(N.S.)
CC_mid-posterior	431.92 ± 98.07	435.34 ± 104.08	0.868(N.S.)
CC_central	502.99 ± 138.19	517.19 ± 120.47	0.586(N.S.)
CC_mid-anterior	459.74 ± 118.25	469.97 ± 97.08	0.637(N.S.)
CC_anterior	746.52 ± 120.49	787.42 ± 116.37	0.090(N.S.)
lhSurfaceHoles	60.77 ± 17.26	58.90 ± 12.54	0.535(N.S.)
rhSurfaceHoles	62.24 ± 12.29	64.75 ± 17.18	0.413(N.S.)
SurfaceHoles	123.02 ± 27.31	123.66 ± 26.50	0.906(N.S.)

CSF, cerebrospinal fluid; WM, white matter; inf, inferior; lat, lateral; DC, dorsal cortex; CC, cerebral cortex; lh, left hemisphere; rh, right hemisphere; N.S., not significant.

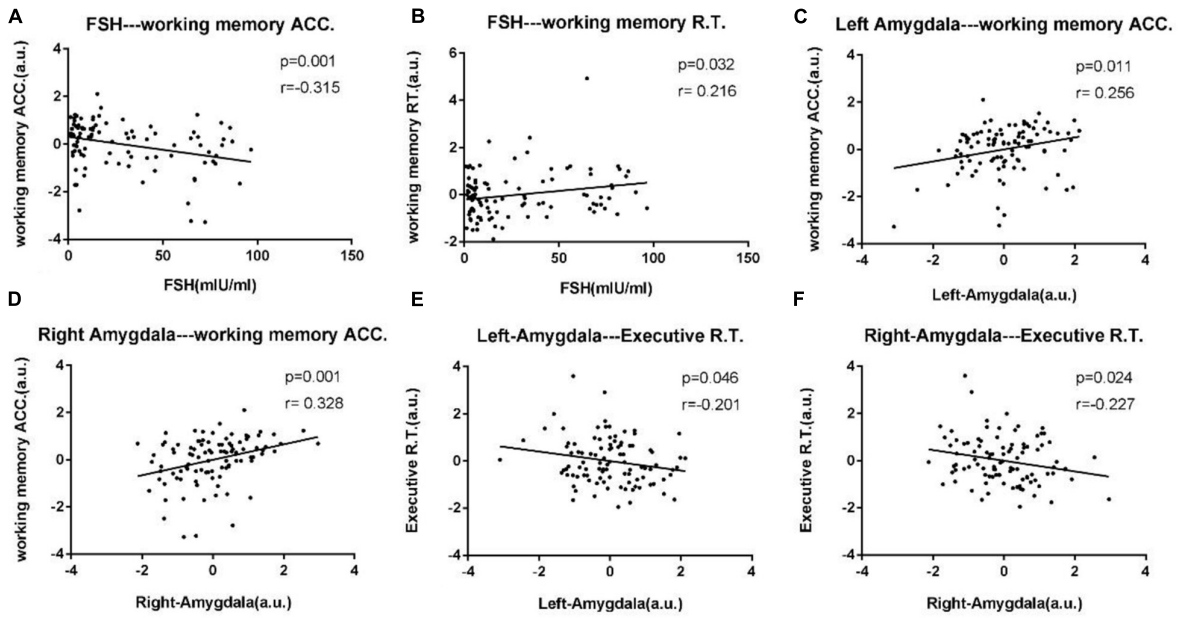

FIGURE 1 | Scatter diagrams exhibit significant pair-wise correlations among bilateral amygdala, clinical data, and neuropsychological data from all experimental participants in **(A–F)**. **(A)** Increased serum FSH levels were negatively correlated with working memory accuracy ($p = 0.001$, $r = –0.315$). **(B)** Increased serum FSH levels were positively correlated with working memory RT ($p = 0.032$, $r = 0.216$). **(C,D)** The Two-back working memory accuracy rate was positively correlated with left amygdala ($p = 0.011$, $r = 0.256$) and right amygdala ($p = 0.001$, $r = 0.328$). **(E,F)** Executive reaction time was negatively correlated with left amygdala ($p = 0.046$, $r = –0.201$), right amygdala ($p = 0.024$, $r = –0.227$).

executive function. In addition, Pearson's correlation analysis revealed that increased FSH levels were correlated with working memory. Decreased subcortical volume in the bilateral amygdala was also associated with working memory and executive capacity. These results suggest that the subcortical structural alterations were closely correlated with clinical symptoms in early menopausal women, which may provide direction for future studies on biomarkers and medication guidelines in early menopausal women.

Regarding sex hormones, the early menopausal women showed declines in naturally circulating levels of sex hormones (i.e., estradiol, progesterone) as well as a dysregulation of gonadotropin feedback loops, characterized by increasing levels of serum FSH and luteinizing hormone (LH) (Gillies and Mcarthur, 2010; Merlo et al., 2017). Meanwhile, in previously published reports, many estradiol response subtypes have been found in brain regions correlated with memory and executive capacity (i.e., amygdala, hippocampus, cerebral cortex, and basal forebrain) (Rettberg et al., 2014; Velázquez et al., 2021). Besides, endogenous hormones can induce permanent changes in certain tissue throughout the lifespan (Raz et al., 2004; Erickson et al., 2010b; Herting et al., 2015).

In this study, the early menopausal women had lower estradiol levels in contrast to the controls. The exact level of estradiol in some subjects was too low to be detected; thus, there were no statistical or correlation analyses for this crucial hormone in that cohort, so the value of serum FSH was indirectly used to reflect this condition. Furthermore, increased serum FSH levels were negatively correlated with lower working memory accuracy and positively correlated with longer working memory reaction time. This suggests that memory function may decrease as serum FSH levels increase, which is consistent with previous studies.

The amygdala encompasses several subregions with distinct functional and connectional characteristics in humans (Erickson et al., 2010a). Previous studies have shown that the amygdala is related to either pleasant or unpleasant emotions (i.e., fear, anxiety, and depression) (Barrett et al., 2007; Lanteaume et al., 2007; Williams et al., 2010). Other evidence has proven that the left amygdala is involved in the regulation of memory consolidation by emotional arousal. During this period, synaptic plasticity is promoted by increasing interactions between neocortical storage sites and temporal lobe structures, which involved in declarative memory (Paré et al., 2002; László et al., 2010).

In addition, our prior research on postmenopausal women displayed functional connections between the amygdala and bilateral prefrontal cortex, which may be involved in the neuropathological mechanisms underlying executive function impairments. In this study, decreased subcortical volume in the bilateral amygdala in early menopausal women were associated with prominently higher scores on depression scales, lower accuracy rate in the Two-back working memory test, and longer executive reaction time in an executive function exam. Meanwhile, Pearson's correlations analyses showed that reduced subcortical volume in the bilateral amygdala was positively associated with lower working memory accuracy and negatively associated with longer executive reaction time. These results are consistent with the corresponding preceding results, which indicate that the amygdala plays a key role in modulating mental and cognitive functions in early menopausal women.

Two limitations in this research should be noted. First, this is a prevalence survey, and whether the alterations in brain structure and function are reversible after estradiol replacement therapy remains to be investigated using another experimental design. Second, no correlation results between sex hormones and the changes in brain structure were found since this experiment was performed in hospital, so the exact level of estradiol was unattainable if the exact value was less than 10. Taking these two limitations into consideration, the exact levels of sex hormones should be tested in a biochemical laboratory. Finally, the early menopausal women who undergo hormone therapy should be added as another control group to confirm whether the changes in brain structure and function are reversed after estradiol replacement therapy in following studies.

CONCLUSION

This study, for the first time, clarifies the effects of sex steroid hormones on brain structure in early menopausal women. The study showed that shrinkage in the left amygdala and right amygdala were the most obvious alterations in brain structure. Furthermore, subcortical structural alterations were closely correlated with clinical symptoms in early menopausal women, which may guide the potential use of estradiol for prevention of cognitive impairment and structural brain alterations in menopausal women.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Research Ethics Committee of Xinqiao Hospital (Chongqing, China). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DZ, LW, SZ, WF, HH, MG, BL, JH, and GL conceptualized and designed the study. HH, MG, BL, JH, and GL conducted the research. SZ and WF analyzed the data and wrote the manuscript. All authors provided critically revisions to the manuscript.

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The Menstrual Cycle Modulates Whole-Brain Turbulent Dynamics

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Brain dynamics have recently been shown to be modulated by rhythmic changes in female sex hormone concentrations across an entire menstrual cycle. However, many questions remain regarding the specific differences in information processing across spacetime between the two main follicular and luteal phases in the menstrual cycle. Using a novel turbulent dynamic framework, we studied whole-brain information processing across spacetime scales (i.e., across long and short distances in the brain) in two open-source, dense-sampled resting-state datasets. A healthy naturally cycling woman in her early twenties was scanned over 30 consecutive days during a naturally occurring menstrual cycle and under a hormonal contraceptive regime. Our results indicated that the luteal phase is characterized by significantly higher information transmission across spatial scales than the follicular phase. Furthermore, we found significant differences in turbulence levels between the two phases in brain regions belonging to the default mode, salience/ventral attention, somatomotor, control, and dorsal attention networks. Finally, we found that changes in estradiol and progesterone concentrations modulate whole-brain turbulent dynamics in long distances. In contrast, we reported no significant differences in information processing measures between the active and placebo phases in the hormonal contraceptive study. Overall, the results demonstrate that the turbulence framework is able to capture differences in whole-brain turbulent dynamics related to ovarian hormones and menstrual cycle stages.

Keywords: menstrual cycle, turbulence, brain information processing, whole-brain dynamics, resting-state fMRI

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1. INTRODUCTION

The brain is one of the most complex systems, and it is intrinsically modulated by sex steroid hormone fluctuations (for a review, see Beltz and Moser, 2020). Ovarian hormones represent the neuroendocrine milieu throughout the female lifespan (McEwen et al., 2012). For instance, previous studies have demonstrated the significant influence of estradiol and progesterone in cognitive, emotional and social functioning (Toffoletto et al., 2014; Barth et al., 2015; Galea et al., 2017). Estradiol and progesterone concentrations are related to hippocampal connectivity, upholding memory retrieval (Jacobs et al., 2016, 2017). Psychosocial stress response seems to be also modulated by estradiol as high concentrations are related to deactivation of the limbic system in contrast with lower estradiol levels (Albert et al., 2015). In naturally-cycling women, a typical menstrual cycle occurs every 25–32 days (Lenton et al., 1984, for a meta-review, see Fehring et al., 2006) and comprises two main broad phases, the follicular and the luteal phases (Guyton and Hall, 2006; Song et al., 2017; Garcia et al., 2018; Olatunji et al., 2020; Schmalenberger et al., 2020). The follicular phase begins with the onset of menses, and it is characterized by a progressive increase in estradiol concentrations that reach the maximum peak during the pre-ovulatory phase, during which progesterone levels are at their lowest. In contrast, the luteal phase, which spans after ovulation occurs until the last day of the menstrual cycle, is marked by an increment in progesterone levels that reach their peak near the middle of the phase, when estradiol typically experiences a secondary mid-luteal peak (Reed and Carr, 2000; Bull et al., 2019).

Exploring differences in the brain as a function of the menstrual cycle phases and ovarian hormone levels has been undertaken using several imaging modalities (Witte et al., 2010; Jacobs and D'Esposito, 2011; Rapkin et al., 2011; Petersen et al., 2014; Arélin et al., 2015; Lisofsky et al., 2015b; Catenaccio et al., 2016; Engman et al., 2018; Weis et al., 2019; for a review, see Dubol et al., 2021). To date, human brain imaging studies have typically sampled women across a limited set of days, which do not represent the whole rhythmic variability of hormone production over an entire menstrual cycle. More recently, a dense-sampling neuroimaging study has shown positive associations between estradiol and dynamic, spatially-diffuse changes in resting-state networks from a 30 consecutive days assessment of a young woman, thus enabling the study of functional connectivity over a complete menstrual cycle (Pritschet et al., 2020; Mueller et al., 2021).

Yet, the effects of the two main phases of the menstrual cycle and day-to-day hormonal changes on whole-brain information processing remain unclear. Recently, a novel framework has been proposed to describe information processing at the macroscale level by demonstrating the presence of turbulent dynamics in the human brain (Deco and Kringelbach, 2020; Deco et al., 2021a). Turbulent brain dynamics have been explored in a large sample of healthy subjects, where enhanced transmission of information across the whole brain was determined by the local synchronization between brain regions (Deco and Kringelbach, 2020). Inspired by the advances in corroborating the presence of turbulent behavior in fluid dynamics (Kuramoto, 1984; Kolmogorov, 1991a,b), this framework has sought to demonstrate the link between this level of local synchronization among brain areas with the rotational vortices found in fluid dynamics. Therefore, the novelty of the method is that it allows analyzing the brain's information processing across spacetime scales, given that the size of rotational vortices determines various scales of information transmission. This framework has been successfully applied to characterize turbulent behavior in the brain's information processing during rest and different cognitive tasks (Deco and Kringelbach, 2020), demonstrated that different levels of turbulent dynamics describe and differentiate between unconscious and conscious brain states (Escrichs et al., 2021), and showed how large-scale connections enhance the transmission of information across the whole-brain network (Deco et al., 2021b). Therefore, the turbulent framework may advance our understanding of the effect of the two main phases of the menstrual cycle (i.e., follicular and luteal) on large-scale brain network communication.

Here, we applied the turbulent framework (Deco and Kringelbach, 2020; Deco et al., 2021b; Escrichs et al., 2021) to the same deep-sampling datasets used in Pritschet et al. (2020) and Mueller et al. (2021) to investigate the brain's information processing across spacetime scales during the follicular and luteal phases of a woman's menstrual cycle. First, we applied a turbulent dynamic analysis while the participant, scanned daily over an entire menstrual cycle ($N = 30$ days), was freely cycling (NaturalMenstrualCycle Study). In addition, we investigated turbulent dynamics while the same participant was under a hormonal-based regime to compare differential endocrine states on the brain's information processing (HormonalContraceptive Study). Moreover, we computed multi-level models with measures of information processing as output, and estradiol and progesterone as predictors to understand the relationship between day-by-day ovarian hormone levels and whole-brain turbulent dynamics.

2. METHODS

2.1. Participant and Study Design

A healthy woman was scanned during 30 consecutive days over a complete natural menstrual cycle and while taking a hormonal contraceptive pill (aged 23 and 24, respectively). The participant underwent time-locked blood sampling to determine hormone levels before each MRI session. The participant had no history of psychiatric nor endocrine disorders. During the NaturalMenstrualCycle Study, the participant was naturally-cycling and had not undergone any hormonal treatment 12 months before the study. In the HormonalContraceptive Study, the subject was under a hormonal-based regime (0.02 mg ethinylestradiol, 0.1 mg levonorgestrel, Aubra, Afaxys Pharmaceuticals) for 10 months before acquiring the data. This hormonal regime selectively suppressed progesterone levels, allowing estradiol to fluctuate at comparable levels to the NaturalMenstrualCycle Study. For further information about

the dataset, we refer readers to Pritschet et al. (2020) and Mueller et al. (2021).

2.2. MRI Data Acquisition

Raw data of daily scanning for both studies were obtained from the open-access project "28andMe" (https://openneuro.org/datasets/ds002674/versions/1.0.5). Anatomical and functional data were acquired on a Siemens 3T Prisma scanner with a 64-channel phased-array head coil. High-resolution T1-weighted images were acquired with magnetization prepared rapid gradient echo (MPRAGE) sequence (TR = 2,500 ms, TE = 2.31 ms, TI = 934 ms, flip angle = 7°, 0.8 mm thickness) with a gradient echo fieldmap (TR = 758 ms; TE1 = 4.92 ms; TE2 = 7.38 ms; flip angle = 60°). Resting-state data were acquired using a T2*-weighted multi-band echo-planar imaging (EPI) sequence (72 oblique slices, TR = 720 ms, TE = 37 ms, voxel size=$2mm^3$, flip angle = 56°, multiband factor = 8) for a total of 820 volumes (=10 min). A complete description of the MRI acquisition can be consulted in Pritschet et al. (2020) and Mueller et al. (2021).

2.3. Resting-State fMRI Preprocessing

We computed preprocessing of functional data using the Data Processing Assistant for Resting-State fMRI (DPARSF) (Yan and Zang, 2010). Before preprocessing, functional and anatomical images were manually reoriented. Then, resting-state data were corrected for differences in slice acquisition time, and the first 5 time-points were discarded to allow for signal stabilization. Further preprocessing steps included realignment for motion correction across volumes and co-registration of functional images to T1-weighted images using unified segmentation. Then, a regression of nuisance covariates was applied to correct data for six head movement parameters, for global mean signal, the white matter, and cerebrospinal fluid signal. Subsequent steps included spatial normalization in MNI space, smoothing using a 6-mm full-width-at-half-maximum Gaussian kernel, and band-pass temporal filtering of 0.01–0.1 Hz. Lastly, time series were extracted using the Schaefer parcellation comprising 1,000 regions and 17 resting-state networks (Schaefer et al., 2018).

2.4. Probabilistic Tractography Analysis

We computed the T2-weighted and diffusion spectrum images of 32 participants from the Human Connectome Project (HCP) as reported in Deco and Kringelbach (2020). The acquisition parameters are described in detail on the HCP website (Setsompop et al., 2013). The openly Lead-DBS software (https://www.lead-dbs.org/) provides the preprocessing described in Horn et al. (2017). In brief, data were pre-processed using a q-sampling imaging algorithm performed in DSI studio (http://dsi-studio.labsolver.org). A white-matter mask was computed by segmenting the T2-weighted anatomical images and co-registering the images to the b0 of the diffusion MRI data by using SPM12. For each participant, 200,000 fibers were sampled within the white-matter mask. Fibers were converted to MNI space using Lead-DBS (Horn and Blankenburg, 2016). Finally, we applied the methods in Lead-DBS to obtain the structural connectomes from the Schaefer 1,000 parcellation (Schaefer et al., 2018).

2.5. Turbulent Framework

To understand the whole-brain turbulent dynamics underlying the menstrual cycle, we applied the turbulent framework (Deco and Kringelbach, 2020; Deco et al., 2021b). We investigated information processing across spacetime scales (i.e., over long and short distances in the brain) between the two main phases (i.e., follicular and luteal) of the participant's menstrual cycle. In particular, the information processing measures (**Figures 1A–D**) are based on Kuramoto's studies of coupled oscillators describing turbulent fluid dynamics (Kuramoto, 1984). We computed four empirical measures for each condition using a range of five spatial scales, λ, namely $\lambda = 0.24$, $\lambda = 0.21$, $\lambda = 0.18$, $\lambda = 0.15$ and $\lambda = 0.12$, where higher λ values represent shorter distances in the brain, and lower λ values reflect long distances. Moreover, we computed the same empirical measures for a second dataset of the same woman undergoing hormonal treatment as a comparison between hormonal states. A complete description of the methods can be consulted in Deco and Kringelbach (2020) and Deco et al. (2021b).

2.5.1. Kuramoto Local Order Parameter

We computed the amplitude turbulence, $R_\lambda(\bar{x}, t)$, as the modulus of the Kuramoto local order parameter for a given node as a function of time as follows:

$$R_\lambda(\bar{x}, t)\, e^{i\vartheta_\lambda(\bar{x},t)} = k \int_{-\infty}^{\infty} d\bar{x}' G_\lambda\left(\bar{x} - \bar{x}'\right) e^{i\varphi(\bar{x}',t)} \quad (1)$$

where $\varphi(\bar{x}, t)$ represents the phases of the BOLD signal data, G_λ refers to the local weighting kernel $G_\lambda(\bar{x}) = e^{-\lambda|\bar{x}|}$, k represents the normalization factor $[\int_{-\infty}^{\infty} d\bar{x}' G_\lambda\left(\bar{x} - \bar{x}'\right)^{-1}]$, and λ defines the spatial scaling.

2.5.2. Amplitude Turbulence

The level of amplitude turbulence is represented by the standard deviation of the modulus of the Kuramoto local order parameter (R_λ):

$$D = < (R_\lambda)^2 >_{(n,t)} - < R_\lambda >^2_{(n,t)} \quad (2)$$

and the brackets $<>_{(n,t)}$ represent average values across nodes and time.

2.5.3. Information Cascade Flow and Information Cascade

The information cascade flow is computed as the time correlation between the Kuramoto local order parameter in two consecutive scales and time points:

$$IF(\lambda) = < corr_t(R_\lambda(\bar{x}, t + \Delta t), R_{\lambda-\Delta\lambda}(\bar{x}, t)) >_n \quad (3)$$

This equation describes how information is transmitted across different scales $\lambda - \Delta\lambda$, where $\Delta\lambda$ represents the scale step in consecutive time steps (t and $t + \Delta t$) and the brackets $<>_n$ represent average values across nodes.

Then, the information cascade is computed by averaging the information cascade flow across scales λ, thus capturing the whole information processing profile across all scales.

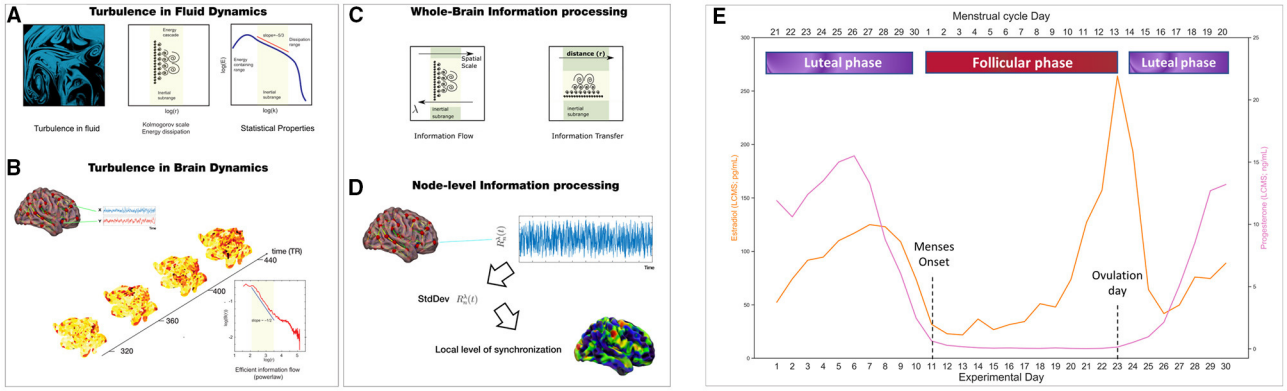

FIGURE 1 | Methods: Turbulent framework and menstrual cycle parsing into phases. We studied how the brain information processing during resting-state varies between the follicular (red) and luteal (purple) phases of a woman menstrual cycle by applying the Turbulent Framework (Deco and Kringelbach, 2020). We computed 4 measures: the level of amplitude turbulence, information cascade flow, information cascade, and information transfer. **(A)** These measurements are based on Kolmogorov's studies demonstrating the presence of turbulence in fluid dynamics. **(B)** More recently, turbulent dynamics have been found in the human brain, for which the level of amplitude turbulence can be calculated. **(C)** Information cascade flow and information transfer are two measurements of information transmission across the whole-brain at different spatial scales by taking into account the Euclidean distance between brain areas. **(D)** This panel shows how turbulence levels, which reflect information processing capability, can be calculated at the node-level to understand which brain areas show the highest values in node-level information processing. **(E)** The lower x-axis shows the days of the experiment (1-30), whereas the upper x-axis indicates the division of the menstrual cycle phases. The phases were divided as follows: Follicular phase (days of the experiment: 11 to 23, where 11 is the onset of menses and 23 is the day of ovulation). The luteal phase included the days after ovulation (days of the experiment: 24-30) and days before menses (days of the experiment: 1-10). For 30 consecutive days, the subject provided blood samples to examine serum hormone concentrations (Estradiol, orange line in pg/mL; Progesterone, purple line in ng/mL). LC-MS, Liquid Chromatography-Mass Spectrometry. **(A–D)** adapted from Deco and Kringelbach (2020), Deco et al. (2021a) and Escrichs et al. (2021)

2.5.4. Information Transfer

The spatial information transfer describes how information is transmitted across the whole-brain at a given scale (i.e., λ) and is computed as the slope of a linear fitting in a log-log scale of the time correlation between the Kuramoto local order parameter in pairs of nodes as a function of the Euclidean distance (r) within the inertial subrange:

$$log(corr_t(R_n^\lambda, R_p^\lambda)(r)) = A * log(r) + B \tag{4}$$

A and B are the fitting parameters and A (i.e., the negative slope) represents the spatial information transfer.

2.5.5. Node Variability of Local Synchronization

The node variability, NVLS, of the local synchronization is defined as standard deviation across time of the local Kuramoto order parameter as:

$$NVLS(n) = < R_n^\lambda(t)^2 >_t - < R_n^\lambda(t) >_t^2 \tag{5}$$

where brackets $<>_t$ represent average values across time.

We estimated the discrete version of the node-level Kuramoto order parameter, with modulus R and phase ν, which represents a spatial average of the complex phase factor of the local oscillators weighted by the coupling, measured as:

$$R_n^\lambda(t)\, e^{i\nu_n(t)} = \sum_p \left[\frac{C_{np}^\lambda}{\sum_q C_{nq}^\lambda} \right] e^{i\varphi_p(t)} \tag{6}$$

where $\varphi_p(t)$ represents the phases of the BOLD signal data, and C_{nq}^λ refers to the local weighting kernel between nodes n and p:

$$C_{np} = e^{-\lambda(r(n,p))} \tag{7}$$

where $r(n,p)$ is the Euclidean distance between nodes n and p in MNI space, and λ is the scaling of the local weighting obtained by adjusting the structural connectome matrix.

2.6. Statistical Analysis

We investigated the two main phases of the menstrual cycle (i.e., follicular and luteal). To this end, we divided the phases for the NaturalMenstrualCycle Study as follows: Follicular phase (days of the experiment: 11–23, where 11 is the onset of menses and 23 is the ovulation day). In the luteal phase, we included the days after ovulation (days of the experiment: 24–30) and days before menses (days of the experiment: 1–10). Please see **Figure 1E**. This division follows regular standards to divide the menstrual cycle into two phases (Olatunji et al., 2020; Schmalenberger et al., 2020). For statistical comparisons, we applied the Wilcoxon rank-sum method to test the differences between phases. We also performed group analyses removing the ovulation window (days of the experiment 12–14), which is characterized by a strong peak in estradiol levels (Pritschet et al., 2020). Concerning the HormonalContraceptive Study, we tested differences when the participant took exogenous hormonal contraceptive pills (active condition) and placebo inactive pills (placebo condition). Finally, to understand the relationship between changes in hormone concentrations and turbulent brain dynamics, we carried out multi-level models for each dependent

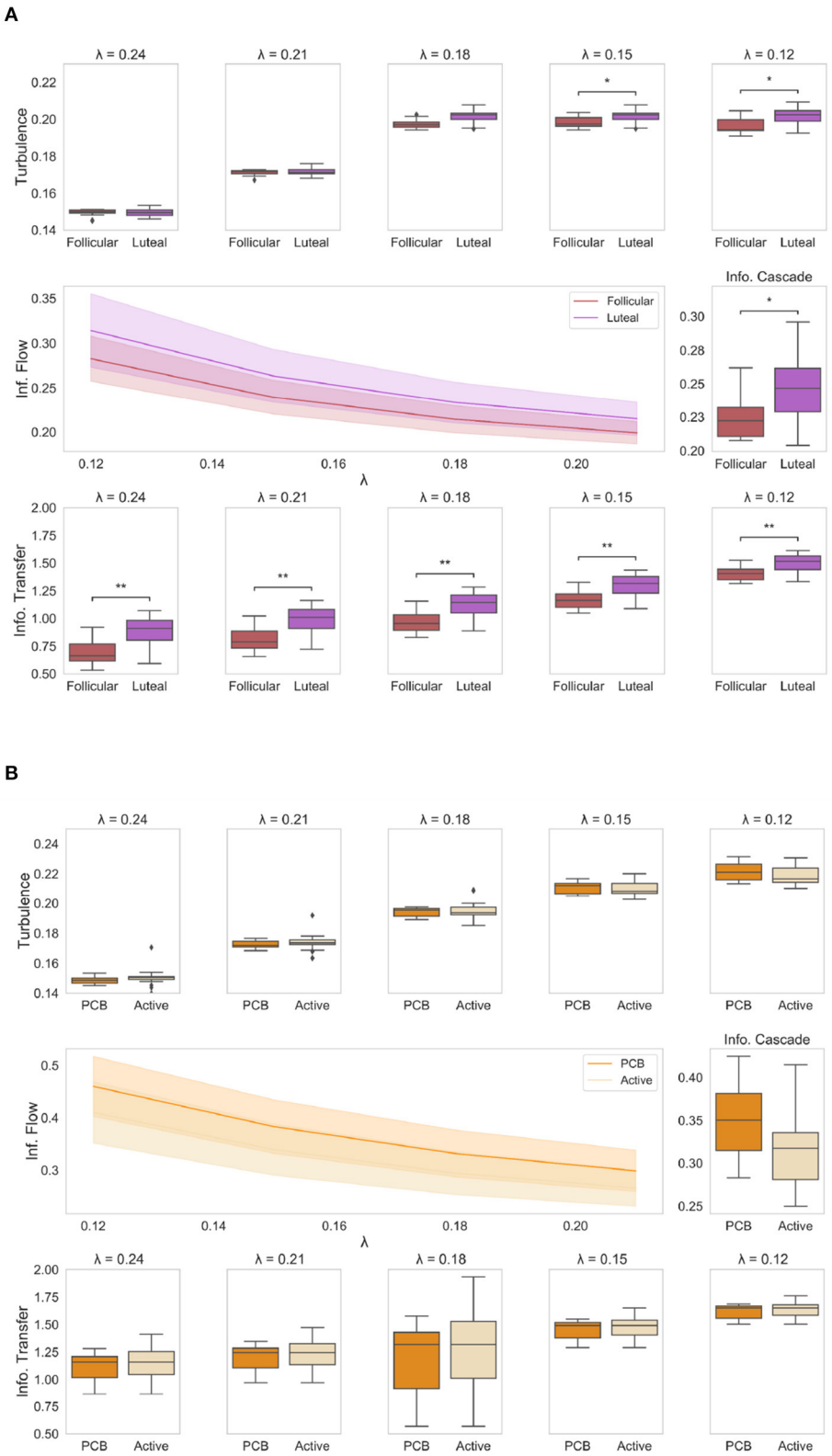

FIGURE 2 | Results of information processing analysis: **(A)** The boxplots show the level of amplitude turbulence between the two phases of the NaturalMenstrualCycle Study (red: follicular, purple: luteal) across different spatial scales (i.e., *lambda*). We show that the turbulence level (upper plots) is significantly higher in lower scales

(Continued)

FIGURE 2 | (i.e., long distances) in the luteal phase than in the follicular ($p < 0.05$). The plot in the middle shows how the luteal phase (purple line) is characterized by higher information cascade flow across all scales than the follicular phase (red line). This is clearly displayed in the middle boxplot representing a higher average value of information cascade flow across scales (i.e., information cascade) for the luteal phase compared to the follicular ($p < 0.05$). Similarly, the boxplots at the bottom of the figure show how the information transfer is significantly reduced in the follicular phase compared to the luteal across all scales ($p < 0.001$). **(B)** The boxplots represent the four empirical measures (amplitude turbulence at the top; information cascade flow and information cascade in the middle; information transfer at the bottom) for HormonalContraceptive Study, comparing placebo (dark orange) and active (light orange) phases. We show that there is no difference in any of the four measures under the Turbulence framework between the active and placebo phases when the participant is under a hormone-based regime. *represents $p < 0.05$ while ** represents $p < 0.01$.

variable (brain information processing measures) with hormone levels (estradiol and progesterone) as fixed effects and participant and session day of testing as random effects.

3. RESULTS

Results of information processing analysis are presented in **Figure 2**.

3.1. Amplitude Turbulence

First, we computed the amplitude turbulence across different spatial scales, which is defined as the standard deviation of the modulus the local Kuramoto order parameter applied to the empirical functional data (Kawamura et al., 2007). We found that the amplitude turbulence level (**Figure 2A**, upper panel) was significantly higher for the luteal phase compared to the follicular ($p < 0.05$) in lower values of λ, i.e., in long distances. In particular, we found significant higher levels of amplitude turbulence in the luteal phase at $\lambda = 0.15$ ($p = 0.01$) and $\lambda = 0.12$ ($p = 0.01$), whereas differences between follicular and luteal phases were not significant at λ=0.24, $\lambda = 0.21$ and $\lambda = 0.18$ ($p > 0.05$). This suggests that when naturally-cycling, the luteal phase is characterized by higher levels of amplitude turbulence across long distances. In contrast, we did not find any significant difference between the active and placebo conditions of the HormonalContraceptive Study (**Figure 2B**, upper panel) at any scale ($p > 0.05$).

3.2. Information Cascade Flow and Information Cascade

Next, we calculated information cascade flow, which indicates how information is transmitted over time from one given scale to another. This measure is defined as the time correlation between the Kuramoto order parameter into two sequential spatial scales. Then, to understand the efficiency of information transmission, we computed the information cascade by averaging the information cascade flow across all λ scales. The information cascade flow and information cascade results for each condition are presented in **Figure 2**, middle panel. The information cascade flow (**Figure 2A**, left panel) was significantly higher in the luteal phase compared to the follicular ($p < 0.05$) across all scales. The information cascade (**Figure 2A** middle, right panel) shows significantly higher values for the luteal phase compared to the follicular ($p = 0.04$). This result indicates that the information transmission across scales is enhanced in the luteal phase compared to the follicular phase. By contrast, we reported no significant differences in information cascade flow and information cascade between the active and placebo phases of the HormonalContraceptive Study ($p > 0.05$). Together, our results show that being on a hormonal contraceptive regime leads to a more stable information transmission pattern across the menstrual cycle, while during naturally cycling, information transmission is enhanced across the whole-brain network in the luteal phase.

3.3. Information Transfer

Lastly, we calculated the information transfer (**Figure 2A**, lower panel), which reflects how information travels across space at a particular scale, λ, computed as the slopes in the decay of the information transmission. The information transfer was significantly reduced during the follicular phase compared to the luteal at all λ scales ($p < 0.05$), meaning that the luteal phase leads to better information transfer than the follicular phase across all scales. Interestingly, being on a hormone-based regime appeared to cancel this effect with active and placebo phases showing similar levels of information transfer across all spatial scales ($p > 0.05$ for all λ values, **Figure 2B**, lower panel).

Remarkably, removal of the ovulation window in the NaturalMenstrualCycle Study did not change the results of any of the four measures. To compare the consistency of our findings, we also performed the analysis of the HormonalContraceptive Study by splitting it up into a simulated "follicular" and "luteal" phases as in NaturalMenstrualCycle Study. Once more, we found no differences between the two phases in the HormonalContraceptive Study for any of the four measures ($p > 0.05$).

3.4. Node Variability of Local Synchronization

Following the results of the information processing analysis, we aimed to investigate which brain areas show the highest difference between the two phases of the NaturalMenstrualCycle Study. Therefore, we calculated the difference in the variability of local synchronization across nodes between the luteal and the follicular phases (**Figure 3**). We found that the default mode network (DMN) was the one showing the highest increase in variation of local synchronization during the luteal phase. Furthermore, brain areas belonging to the salience/ventral attention, somatomotor, control and dorsal attention (DAN) networks also showed increased variability of

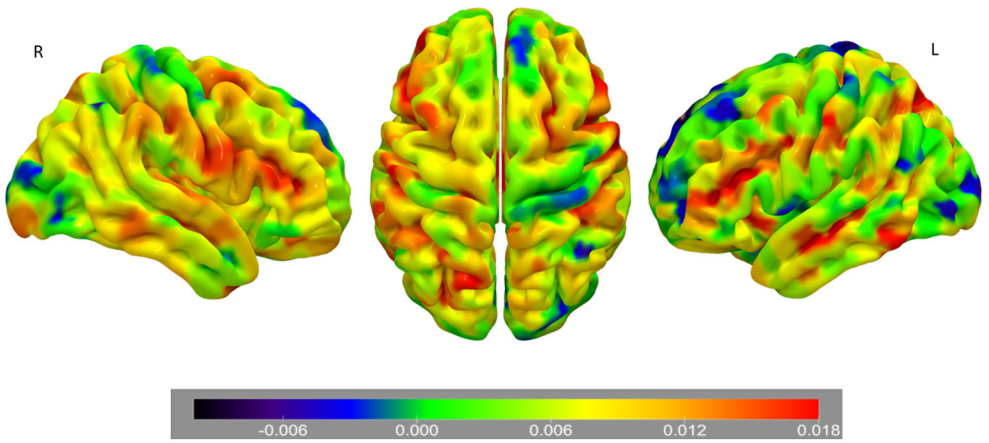

FIGURE 3 | Differences in node-level information processing across the whole-brain network between the luteal and follicular phases of the NaturalMenstrualCycle Study. Rendered brains show the difference in variation of local synchronization levels for each brain area between phases. Colder colors (i.e., back-blue extreme) represent brain areas showing decreased levels of variation during the luteal phase than the follicular, while warmer colors (i.e., red extreme) mark the areas with the most significant increase in variation of local synchronization during the luteal phase. Areas marked in green are the ones showing similar variation levels across both phases. Areas belonging to the default mode, the salience/ventral attention, the somatomotor, the control, and the dorsal attention networks show the highest values of node-level synchronization during the luteal phase. The rendered brain was plotted using the Surf Ice software (https://www.nitrc.org/projects/surfice/).

local synchronization at $\lambda = 0.15$ and $\lambda = 0.12$ during the luteal phase. This result indicates that the brain's information transmission is enhanced across large-scale networks at long distances during the luteal phase.

3.5. Multi-Level Model With Hormone Levels and Turbulence

Finally, we explore the relationship between day-to-day hormonal concentrations when naturally-cycling (NaturalMenstrualCycle Study) and turbulence levels (i.e., where we found significant differences between phases). To this end, we carried out multi-level models for each dependent variable (turbulence at $\lambda = 0.12$ and $\lambda = 0.15$ scales) with sex hormone levels (estradiol and progesterone) as fixed effects. As random effects we included subject ($N = 1$) and testing day (observations days = 30).

The resulting model' lmer syntax for turbulence at λ 0.12 was: *turbulence* λ *0.12* $\sim$ *1 + Estradiol levels + Progesterone levels + (1 + session | subject)*. The parameter estimates for the model of turbulence 0.12 showed significant main effects of estradiol (p=0.047) and progesterone levels (p=0.005). In addition, the model output for turbulence at $\lambda = 0.15$ [model syntax: *turbulence* λ *0.15* $\sim$ *1 + Estradiol levels + Progesterone levels + (1 + session | subject)*] showed the significant effect of progesterone (p=0.015), however, the estradiol did not show a significant effect at λ=0.15 scale ($p = 0.086$). The full outputs of the multi-level models are presented in **Figure 4A**. These results suggest that changes in concentrations of ovarian hormone modulate whole-brain turbulent dynamics in long distances (see **Figure 4B** for a visual representation of the interplay between estradiol, progesterone, and turbulence levels controlling for the experimental session).

4. DISCUSSION

In the present work, we aimed to explore how the brain's information processing changes across spacetime scales between the two main phases of the menstrual cycle, namely the luteal and the follicular. Furthermore, we used a second dataset (HormonalContraceptive Study) as a comparison condition in which the same participant was on hormonal contraception. We demonstrated that information processing across scales changes significantly between the luteal and follicular phases when naturally-cycling but not under selective hormonal suppression. At the node-level, we found that the DMN, salience/ventral attention, somatomotor network, DAN, and control networks showed an increased variation of local synchronization during the luteal compared to the follicular phase. Moreover, we found that changes in estradiol and progesterone concentrations modulate whole-brain turbulent dynamics in long distances.

We showed that the luteal phase was characterized by higher turbulence levels than the follicular phase across lower spatial scales (i.e., long distances in the brain). Moreover, the analysis of information cascade flow and information cascade measures showed that efficiency in information transmission across different scales was enhanced during the luteal phase compared to the follicular. In the same way, we reported increased information transfer across the whole-brain at lower spatial scales for the luteal phase compared to the follicular

A

Output of the model for turbulence at $\lambda = 0.12$

	Estimate	SE	p value
Intercept	0.2000	0.0016	<.001
Estradiol	0.0003	0.0001	0.047
Progesterone	0.0004	0.0001	0.005

Output of the model for turbulence at $\lambda = 0.15$

	Estimate	SE	p value
Intercept	0.1985	0.0010	<.001
Estradiol	0.0001	0.0001	0.086
Progesterone	0.0002	0.0001	0.015

B

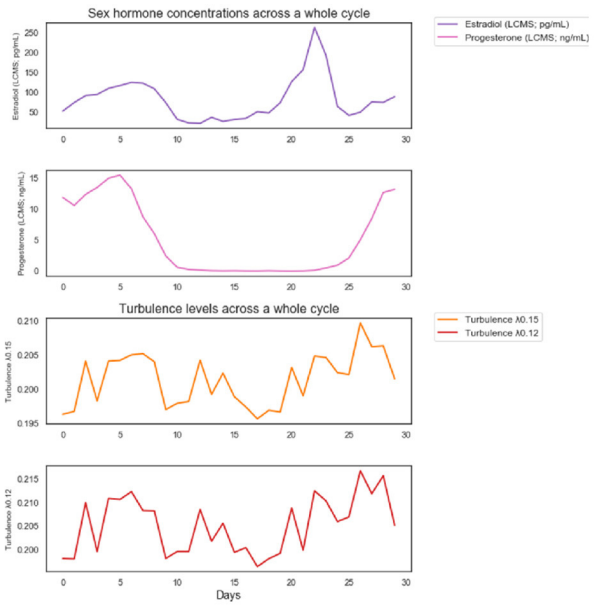

FIGURE 4 | Exploratory analysis. **(A)** Multi-level models outputs of whole-brain turbulence levels predicted by day-to-day ovarian hormone concentrations for the NaturalMenstrualCycle Study. We found that estradiol and progesterone levels predicted brain turbulence at $\lambda = 0.12$. Similarly, progesterone levels predicted brain turbulence at $\lambda = 0.15$, however, estradiol was not significant at this λ scale. These results indicate that hormonal changes modulate turbulent dynamics in long distances in the brain. **(B)** Plot of estradiol (purple line) and progesterone (pink line) daily concentrations and turbulence levels at $\lambda = 0.15$ (orange line) and at λ=0.12 (red line) across the 30 days of the experiment (x-axis).

when the participant was freely cycling. In contrast, we showed that being under a hormone-based regime led to a more stable pattern of turbulence levels across the whole cycle, with no significant shifts in information processing, as found for all four empirical measures of turbulent dynamics (i.e., amplitude turbulence, information cascade flow, information cascade, and information transfer).

We further computed the differences in the variability of synchronization levels across the whole-brain between the luteal and the follicular phases to highlight cycle-dependent changes in large-scale brain networks. We found that the DMN was the network presenting the most significant dynamic variation depending on the menstrual cycle phase, showing increased levels of variation in local synchronization during the luteal phase. Together with the DMN, we found that other large-scale networks such as the salience/ventral attention, somatomotor, control, and DAN showed the highest cycle-dependent changes in this measure. These results are in line with recent findings using the same dataset (Pritschet et al., 2020; Mueller et al., 2021). The work of Pritschet et al. (2020) used a time-lagged analysis and found that fluctuations in estradiol levels led to an enhancement of network efficiency, particularly for the DMN and the DAN. Accordingly, using dynamic community detection, Mueller et al. (2021) found that peaks in estradiol were reflected in a localized and transient reorganization of large-scale networks, particularly of the DMN and a control subnetwork. The authors also found that other brain networks, such as the temporoparietal, limbic, subcortical networks, showed the highest flexibility values in response to a rise in estradiol concentration (Mueller et al., 2021). Here, we expand previous findings from this dataset by demonstrating that the effects of the cycle stage were not only reflected at the spatiotemporal level of the brain's information processing but also in the transmission of information across different scales. Previous human neuroimaging studies investigating the effects of hormone fluctuation and cycle stage on the brain's functional connectivity dynamics also provided compelling evidence of hormone-related modulation of several networks (Petersen et al., 2014; Arélin et al., 2015; Lisofsky et al., 2015a; Weis et al., 2019). For example, Arélin et al. (2015), using a deep-sampling approach, demonstrated that inter-regional connectivity changed in association with progesterone levels and that increases in this sex hormone led to enhanced connectivity between the dorsolateral prefrontal cortex, hippocampus, and sensorimotor cortex. A cross-sectional study conducted by Petersen et al. (2014) found that the follicular phase compared to the luteal was associated with higher within-network connectivity for the DMN and the control network. Here, we extend these findings by demonstrating that the effects of the cycle stage are reflected in the transmission of information across different spatial scales, both for large-scale networks and across the whole-brain network.

Finally, we were interested in examining the relationship between sex hormone levels and differences in the brain's turbulence levels. Results of multi-level models controlling for the experimental session showed that estradiol and progesterone concentrations modulate information transmission across long distances in the brain when the participant was naturally-cycling. This result suggests an interplay between progesterone and estradiol with the brain's information transmission, for which variations in ovarian hormone levels alter information processing across the whole-brain network. This trend is in line with previous literature reporting an association between cortico-cortical and subcortical-cortical functional connectivity and higher concentrations of estradiol and progesterone (Peper et al., 2011). Additionally, Weis and Hausmann (2010) found that higher levels of both ovarian hormones are associated with lower interhemispheric inhibition, thus increased functional communication between the two hemispheres.

We want to highlight some limitations of the current study. We used a dense-sampling dataset from a single subject. Future studies could benefit from a dense-sampling design with a larger sample size of participants to study fine-grained phases (e.g., early- and late-follicular, ovulatory, mid-, and late-luteal).

In addition, the empirical measures applied in this study do not offer a causal explanation of cycle-dependent changes in turbulent dynamics in the human brain. A future direction would be to apply whole-brain computational modeling to shed light on the mechanisms behind sex hormone fluctuations and changes in whole-brain dynamics. A handful of studies have provided evidence of the impact of hormonal transition or suppression on the risk of developing mood disorders (Bloch et al., 2000; Young et al., 2007; Taylor et al., 2019). Therefore, elucidating the mechanisms underlying the relationship between sex hormone concentrations and brain functioning and dysfunction may foster a deeper understanding of mood disorders related to neuroendocrine change.

5. CONCLUSIONS

In this work, we showed how the menstrual cycle modulates whole-brain turbulent dynamics. We applied a novel turbulence framework to study whole-brain information processing across spacetime scales (i.e., over long and short distances in the brain) during both a naturally occurring menstrual cycle and under a hormonal contraceptive regime. We demonstrated that the luteal phase is characterized by higher turbulence levels at lower scales (i.e., long distances in the brain) and higher information transmission across scales. By contrast, under hormonal-based regime showed no differences in information processing across the whole menstrual cycle. Furthermore, we found that the DMN, salience/ventral attention, somatomotor, control, and dorsal attention were the large-scale networks showing the most significant increases during the luteal vs. the follicular phases. Finally, we found an interplay between progesterone and estradiol with the brain's information transmission, showing that ovarian hormone levels alter information processing across the whole-brain network. Overall, our results show that ovarian hormones and menstrual cycle stages modulate whole-brain turbulent dynamics.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study can be found in the "28andMe" open repository at https://openneuro.org/datasets/ds002674/versions/1.0.5.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of California, Santa Barbara Human Subjects Committee. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

GD and AE designed the study. ED, MK, YS, GD, and AE developed methodology and software. EJ, LP, and TS provided the MRI data. ED and AE preprocessed and analyzed the datasets. ED, CU, YS, and AE wrote the original draft. VG contributed to analysis and interpretation of results during the review process. All authors interpreted and discussed the results, reviewed, edited, and approved the last version of the manuscript.

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Adaptation in Gait to Lunar and Martian Gravity Unloading During Long-Term Isolation in the Ground-Based Space Station Model

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The aim of the experiment was to evaluate the adaptive responses of biomechanical and electromyographic parameters to vertical unloading (Lunar—0.15 G and Martian—0.35 G) when walking during the 4-month isolation experiment SIRIUS-19 in the ground-based space station model (GBI). The study involved 6 healthy international crew members of the SIRIUS-19 project aged 34 ± 6.2 years (3 women and 3 men). Body Weight Unloading (BWU) conditions was created by the h/p/cosmos airwalk system. The locomotor test included walking (3.5 ± 0.3 km/h) with a sequential change of BWU modes: 5-min walking with 0% BWU (1 G), 5-min walking with 65% BWU (0.35 G) and 5-min walking with 85% BWU (0.15 G). Ground Reaction Force was recorded by the h/p/cosmos treadmill device. Muscle Lab Model 4000e device was used to record the electromyographic signals of the hip and shin muscles. The locomotor test was performed twice before GBI, monthly during GBI and 1 week after leaving isolation. The results obtained before GBI demonstrate that the changes of support and proprioceptive afferentation signals play significant role in reorganizing of the biomechanical structure of motor acts and the development of new movement patterns. The results of the study are consistent with the previously obtained results of other studies in this direction. Despite the fact that during the GBI the participants of the experiment performed regular physical training, a decrease in the performance indicators values was detected, especially pronounced after 100 days of GBI. This is probably due to limited space of a space station model, as well as the development of a special motor stereotype in it. Noteworthy are the results obtained after the 4th session of the experiment, indicating the effect of sensorimotor learning. We think that the data obtained in this study will be useful in research both in gravitational physiology and in clinical medicine.

Keywords: motor adaptation, gait, electromyography (EMG), body weight unloading, ground reaction (forces), ground based isolation

INTRODUCTION

Sensory-motor adaptation is the result of the coordinated activity of sensory and motor systems, as well as central integration processes. It is the ability to maintain the accuracy and control of movements, to modify motor commands when the sensory signals change. Any changes in the signals from the leading sensory inputs induce the generation of the corresponding motor commands in the central nervous system, which are transmitted along the descending pathways to different body segments (Shadmehr et al., 2010). In particular, studies in the field of gravitational physiology have demonstrated a significant effect of the level of support and proprioceptive afferentation on the regulation of tonic muscle activity (Kozlovskaya et al., 2007). The withdrawal of the support afferentation leads to the decline of the tonic motor units activity in extensor muscles and the alteration of the motor units recruitment patterns in the spinal cord (Shenkman et al., 2017). Also previously shown that load-related afferent feedback modulates vestibular input and can be used by the central nervous system to modify performance of motions (Fredrickson et al., 1966; Jian et al., 2002; Marsden et al., 2003). This is a key factor in the modification of human locomotor patterns under real (Cavanagh et al., 2010; De Witt and Ploutz-Snyder, 2014; Saveko et al., 2020) and simulated (McCrory et al., 2004, 1997; Sylos-Labini et al., 2013) microgravity conditions. For the first time the concept of influence the plantar cutaneous afferents to modulate the postural-tonic system of mammals evolved from experiments performed in microgravity and dry immersion studies (Grigor'ev et al., 2004; Kozlovskaya et al., 2007). Kozlovskaya et al. (2007) demonstrated that dynamic plantar pressure stimulation during a prolonged absence of weight bearing can attenuate the functional neuromotor degradation associated with chronic unloading (Litvinova et al., 2004). With the use of functional magnetic resonance tomography, plantar pressure stimulation was further shown to activate the primary sensorimotor cortex associated with load-bearing stepping (Kremneva et al., 2013), thus implying a convergence of plantar cutaneous stimuli with the supraspinal control of locomotion.

Currently, studies with a support unloading are especially relevant for space medicine in order to predict movement disorders in partial gravity conditions (Richter et al., 2017). The only experience of locomotion and operator activity on the surface of other planets with different gravity level from the Earth's gravity level revealed disorders in the movements coordination and functional performance of astronauts: the performance of any motor task was characterized by a significant loss of accuracy and control of movements, an increase in the time of performance of operator tasks, etc. (Scheuring et al., 2008). The reorganizing of the biomechanical structure of motor acts in such conditions, among other things, is caused by the alteration of the ratio between body mass and effort values required for its movement performance, a decrease in the activity of postural muscles, disused earth-specific movements, and the development of new movement patterns with a change in the nature of motor activity in general (Kozlovskaya et al., 1988). To date, there are not enough quantitative data characterizing sensory-motor reactions in conditions with different gravity level from the Earth's gravity level. At the same time, such knowledge is important in the future interplanetary missions, including to the Moon and Mars. Thus, the study of movement patterns while Lunar (0.1–0.20 G) and Martian (0.30–0.40 G) weight unloading is of particular practical importance (Richter et al., 2017). Moreover, as part of the prospects for interplanetary expeditions, crew members will have to adapt not only to the change in the level of gravity, but also to stay in the specific conditions of the limited space of the spacecraft for a long time, which is accompanied by a change in boundaries of the environment space, which can also effect the manipulation of motor representations (Gangopadhyay et al., 2010; Kim et al., 2018).

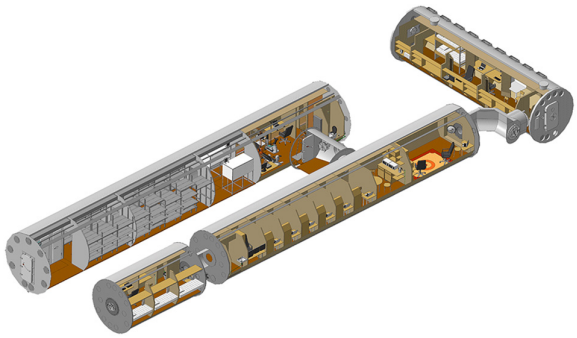

FIGURE 1 | Main view of medical-technical experimental facility. Right side. IBMP/Haider Hobihozin.

We have suggested the hypothesis that the factors of long-term isolation in limited space of a space station model can elicit change in the adaptive responses of biomechanical and electromyographic parameters to partial vertical unloading while walking.

One of the ways to change sensory information related to the support load separately is the Body Weight Unloading (BWU). In this connection, the measurement of biomechanical parameters of natural locomotions (for example, walking) while BWU is used to study the mechanisms of adaptive reactions of the sensory-motor system both in gravitational physiology (Sylos-Labini et al., 2013; Pavei and Minetti, 2016) and in clinical medicine (Apte et al., 2018).

In connection with the above, the purpose of the experiment was to assess the adaptive responses of biomechanical and electromyographic parameters to Lunar (0.15 G) and Martian (0.35 G) vertical unloading while walking during the 4-month isolation in the ground model of the space station.

MATERIALS AND METHODS

Subjects

The study involved 6 healthy crew members (3 women and 3 men) of the international project SIRIUS-19 (Scientific International Research in Unique Terrestrial Station) implemented by IMBP RAS and NASA HRP aged 34 ± 6.2 years

FIGURE 2 | Equipment used during a session of experiment.

(height: 175.0 ± 9.6 cm; weight: 68.8 ± 13.1 kg; body mass index: 22.3 ± 2.9 kg/m2). Inclusion criteria: the crew was to consist of representatives of both sexes, several nationalities and different cultures, lack of experience of space flights. Exclusion criteria: the presence of pathologies of the respiratory, digestive, urinary, sensorimotor, circulatory, nervous and integumentary systems that cause additional risks to health and life during 4 months of isolation in the space station model. The number of participants in the experiment was limited by the planned scenario of a flight to the moon and back of an international crew of six people[1] (National Aeronautics and Space Administration, 2021). One of the study participants had a previous experience of 3 months of isolation in a mock-up of a Martian research station. Crew members were allowed to participate in the experiment by the medical expert commission of the Institute of Biomedical Problems of RAS and signed an Informed Consent to participate in the study in accordance with the provisions of the Helsinki Declaration of Human Rights. The research procedure was preliminarily reviewed and approved by the Biomedical Ethics Commission of the Institute of Biomedical Problems of RAS. The participants provided their written informed consent to participate in this study.

[1]https://www.nasa.gov/content/sirius

Ground-Based Space Station Model

The crew members of the SIRIUS-19 project spent 4 months in the medical-technical facility of IBMP RAS what meant for simulation of conditions of life and activity of the crew, that are maximally close to the conditions of real spaceships. Ground-based Isolation (GBI) was accompanied by the control of the experimental parameters (work and rest regime, habitat parameters, nutrition, etc.). It is important to note that during GBI, the crew members performed physical training like on the International Space Station (Kozlovskaya et al., 2015) and were in Earth gravity. The total volume of the modules was 550 m^3 (**Figure 1**). The scenario for the 4-month mission SIRIUS-19 included a simulation of a real manned flight to the Moon with a landing on the Lunar surface and return to the Earth. It is worth noting that studies using this model are relevant not only for predicting risks in long-term space flights, but also for patients who are in prolonged isolation and/or conditions of reduced motor activity (Mitrokhin et al., 2020).

Body Weight Unloading System

Walking was carried out on the treadmill H/P/Cosmos Mercury 4.0 (H/P/Cosmos sports & medical gmbh, Germany) with a canvas size of 150 × 50 cm. The body weight of the subjects was determined before each session of the experiment using Kistler

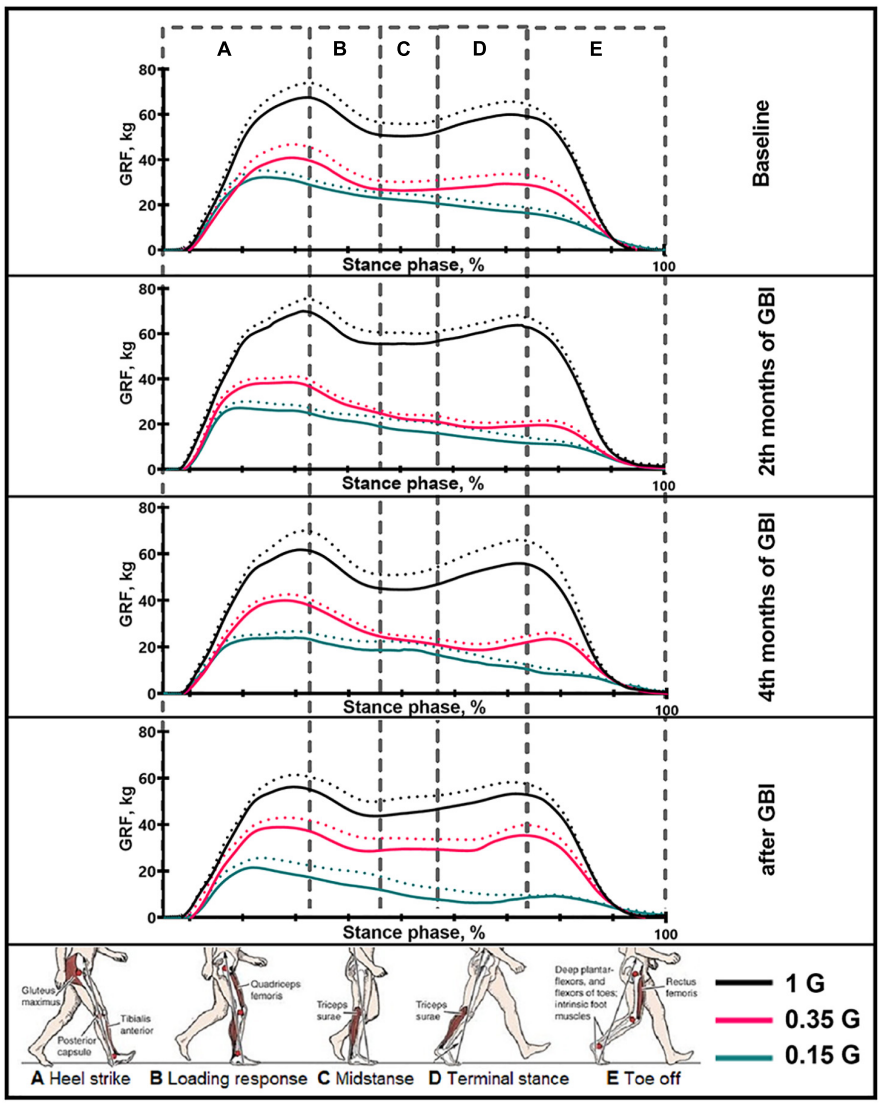

FIGURE 3 | The average podogram of 6 SIRIUS-19 crew members in different sessions of the experiment. On the ordinate axis: ground reaction force, kg. On the abscissa axis: stance phase, %. 1 G, 0.35 G, 0.15 G—body weight unloading modes—0%, 65%, 85% of the Earth weight, respectively. «Baseline»—results obtained before isolation. «2 months of GBI» and «4 months of GBI»—results obtained on the 50th and 100th day of isolation respectively. «1 week after GBI»—results obtained a week after leaving isolation. The dotted lines display the error bars (SEM).

force plate (Kistler Group, Switzerland) installed under the treadmill canvas. The change and regulation of the gravitational load on the musculoskeletal system was carried out by vertical unloading using the H/P/Cosmos-Air Walk system (H/P/Cosmos sports and medical gmbh, Germany), consisting of the Jun-Air Blue line Model 4-4 compressor, a frame structure and a special H/P/Cosmos-Air Walk vest (**Figure 2**). Locomotor test included different BWU mode (Martian gravity mode—35% of the Earth's weight and Lunar gravity—15% of the Earth's weight).

Procedure

The locomotor test included walking with sequential changes in BWU modes: 5 min walking with 0% BWU (1 G), 5 min walking with 65% BWU (0.35 G) and 5 min walking with 85% BWU (0.15 G). Walking in the active mode of the treadmill was carried out at a constant speed of 3.5 ± 0.3 km/h (0.97 ± 0.08 m/s; 2.17 ± 0.19 mph). This locomotor test was performed 20 and 7 days before GBI (baseline sessions), monthly during GBI and 7 days after GBI. Locomotor test data during GBI were recorded only on the 50th and 100th day of GBI (on the 2nd and 4th months of isolation). The mode change was carried out and controlled by the performer of the experiment (before and after GBI—by the researcher, during GBI—by one of the crew members). There was no rest period between mode changes. All experiment sessions were conducted in the space station model module. Each experimental session was accompanied by a video recording of the performance of the locomotor test in order to control the protocol of the study.

Quantitative evaluation of video data was not carried out, but it was used for observational evaluation of movements based on the obtained quantitative data of biomechanical parameters and electromyographic activity of muscles.

Biomechanical Parameters Measurement

Ground Reaction Force (GRF) were recorded by the Kistler force plate using the "Kistler Gateway" software (Kistler Group, Switzerland). The treadmill was equipped with two force plate under the canvas, arranged in the front and back of area of movement. Each force plate contained four piezoelectric uniaxial load sensors that measure the vertical component of the support reaction (Ivanenko et al., 2002). In our study, data from load sensors were recorded at a frequency of 100 Hz using software provided by the treadmill developer. At the end of the 3rd minute from the beginning of the unloading mode change, biomechanical parameters were recorded 3 times with a duration of 30 s. Based on the data obtained, GRF (in kg) parameters were calculated using the "Kistler Gateway" software at different moments of the stance phase: Heel strike, Loading response, Midstance, Terminal stance, Toe off (**Figure 3**). The value of the amplitude of Midstance was considered as the difference between the GRF value of the minimum vertical pressure at the moment of Midstance and the average value of the GRF of the maximum vertical pressure at the moments of Heel strike and Toe off. Stance phase is defined as the period of time where the foot is in contact with the ground. Previous studies show that quantitative assessment at different moments of stance (also known as "inner-stance phases") can bring additional insight into gait assessment (Burnfield, 2010; Mariani et al., 2013; Vaverka et al., 2015). As well as the temporal and spatial characteristics of walking were calculated "Kistler Gateway" software: Gait cycle time (s), Single support time (s), Double support time (s), Step length (cm), Cadence (steps/min). This the temporal characteristics of walking is commonly used in such studies (Awai et al., 2017; Apte et al., 2018).

Electromyography Measurement

The electromyographic activities were recorded by means of disposable surface electrodes bipolar skin electrodes 42 × 24 cm Kendalll, which were located in the middle of the projection of the belly of muscles. The distance between the electrodes was 17 mm. The surface of the skin was carefully cleaned with a sponge, and then degreased with ethanol before applying the electrodes, in order to reduce the electrical resistance. EMGs were recorded using a MuscleLab 4000e telemetric eight-channel hardware—software complex, which allows the EMG-activity of muscles to be recorded at a distance of 100 m. Based on the Bluetooth wireless technology, the data were recorded online on a computer hard drive. The input impedance of each channel of an electromyograph was 2 MΩ at a transmission frequency of an EMG signal of 100 Hz on one Bluetooth channel. The MuscleLab 4000e hardware allows inverting and averaging the electromyographic signal during a 10-ms exposure. During processing the inverted EMG was smoothed using a Butterworth low-pass filter of the second order. The electromyographical signals of the leg muscles were integrated, and the mean and standard deviations were calculated from 13–15 double steps (**Figure 2**). Electromyographic activity was recorded from 4 muscles of the leg and thigh: m. soleus (SL), m. gastrocnemius lateralis (GL), m. tibialis anterior (TA) and m. rectus femoris (RF). Based on the results of similar studies, muscle activity was considered in terms of the magnitude of the EMG signal as an average value over the entire gait cycle. Also the profiles of EMG activity of each muscles during the gait cycle were analyzed (Awai et al., 2017; Apte et al., 2018; **Figures 4**, **7**).

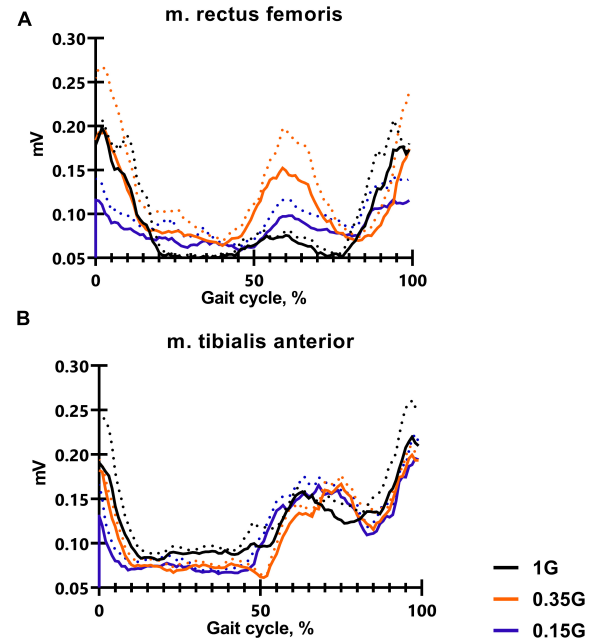

FIGURE 4 | The average EMG activity profile of 6 SIRIUS-19 crew members in baseline sessions: m. rectus femoris **(A)**, m. tibialis anterior **(B)**. On the ordinate axis: EMG amplitude, mV. On the abscissa axis: gait cycle, %. 1 G, 0.35 G, 0.15 G—body weight unloading modes—0%, 65%, 85% of the Earth weight respectively. «Baseline»—results obtained before isolation. «2 months of GBI» and «4 months of GBI»—results obtained on the 50th and 100th day of isolation, respectively. «1 week after GBI»—results obtained a week after leaving isolation. The dotted lines display the error bars (SEM).

Statistical Analysis

When analyzing the results of the study, the difference of parmaterials in both between the experiment sessions (before GBI, on the 2nd and 4th month of GBI, after GBI) and between different BWU modes (1 G, 0.35 G, 0.15 G) was estimated. For data analysis, we used the GraphPad Prism version 8 (GraphPad Software, United States). The data were analyzed using Two Way Repeated Measures ANOVA, *post hoc* Bonferroni. All significance levels were set at $p \leq 0.05$. All data were tested for normality using Shapiro–Wilk test (all data are normally distributed—$p > 0.05$). The results of two baseline (pre-GBI) experimental sessions were averaged and had no statistical difference between them. The values that have reached a significant difference compared with

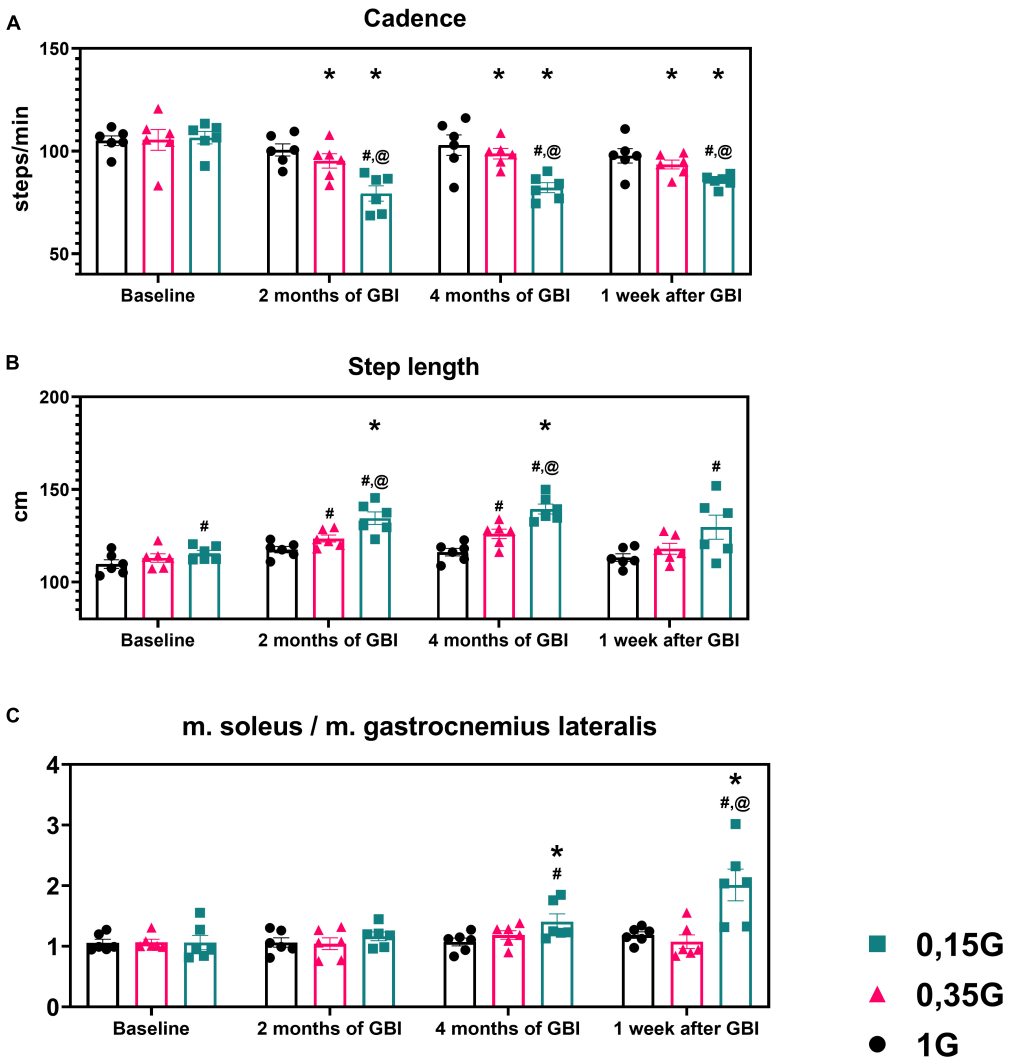

FIGURE 5 | Kinematic characteristics of walking in different sessions of the experiment: Step length **(A)** in centimeters, Cadence **(B)** in steps per minute. **(C)** The ratio of the average amplitude of EMG activity of m. soleus to the average amplitude of EMG activity of m. gastrocnemius lateralis. 1 G, 0.35 G, 0.15 G—body weight unloading modes—0%, 65%, 85% of the Earth weight respectively. «Baseline»—results obtained before isolation. «2 months of GBI» and «4 months of GBI»—results obtained on the 50th and 100th day of isolation, respectively. «1 week after GBI»—results obtained a week after leaving isolation. *A significant difference compared to the baseline values. $^{\#}$A significant difference compared with values at 1 G. $^{@}$A significant difference compared with values at 0.35 G. ^{No}A significant difference compared with values at 0.15 G. Data presented as MEAN with SEM + individual results.

the baseline values in the text are marked with a "*" sign, compared with the values at 0% BWU (1 G)—"$^{\#}$," compared with the values at 65% BWU (0.35 G)—"$^{@}$," compared with values at 85% BWU (0.15 G)—"^{No}."

RESULTS

0% Body Weight Unloading (1 G)

The results obtained in the baseline studies were completely consistent with normal walking in both EMG profile of muscle activity and biomechanical characteristics (Latash and Zatsiorsky, 2015). All the moments of the step were well expressed on the walking podogram (**Figure 3**). At the same time, GRF values at the moment of Heel strike were higher than GRF values at the moment of Toe off by 7.49 ± 5.42 kg, and Midstance amplitude was 13.29 ± 4.86 kg (**Figure 3**). Gait cycle time was 1.13 ± 0.08 s, Cadence was 105.05 ± 3.54 steps/min, Step length was 109.69 ± 2.11 cm, Double support time was 0.27 ± 0.03 s, and Single support time was 0.40 ± 0.11 s (**Figures 5A,B**, **6**). Equal work of the flexor and extensor muscles of the lower extremities was also observed (**Figures 4**, **7**).

During GBI, there was a tendency toward an increase in stride length, a decrease in stride frequency (**Figures 5A,B**). At the same time, the difference between GRF values at Heel strike moment and Toe off moment decreased: on the 2nd month this difference was 6.46 ± 4.16 kg, and on the 4th month—2.65 ± 1.05* kg (p = 0.0384; F = 7.843; Cohen's d = 2.523). It is interesting to

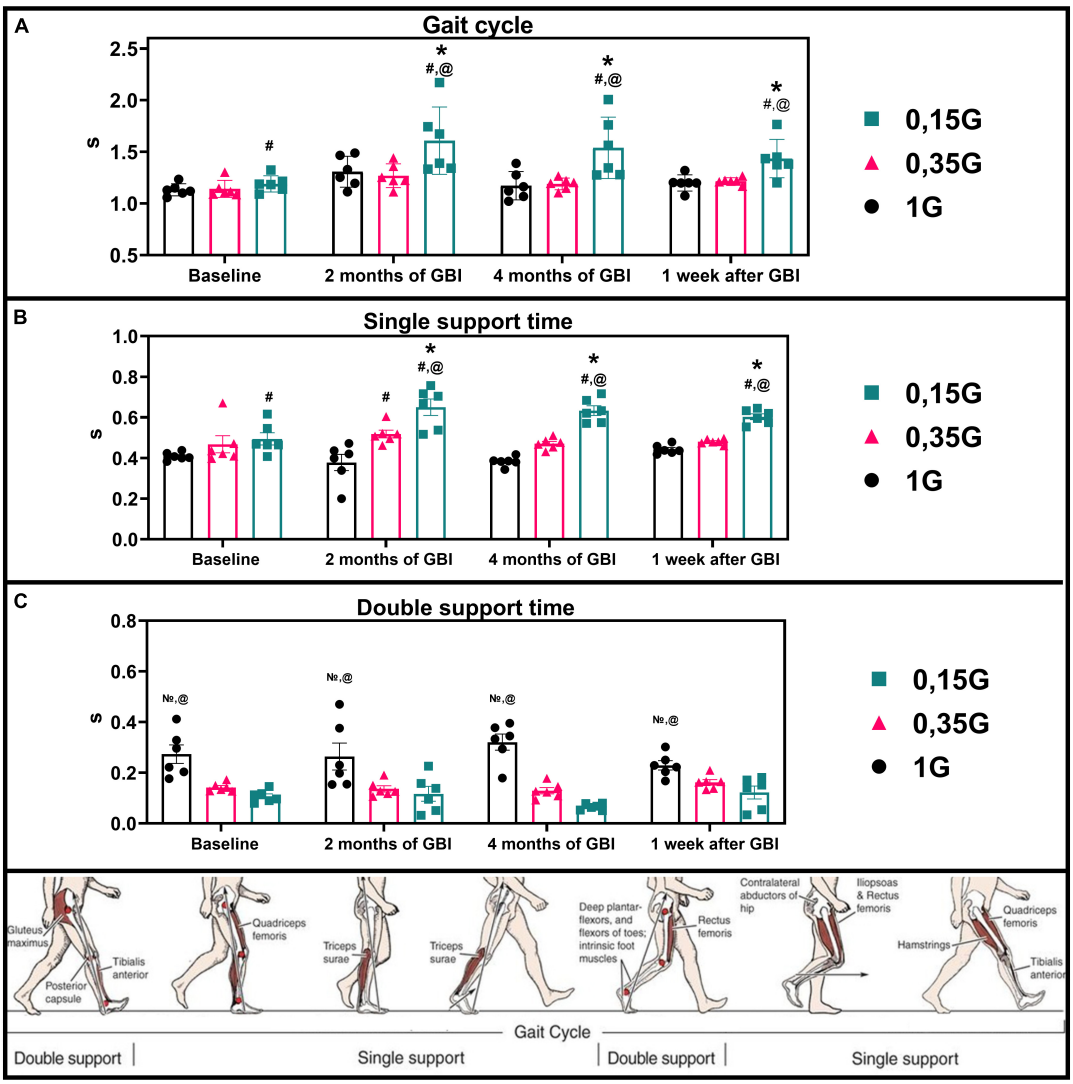

FIGURE 6 | Kinematic characteristics of walking in different sessions of the experiment: Gait cycle **(A)**, Single support **(B)**, Double support **(C)** in seconds. 1 G, 0.35 G, 0.15 G—body weight unloading modes—0%, 65%, 85% of the Earth weight, respectively. «Baseline»—results obtained before isolation. «2 months of GBI» and «4 months of GBI»—results obtained on the 50th and 100th day of isolation respectively. «1 week after GBI»—results obtained a week after leaving isolation. *—a significant difference compared to the baseline values. #—a significant difference compared with values at 1 G. @—a significant difference compared with values at 0.35 G. №—a significant difference compared with values at 0.15 G. Data presented as MEAN with SEM + individual results.

note that on the 2nd month of GBI, the value of the amplitude of Midstance decreased to 10.91 ± 3.53 kg against the background of a tendency toward a decrease in EMG activity of SL and an increase in Gait cycle time. Also on the 4th month the value of the amplitude of Midstance increased to 17.57 ± 3.12* kg ($p = 0.0015$; $F = 39.23$; Cohen's $d = 5.615$) against the background of a tendency toward increased EMG activity of SL (**Figures 3**, **6**, **7**).

After GBI, EMG activity of SL and GL muscles increased (**Figure 7**), while the overall GRF values decreased by 6.54 ± 2.17* kg ($p = 0.0071$; $F = 19.32$; Cohen's $d = 3.013$). In particular, GRF values at Heel strike moment was 11.37 ± 2.23* kg ($p = 0.0262$; $F = 9.854$; Cohen's $d = 5.098$) lower and GRF values at Toe off moment—was 6.52 ± 1.87* kg ($p = 0.047$; $F = 6.824$; Cohen's $d = 3.486$) lower compared to pre-GBI data. The difference between the GRF values at these moments was 3.01 ± 2.01* kg ($p = 0.0474$; $F = 6.867$; Cohen's $d = 1.497$), and the value of the amplitude of Midstance was 10.34 ± 3.91 kg. It is worth noting that during the experiment, the moment of the minimum vertical pressure at Midstance gradually shifted closer to the beginning of the gait cycle. This phenomenon is most pronounced on the 4th month of GBI and on the 7th day after it. Also on the 7th day after GBI, the moment of maximum vertical pressure at Heel Strike occurred faster than in the baseline studies (**Figure 3**).

65% Body Weight Unloading (0.35 G)

In the background sessions, the most notable changes in comparison with 1 G were recorded in the total GRF values and

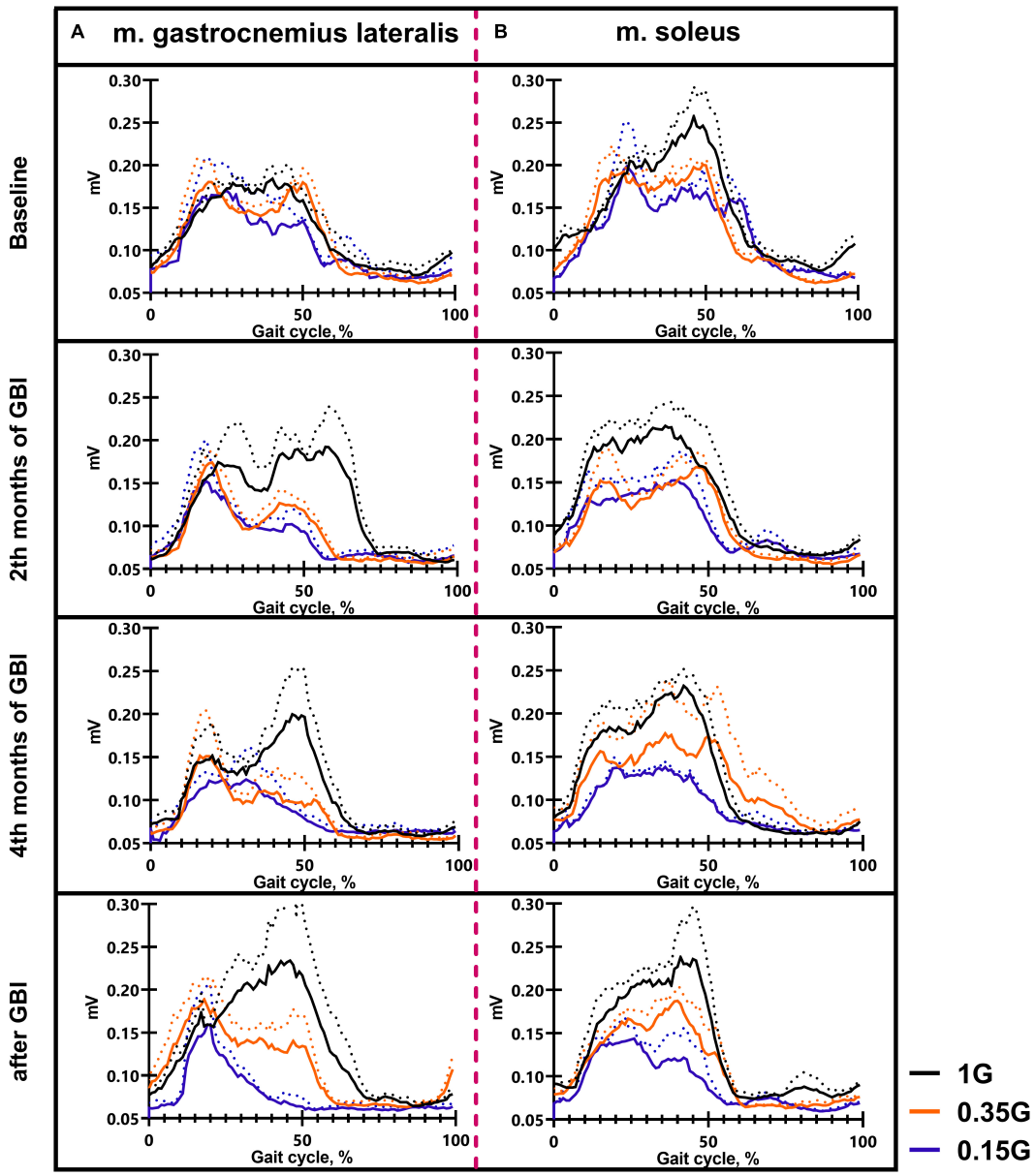

FIGURE 7 | The average EMG activity profile of 6 SIRIUS-19 crew members in different sessions of the experiment: m. gastrocnemius lateralis **(A)**, m. soleus **(B)**. On the ordinate axis: EMG amplitude, mV. On the abscissa axis: gait cycle, %. 1 G, 0.35 G, 0.15 G—body weight unloading modes—0%, 65%, 85% of the Earth weight, respectively. «Baseline»—results obtained before isolation. «2 months of GBI» and «4 months of GBI»—results obtained on the 50th and 100th day of isolation, respectively. «1 week after GBI»—results obtained a week after leaving isolation. The dotted lines display the error bars (SEM).

EMG activity of the extensor muscles—SL and GL. The total GRF values at 0.35 G were lower than the total GRF values at 1 G by 22.10 ± 3.44$^{\#}$ kg/45.29 ± 6.32$^{\#}$% (p = 0.0003; F = 81.15; Cohen's d = 6.424). At the same time the difference between GRF values at Heel strike moment and Toe off moment was higher compared to 1 G and amounted to 11.56 ± 4.76 kg, also the value of the amplitude of Midstance was lower and amounted to 8.22 ± 2.13$^{\#}$ kg (p = 0.0050; F = 22.78; Cohen's d = 3.859). All the moments of gait were well expressed on the walking podogram, as at 1 G, however, the moment of maximum vertical pressure at Heel Strike came earlier (**Figure 3**). Gait cycle time was 1.13 ± 0.03 s, Cadence was 105.45 ± 11.01 strides/min, Step length was 112.98 ± 7.23 cm, Double support time was 0.14 ± 0.01$^{\#}$ s (p = 0.0174; F = 12.22; Cohen's d = 5.813), and Single support time was 0.47 ± 0.02 s (**Figures 5A,B**, **6**).

During GBI, a significant decrease in GRF values at Heel Strike moment wasn't revealed, however, GRF values at Toe Off moment were lower than in the baseline sessions at 0.35 G by 9.5 ± 1.76$^{\#}$ kg (p = 0.0269; F = 6.902; Cohen's d = 5.397), 5.98 ± 2.04$^{\#}$ kg (p = 0.0217; F = 7.653; Cohen's d = 2.931) and

1.08 ± 0.94$^{\#}$ kg (p = 0.0383; F = 5.790; Cohen's d = 1.148) on the 2nd, 4th month of GBI and after it, respectively. At the same time, the difference between GRF values at Heel strike moment and Toe off moment gradually decreased, and on the 7th day after GBI difference was 11.04 ± 6.03 kg. Midstance amplitude was practically unnoticeable on podogram in the 2nd and 4th months of GBI and was noticeably shifted toward the end of the gait cycle. On the 7th day after GBI Midstance amplitude was 10.01 ± 3.11 kg (**Figure 3**).

The dynamics of kinematic and electromyographic characteristics during GBI at 0.35 G was similar to the dynamics of characteristics at 1 G, but was more pronounced. So for example, on the 2nd month GBI Step length increased by 10.50 ± 3.67$^{\#}$ cm (p = 0.0337; F = 8.425; Cohen's d = 2.861), Cadence decreased by 10.23 ± 2.14* steps/min (p = 0.0164; F = 12.59; Cohen's d = 4.780), and Single support time significantly increase by 0.05 ± 0.01* s (p = 0.0149; F = 13.26; Cohen's d = 5.364) compared to pre-GBI results (**Figures 5A,B**, **6**).

The dynamics of EMG activity of the muscles at 0.35 G is of particular interest. In the baseline sessions EMG activity of RF increased by 5.61 ± 8.13$^{\#}$% (p = 0.0173; F = 11.82; Cohen's d = 0.690), while EMG activity of the extensor muscles SL and GL decreased by 6.57 ± 4.23% and 14.4 ± 3.41$^{\#}$% (p = 0.0395; F = 7.657; Cohen's d = 4.223), respectively. During GBI and after it, EMG activity of the flexor muscles RF and TA remained practically unchanged; however, EMG activity of the extensor muscles SL and GL sharply decreased. On the 4th months of GBI, EMG activity of SL decreased by 31.09 ± 9.12$^{*\#}$% ($^{*}p$ = 0.0019; F = 11.61; Cohen's d = 3.409 and $^{\#}p$ = 0.0141; F = 13.61; Cohen's d = 1.369), and EMG activity of GL decreased by 22.87 ± 5.89$^{*\#}$% ($^{*}p$ = 0.0396; F = 7.651; Cohen's d = 3.882 and $^{\#}p$ = 0.0010; F = 47.87; Cohen's d = 4.276) compared to 1 G values. After GBI, there was a tendency to an increase in EMG activity of the extensor muscles like at 1 G (**Figures 4**, **7**).

85% Body Weight Unloading (0.15 G)

At 0.15 G, the mentioned BWU effects was more pronounced. In baseline sessions the total GRF values sharp decreased by 28.52 ± 2.42$^{\#}$ kg/58.44 ± 4.11$^{\#}$% (p < 0,0001; F = 159.4; Cohen's d = 11.785) compared to 1 G results and by 6.42 ± 1.86$^{@}$ kg/13.15 ± 2.75$^{@}$% (p = 0.0039; F = 25.61; Cohen's d = 3.451) compared to 0.35 G results. The podogram was noticeable only at Heel Strike moment, that shifted to the beginning of the gait cycle. Toe off and Midstance were not expressed on the podogram (**Figure 3**). Gait cycle time was 1.19 ± 0.21$^{\#}$ s (p = 0.0355; F = 8.169; Cohen's d = 0.377), Cadence was 106.51 ± 8.56 steps/min, Step length was 116.55 ± 9.03$^{\#}$ cm (p = 0.0116; F = 15.07; Cohen's d = 1.046), Double support time was 0.10 ± 0.03$^{\#}$ s (p = 0.0067; F = 19.87; Cohen's d = 5.666), and Single support time was 0.49 ± 0.02$^{\#}$ s (p = 0.0413; F = 7.448; Cohen's d = 1.138) (**Figure 6**).

During GBI, the total GRF values gradually decreased in comparison with the baseline values, and on the 7th day after GBI, the total GRF values were lower than the baseline ones by 9.09 ± 2.08* kg (p = 0.0061; F = 20.65; Cohen's d = 4.370) (**Figure 3**). At the same time, during GBI, the kinematic characteristics of walking significantly changed compared to the baseline values. On the 2nd month of GBI, Step length sharply increased by 18.68 ± 8.84$^{*\#@}$ cm ($^{*}p$ = 0.0004; F = 70.74; Cohen's d = 2.113; $^{\#}p$ = 0.0077; F = 18.50; Cohen's d = 4.301; $^{@}p$ = 0.0154; F = 13.01; Cohen's d = 3.605), this parameter continued to increase, and on the 4th month of GBI, it was 22.87 ± 6.73$^{*\#@}$ cm ($^{*}p$ = 0.0005; F = 64.98; Cohen's d = 3.398; $^{\#}p$ = 0.0185; F = 11.80; Cohen's d = 1.125; $^{@}p$ = 0.0093; F = 16.82; Cohen's d = 0.410) higher than before the isolation. Cadence values had the reverse tendency to Step length values and decreased on the 2nd month GBI—by 27.14 ± 9.48$^{*\#@}$ steps/min ($^{*}p$ = 0.0005; F = 64.98; Cohen's d = 2.862; $^{\#}p$ = 0.0185; F = 11.80; Cohen's d = 1.125; $^{@}p$ = 0.0093; F = 16.82; Cohen's d = 0.410), on the 4th month GBI—by 24.35 ± 7.11$^{*\#@}$ steps/min ($^{*}p$ = 0.0001; F = 111.7; Cohen's d = 3.424; $^{\#}p$ = 0.0119; F = 14.92; Cohen's d = 1.339; $^{@}p$ = 0.0010; F = 46.74; Cohen's d = 0.683). In addition, Gait cycle time increased mainly due to an increase in Single support time. Gait cycle time increased on the 2nd month GBI—by 0.42 ± 0.12$^{*\#@}$ s ($^{*}p$ = 0.0241; F = 10.22; Cohen's d = 3.513; $^{\#}p$ = 0.0478; F = 5.39; Cohen's d = 0.231; $^{@}p$ = 0.0225; F = 10.62; Cohen's d = 0.325), on the 4th month GBI—by 0.35 ± 0.07$^{*\#@}$ s ($^{*}p$ = 0.0487; F = 6.72; Cohen's d = 5.258; $^{\#}p$ = 0.0407; F = 7.513; Cohen's d = 0.273; $^{@}p$ = 0.0212; F = 10.98; Cohen's d = 0.331). On the 7th day after the isolation, these parameters did not return to pre-GBI values (**Figures 5A,B**, **6**).

In all experimental sessions at 0.15 G, EMG activity of the extensor muscles SL and GL was significantly lower compared to 1 G, and the moment of the maximum EMG activity of these muscles was noticeably shifted toward the beginning of the gait cycle. In baseline sessions EMG activity of the extensor muscles SL and GL was 16.84 ± 7.33$^{\#}$% (p = 0.0132; F = 14.37; Cohen's d = 2.297) and 12.79 ± 8.12$^{\#}$% (p = 0.0216; F = 8.13; Cohen's d = 1.587) lower compared to 1 G results and 2.74 ± 2.87% and 6.67 ± 5.67% compared to 0.35 G results, respectively (**Figure 7**). During GBI, this difference increased, and on the 4th month of GBI, EMG activity of SL and GL was lower by 27.24 ± 4.42$^{*\#}$% ($^{*}p$ = 0.0001; F = 87.11; Cohen's d = 6.162; and $^{\#}p$ = 0.00126; F = 13.43; Cohen's d = 2.143) and 24.19 ± 3.76$^{*\#}$% ($^{*}p$ = 0.0013; F = 56.67; Cohen's d = 6.433; and $^{\#}p$ = 0.0234; F = 7.852; Cohen's d = 1.562) compared to 1 G results, respectively. After GBI EMG activity of SL and GL continued to increase and was lower by 31.17 ± 3.13$^{*\#}$% ($^{*}p$ = 0.0034; F = 27.01; Cohen's d = 9.958; and $^{\#}p$ = 0.0381; F = 6.837; Cohen's d = 1.133) and 42.17 ± 6.17$^{*\#}$% ($^{*}p$ < 0,0001; F = 142.8; Cohen's d = 6.834; $^{\#}p$ = 0.0311; and F = 8.513; Cohen's d = 1.614) compared to 1 G results and by 16.65 ± 6.73$^{*@}$% ($^{*}p$ = 0.0212; F = 7.866; Cohen's d = 2.473; and $^{@}p$ = 0.0411; F = 5.372; Cohen's d = 1.161) and 28.81 ± 6.13$^{*@}$% ($^{*}p$ = 0.0121; F = 14.11; Cohen's d = 4.699; $^{@}p$ = 0.0267 and F = 8.145; Cohen's d = 1.775) compared to 0.35 G results, respectively (**Figures 4**, **7**).

Of interest is the dynamics of the ratio of the values of EMG activity of SL and GL: in baseline studies, this indicator was 1.06 ± 0.29, on the 2nd month GBI—1.16 ± 0.19, on the 4th month GBI—1.42 ± 0.85$^{*\#}$ ($^{*}p$ = 0.0002; F = 101.5; Cohen's d = 10.117; $^{\#}p$ = 0.00181 and F = 11.97; Cohen's d = 3.463), on the 7 days after GBI—2.18 ± 0.89$^{*\#@}$ ($^{*}p$ = 0.0059; F = 21.07; Cohen's

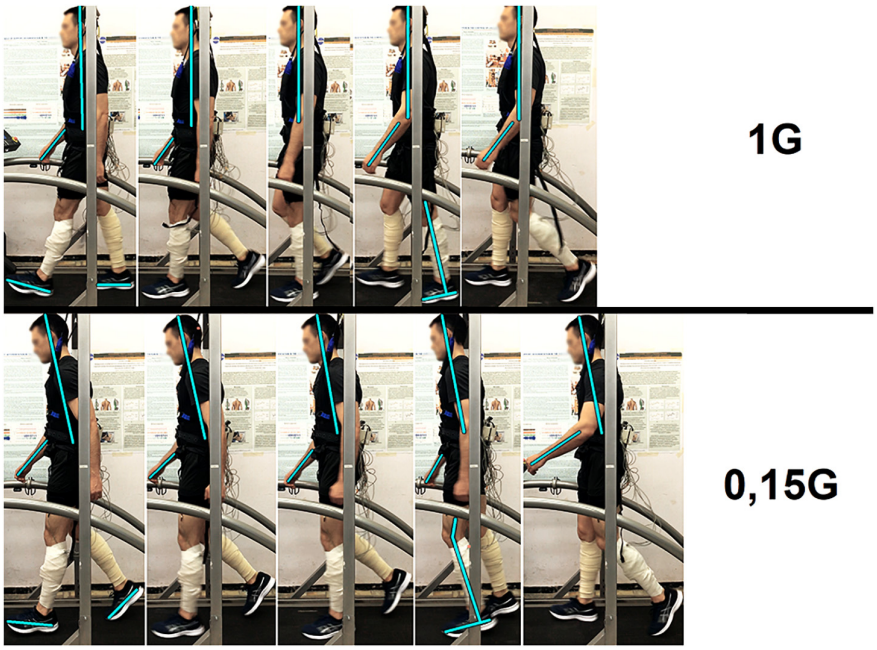

FIGURE 8 | Participant of the experiment at different moments of the step: Heel strike, Loading response, Midstance, Terminal stance, Toe off. 1 G, 0.15 G—body weight unloading modes—0%, 85% of the Earth weight, respectively.

$d = 4.589$; $^{\#}p = 0.00177$; $F = 12.11$; Cohen's $d = 3.489$; $^{@}p = 0.0070$; $F = 19.39$; Cohen's $d = 4.419$) (**Figure 5C**).

DISCUSSION AND CONCLUSION

Pre-ground-Based Isolation Data

The results obtained before GBI while walking in Martian and Lunar BWU modes (0.35 G and 0.15 G) characterize motor responses during G-transitions. **Figure 8** clearly demonstrates the features of the motor strategy when walking in BWU Lunar mode (0.15 G): a tilt of the trunk forward over the support surface, an increased amplitude of the arms swing, an earlier Toe off moment and flexion in the ankle joint at Terminal stance moment. These observations are consistent with changes in the studied parameters while BWU: for example, the shift of the moment of the maximum vertical pressure at Heel Strike to the beginning of the gait cycle, the decrease in EMG activity of the extensor muscles. Despite the significantly decreased repulsion force from the support surface at the Toe off moment, the swing phase increased on the background of a decrease in both single-support and double-support phases due to an increase in the step length and a decrease in the frequency of steps. Also, an increase in the duration of the swing phase is consistent with an increase in the EMG activity of the muscles involved in this phase: mm. tibialis anterior and rectus femoris. Probably, the primary triggering mechanism of these changes is the modification of the signals of the support and proprioceptive afferent, which in turn is a trigger for a decrease in the muscle-tonic activity of the extensor muscles (Kozlovskaya et al., 2007), which leads to the locomotion strategy changes. Interesting that similar strategy was observed during space flight. Thus, the tilt of the body increase over the support surface contributes to an increase in the angular moment speed of falling, and facilitates the work of the muscles (Saveko et al., 2020). Decrease of Cadence also reduces energy expenditure (Zarrugh et al., 1974). Pavei G. and Minetti A.E. have shown a decrease in total external work and range of motion of ankle, hip and knee during walking at a speed of 0.86 m/s and at Lunar level of gravitational unloading using BWU (Pavei and Minetti, 2016). The results of the study are consistent with the previously obtained results of other studies in this direction and complement them (Sylos-Labini et al., 2013; Awai et al., 2017; Richter et al., 2017).

Data Obtained During and After Ground-Based Isolation

The present study involved several limitations. Our results are limited to the small sample sizes inherent to such research and also the absence of the control group. The results obtained confirm the hypothesis, however, the revealed changes in the adaptive reactions of biomechanical and electromyographic parameters to partial vertical unloading when walking during and after GBI are difficult due to these limitations.

Despite the fact that during GBI, the crew members performed physical training as at the International Space Station, after GBI, a decrease in GRF values was observed. Probably, after GBI, the tendency to an increase in EMG activity of the extensor muscles against the background of a decrease in GRF values is result of recruitment of additional motor units, increased firing rates, and/or synchronization (Farina et al., 2004). It is noteworthy

that the ratio of the average amplitude of EMG activity of m. soleus to the average amplitude of EMG activity of m. gastrocnemius lateralis increased significantly during isolation. Since the recruiting order of motor units and the sensory inputs activity interrelated (Shigueva et al., 2015)., we assume that these phenomena may be associated with the presence of crew members in a limited space of a space station model, as well as the formation of a special stereotype of movements in this conditions, which is more likely than the influence of hypokinesia in this case. A decrease in GRF values, in turn, was also observed in real space flight conditions associated with muscle disuse (Saveko et al., 2020). In this case, account should be taken of the fact that a decrease in GRF values can also be the result of motor learning (Zimmermann and Bakker, 2019).

Noteworthy are the results obtained after the 4th session of the experiment—after 50 days of GBI. Sharp significant increase in the gait cycle time, a single support time and a step length with significant decrease in the cadence (frequency of steps) are probably associated with the ability to neuroplasticity and sensory-motor adaptation. It has been shown that people trained to adapt to various sensorimotor disturbances can adapt more quickly to a new sensory environment (Mulavara et al., 2009). Based on this observation, a program of sensorimotor training of astronauts has been developed over the past few years, which also includes BWU method. «Sensorimotor training has been shown to improve locomotor adaptability: increasing stability, lowering cognitive cost and reducing the metabolic expenditure during adaptation to discordant sensory conditions» (Bloomberg et al., 2015). It can be said that during the study participants were trained in motor reactions under the conditions of changed sensory environment. We think that the data obtained in this study will be useful in research both in gravitational physiology in preparation for interplanetary missions and in clinical medicine in the development and evaluation of rehabilitation programs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Biomedical Ethics Commission of the Institute of Biomedical Problems of RAS. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

ASa, VB, VK, and ET designed and conducted the research. ASh helped to process the electromyography data. ASa analyzed the data and drafted the manuscript. ET contributed in the global revision of the manuscript and was a supervisor of the experiment. ASa had primary responsibility for the final content. All authors interpreted the data and have read and approved the final submitted manuscript.

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frontiers in Integrative Neuroscience

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Cognitive Implications of Correlated Structural Network Changes in Schizophrenia

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Background: Schizophrenia is a brain disorder characterized by diffuse, diverse, and wide-spread changes in gray matter volume (GM) and white matter structure (fractional anisotropy, FA), as well as cognitive impairments that greatly impact an individual's quality of life. While the relationship of each of these image modalities and their links to schizophrenia status and cognitive impairment has been investigated separately, a multimodal fusion *via* parallel independent component analysis (pICA) affords the opportunity to explore the relationships between the changes in GM and FA, and the implications these network changes have on cognitive performance.

Methods: Images from 73 subjects with schizophrenia (SZ) and 82 healthy controls (HC) were drawn from an existing dataset. We investigated 12 components from each feature (FA and GM). Loading coefficients from the images were used to identify pairs of features that were significantly correlated and showed significant group differences between HC and SZ. MANCOVA analysis uncovered the relationships the identified spatial maps had with age, gender, and a global cognitive performance score.

Results: Three component pairs showed significant group differences (HC > SZ) in both gray and white matter measurements. Two of the component pairs identified networks of gray matter that drove significant relationships with cognition (HC > SZ) after accounting for age and gender. The gray and white matter structural networks identified in these three component pairs pull broadly from many regions, including the right and left thalamus, lateral occipital cortex, multiple regions of the middle temporal gyrus, precuneus cortex, postcentral gyrus, cingulate gyrus/cingulum, lingual gyrus, and brain stem.

Conclusion: The results of this multimodal analysis adds to our understanding of how the relationship between GM, FA, and cognition differs between HC and SZ by highlighting the correlated intermodal covariance of these structural networks and their differential relationships with cognitive performance. Previous unimodal research has found similar areas of GM and FA differences between these groups, and the cognitive deficits associated with SZ have been well documented. This study allowed us to evaluate the intercorrelated covariance of these structural networks and how these networks are involved the differences in cognitive performance between HC and SZ.

Keywords: multimodal imaging, cognition, gray matter density, fractional anisotropy, schizophrenia

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INTRODUCTION

Schizophrenia is a complex and chronic brain disorder that has severe negative impact on an individual's daily life. The disorder is often diagnosed after an initial psychotic episode in late adolescence to early adulthood (Picchioni and Murray, 2007). This is generally preceded by a prodromal period during which there are subacute changes in general thinking, mood, and social functioning (Picchioni and Murray, 2007). Symptoms of schizophrenia fall into one of three categories: positive, negative, and cognitive. Positive symptoms are generally considered to include hallucinations, delusions, and disordered thinking. Negative symptoms are seen as flattened affect, anhedonia, and apathy (Picchioni and Murray, 2007). Cognitive symptoms manifest in problems with attention, concentration, and memory (Green and Harvey, 2014). The combined impact of these impairments greatly diminish the quality of life experienced by patients with schizophrenia throughout their lifetime. There is no known cure, despite schizophrenia's prevalence of roughly 1% of the population, although anti-psychotic and anti-depressant medications mitigate some of the positive and negative symptoms.

The cognitive deficits associated with schizophrenia are considered one of its core dysfunctions. The degree of impairment is diverse within the disease, impacting many cognitive functions such as sensory processing, inhibition, attention, language function, working memory, episodic memory, and executive function; and it may be also a better predictor of functional recovery and overall outcome than any of the other symptoms patients with schizophrenia suffer (Green and Harvey, 2014). The source of these cognitive deficits is thought to be the pervasive changes in gray and white matter that occur in the transition from prodromal through the first episode of psychosis. These stabilize post-first episode and generally do not decline over the remainder of the illness. The diverse changes to these brain structures disrupt the cortical-subcortical-cerebellar circuit within the brains of patients with schizophrenia, negatively impacting a large number of cognitive domains (Barch and Ceaser, 2012). Because the cognitive deficits are so wide and diverse across the various cognitive domains, we chose to consolidate the cognitive test used in this study into a universal measurement of cognitive performance (g) (Gottfredson, 1998). Neuroscience and neuroimaging studies have shown over time that most higher cortical functions (such as cognition) are distributed widely over the entire brain, rather than localized to particular regions (Barrett and Satpute, 2013). While some regions may be more involved in a particular process than another, there are very few processes that only involve discreet structures of the brain. This understanding of the networked nature of cognition has driven the interest in whole brain analysis to further our understanding of the interconnected interplay (networks) of the entire brain, rather than focusing on specific and isolated structures.

Neuroimaging studies of the disease have shown it to be characterized by wide spread changes in the gray matter volume and the integrity of white matter structures. A large-scale international ENIGMA Schizophrenia Working Group study found that the entire brain showed reduced cortical thickness in patients. Some of the largest effects were seen in the superior temporal gyrus, superior frontal gyrus, middle frontal gyrus, precuneus, and cerebellum (Gupta et al., 2015). A 2016 ENIGMA study also found several subcortical regions in the brain, including the hippocampus, thalamus, and amygdala, had smaller gray matter volumes in patients (van Erp et al., 2016). Smaller studies have commonly found that patients with schizophrenia to also have reduced gray matter volume in subcortical areas such as the hippocampus and thalamus (Bora et al., 2011; Shepherd et al., 2012). Several studies have shown that these areas of reduced gray matter volume have been related to the diminished cognitive performance associated with the disorder. In a study of first-episode patients, the researchers found that cognitive deficits were strongly correlated with the reduced prefrontal and temporo-parietal GM (Minatogawa-Chang et al., 2009). The changes to white matter structure found in schizophrenia are just as profound and pervasive. An ENIGMA study of white matter integrity showed global reduction in FA, with the largest effects seen in the anterior corona radiata, the entirety of the corpus callosum, the cingulum, and the posterior thalamic radiation (Kelly et al., 2018). This is also in keeping with a 2017 review of DTI studies in schizophrenia that shows reduced white matter structural integrity in nearly every tract of the brain (Parnanzone et al., 2017). Smaller studies have shown relationships between lower white matter integrity in patients with schizophrenia and impaired cognitive performance in cognitive domains such as working memory (Karlsgodt et al., 2008; Epstein et al., 2014).

A recent study in animals has shown that MRI GM volume reflects not only the physical volume of the dendrites (gray matter), but also the glial and other support cells, as well as the clustering behaviors of the dendrites (Asan et al., 2021). These changes in the cellular composition within the gray matter have been linked to cognitive performance, although the exact mechanism underlying this is not fully understood. Other studies in animals show that changes in dendritic health and the composition of the glial cells associated with them impair neuroplasticity, which then impairs cognitive performance (Vance et al., 2010; Forrest et al., 2018). Disruptions in white matter integrity are thought to impact cognitive performance on the cellular level due to less stable flow of electrical currents, disruptions of the conduction of action potentials, which then lead to fluctuations in the connectivity of neuronal pathways, reduced efficacy of neurotransmitter systems, and disconnectivity in associative pathways (Fjell et al., 2011). Schizophrenia has been understood to develop from changes within the gray and white matter, as a combination of genetic and environmental factors, that lead to positive and negative symptoms as well as the cognitive impairment associated with it (Bora, 2015).

To date, these structural networks and their relationships to cognition have only been studied unimodally. Newer multimodal analysis tools allow us to consider the simultaneous pattern of these two networks in way previously not possible. The unimodal studies have shown which structural (GM or FA) brain regions differ, but they are limited in that they cannot evaluate the direct relationship between GM and FA. Multimodal studies quantify the simultaneous relationships of different brain

measures in ways that are not possible separately. This study uses a multimodal analysis of magnetic resonance imaging (MRI), to examine how the pattern of simultaneous covariance of gray and white matter changes differ between healthy controls (HC) and patients with schizophrenia (SZ) and the relationship of those changes have with cognitive performance.

Parallel independent component analysis (pICA), a semi-blind multimodal analysis tool, of structural MRI (sMRI) and diffusion MRI (dMRI) is a method that highlights the correlated covariance of these structural networks (gray matter and white matter) in the brain (Sui et al., 2012). This data-driven method allows a whole brain exploration of the relationship between gray matter volume, measured with the sMRI, and the integrity of the white matter, reflected in the fractional anisotropy (FA), measured using dMRI (Calhoun and Sui, 2016). pICA uses independent component analysis (ICA) to identify the maximally independent components of both modalities while simultaneously estimating the degree of correlation between them (Pearlson et al., 2015). pICA can be used to both identify and quantify the relationships between the features, or spatial maps, within the structural networks of gray and white matter. Variance between individual subjects is reflected in the loading coefficients of each feature (Calhoun and Adali, 2009).

A previous multimodal study using joint ICA (jICA) did identify group differences within joint sources of gray and white matter volume between healthy controls (HC) and patients with schizophrenia (SZ) (Xu et al., 2009). This study considered white matter volume, rather than looking at the integrity of white matter structure as reflected in FA. More recently, multimodal studies also used a jICA of GM, FA, and the fractional amplitude of low-frequency fluctuation (fALFF) of patients with SZ, combined with a reference map derived from a multimodal canonical correlation analysis (MCCAR) of cognitive performance, to develop replicable neural markers of the disease (Sui et al., 2013, 2015, 2018). Those studies used spatial maps from a template derived from cognitive performance to predict changes in a jICA of those three modalities, which is the reverse of our approach. Here, we identify correlated GM and FA patterns that are affected by diagnostic status, and then consider the relationship of those components with cognitive function. jICA, while also a fusion analysis technique, differs from pICA considerably. A jICA assumes that the signal sources (for example GM, FA, falff, and scores from specific cognitive domains) will all modulate the same way across the subjects (Calhoun and Adali, 2009). This strong constraint simplifies the estimation of joint information, but does not allow for differentiated covariation between signal sources. pICA does not make this assumption and allows each signal source (in this case, GM and FA) to vary independently and then optimizes the correlated patterns of covariation found between them (Calhoun and Adali, 2009). pICA thus uses a "soft" constraint, whereas jICA uses a "hard" constraint on the inter-modality coupling.

TABLE 1 | Demographic Statistics.

	Schizophrenia	Healthy controls	*p*
N	73	82	—
Sex (%male)	75.34%	75.61%	0.54
Age (mean/range)	37.32/18–65	38.93/18–65	0.44
Education level (median/range)	4/2–8	4/2–8	0.24
Occupation level (median/range)	5/0–7	4/1–7	0.21

A Chi-squared test showed no significant differences regarding sex in patients or healthy controls. A Welch's two sample t-test also found no significant differences between the groups for age. A Mood's median test showed no significant differences between the education and occupation levels between the groups.

MATERIALS AND METHODS

Subjects

The images were collected from 157 subjects, 82 healthy controls (HC) and 75 subjects with schizophrenia (SZ) as part of the COBRE dataset, collected according to the description in Aine et al. (2017). Two subjects with schizophrenia were removed, one for failing the diffusion tensor imaging (DTI) quality control (insufficient number of good gradients), the other for exceeding allowed motion parameters (more than 3 mm). The final sample total was 155, with the HC group comprising 62 males and 20 females, and the SZ group 59 males and 15 females. Both groups ranged from 18 to 65 years in age. The Structured Clinical Interview for DSM Disorders (SCID) was used to gather diagnostic information and subjects were excluded if they had history of substance abuse or dependence within the last 12 months, severe head trauma with more than 5 min loss of consciousness, neurological disorders, or severe cognitive impairment. The range of IQ, as reflected in the WASI Sum IQ metric, was from 65 to 134. Medication dose was calculated according to the methods outlined by Gardner et al. in their 2010 paper, *International Consensus Study of Antipsychotic Dosing* (Gardner et al., 2010). All subjects provided informed consent prior to the study. A Welch's two sample *t*-test and a Pearson's chi-squared test were used to test for group differences in age and gender, respectively, using R version 3.5.0. A Mood's median test was performed to determine group differences in education levels and occupation levels (see **Table 1**).

Image Collection

The imaging data were collected on a Siemens 3T Trio TIM scanner at the Mind Research Network, Albuquerque, NM. The T1-weighted images for GM were collected in the sagittal plane, interleaved, multi-slice mode in a single shot with these parameters: TR/TE/TI = 2,530/[1.64, 3.5, 5.36, 7.22, 9.08]/900 ms, flip angle = 7*, FOV = 256 × 256 mm, slab thickness 176 mm, matrix 256 × 256 × 176, voxel size = 1 × 1 × 1 mm, number of echos = 5, pixel bandwidth = 650 Hz, total scan time = 6 min.

The DTI images for FA were collected using 30 gradient directions and five b = 0, for a total of 72 slices with a slice thickness of 2 mm (isotroptic resolution of 2 × 2 × 2 mm). FOV = 256 × 256 mm, TR/TE = 9,000 ms/84 ms, encoded A-P. Sequence bandwidth was 1,562 Hz/Px and echo spacing was 0.72 ms with an EPI factor of 128. For more details, see Aine et al. (2017).

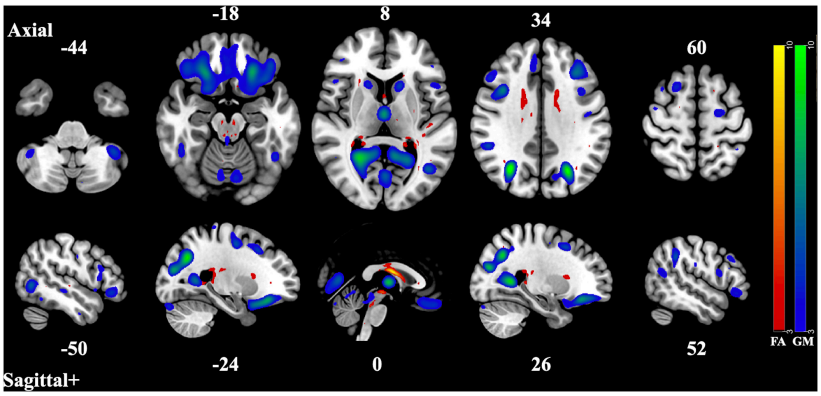

FIGURE 1 | Component Pair 1, Intermodal spatial map highlighting the correlated FA and GM changes that differ significantly (Bonferroni-corrected for multiple comparisons), HC > SZ, z > |3|. pICA correlation between structural networks, $r = 0.61$ ($t = 9.56$, $p = 2.87 \times 10^{-17}$). FA cases vs. controls differences, $t = 3.21$, $p = 0.0016$, GM cases vs. controls, $t = 3.63$, $p = 0.00039$. Red-yellow represents the FA group differences, blue-green represents the GM group differences. See **Supplementary Figures 1–2** for full axial and sagittal images.

Image Processing

Diffusion MRI to Fractional Anisotropy

An FSL v5.0.10 pipeline was used to preprocess the DTI data (Smith et al., 2004). A quality control of the DTI images was done using DTIPrep to ensure that a minimum of 25 gradient directions for each subject were free of artifacts (Liu et al., 2010). Eddy current correction for gradient distortions and head motion were applied to the diffusion-weighted images (Andersson and Sotiropoulos, 2016), after which a brain extraction tool (BET) was used to remove non-brain tissue from the image (Smith, 2002). A diffusion tensor model was fitted to each voxel with DTIFIT (Smith, 2002), creating the fractional anisotropy images. All subjects' FA data were then aligned into a common space using the non-linear registration tool FNIRT (Andersson et al., 2007a,b), which uses a b-spline representation of the registration warp field (Rueckert et al., 1999). Leaving the FA unsmoothed and in 1 × 1 × 1 MNI152 resolution eliminated spurious results due to partial voluming.

Structural MRI to Gray Matter Volume

The T1-weighted sMRI images were reoriented and registered to the MNI152 template and resampled to 2 mm × 2 mm × 2 mm. Using DARTEL in SPM12 (Ashburner and Friston, 2005), the non-brain tissues were stripped and the gray matter, white matter, and cerebral spinal fluid were segmented, leaving normalized, modulated, Jacobian-scaled gray matter images. A QA was performed to ensure that the images produced correlated with the template ($r > 0.85$), and were then smoothed by an 8 mm × 8 mm × 8 mm Gaussian kernel.

Parallel Independent Component Analysis

Parallel ICA was performed using the Fusion ICA Toolbox (FITv2.0a) using Matlab R2017b. The number of principle components for each modality were estimated using a minimum description length (MDL) in the FIT software (4 FA components and 52 GM components when estimated separately, 12 when combined) (Li et al., 2007). The descending trend of entropy was allowed to be −0.001 maximally. ICASSO software was used to ensure cluster stability by retesting each FastICA 10 times. The suggested default of applying the constraint algorithms, which control for over-and under-fitting the data, to the first six component pairs was used (Sui et al., 2018). Spatial maps were calculated and loading coefficients extracted that reflect the decomposition of the subject's data. Z-scores of the spatial maps were thresholded at $|z| > 3$ to identify component clusters. Case vs. control differences in the loading coefficients for the correlated pairs of each of the GM/FA spatial maps were calculated using a two-sample *t*-test, Bonferroni-corrected for multiple tests. The Harvard-Oxford Cortical and Subcortical Structure Atlases were used to identify the gray matter regions. The JHU ICBM-DTI-81 White Matter Labels were used to identify the white matter regions, except where the pICA identified white matter regions outside the 81 tracts provided, in which case, the corresponding gray matter region was labeled as above.

Cognitive Performance

All individuals in the study were administered a battery of neuropsychological tests. All cognitive tests were collected within 1 week of every neuroimaging assessment (Aine et al., 2017). These included: the Continuous Performance Test-Identical Pairs (CPT-IP), the Neuropsychological Assessment Battery (NAB), the Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS), Wechsler Abbreviated Scale of Intelligence (WASI_IV), the Processing Speed Index of the Wechsler Adult Intelligence Scale_IV including symbol search and coding (WAIS_IV), and the Wechsler Test of Adult Reading (WTAR) (Aine et al., 2017). Data reduction was performed using a principal component analysis (PCA) using R version 3.5.0, of which the first component reflects a composite of the cognitive performance (g).

Statistical Analyses

A multiple analysis of covariance (MANCOVA) was performed using R version 3.5.0 to test the significance of the relationships

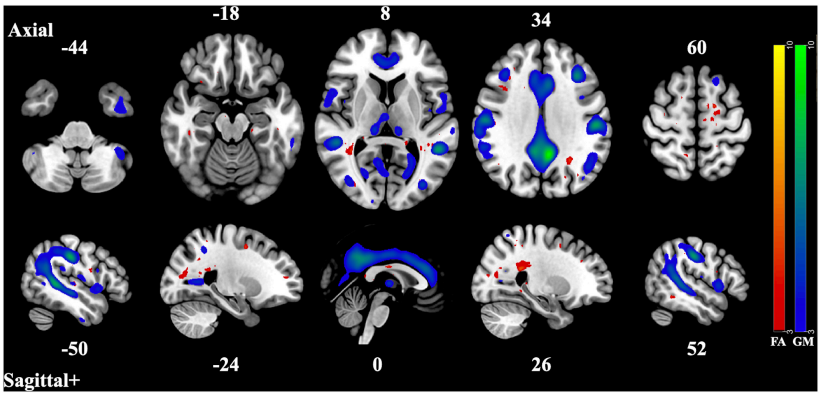

FIGURE 2 | Component Pair 2, Intermodal spatial map highlighting the correlated FA and GM changes that differ significantly (Bonferroni-corrected for multiple comparisons), HC > SZ, z > |3|. pICA correlation between structural networks, $r = 0.59$ ($t = 9.05$, $p = 6.18 \times 10^{-16}$). FA cases vs. controls differences, $t = 2.99$, $p = 0.0032$, GM cases vs. controls, $t = 2.67$, $p = 0.0082$. Red-yellow represents the FA group differences, blue-green represents the GM group differences. See **Supplementary Figures 3**, **4** for full axial and sagittal images.

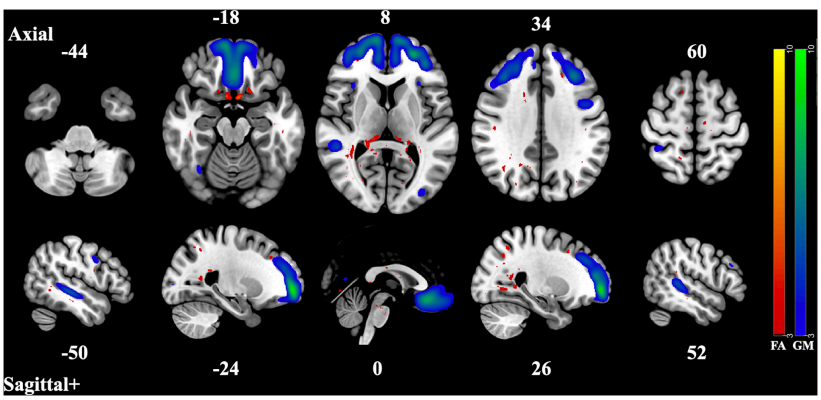

FIGURE 3 | Component Pair 3, Intermodal spatial map highlighting the correlated FA and GM changes that differ significantly (Bonferroni-corrected for multiple comparisons), HC > SZ, z > |3|. pICA correlation between structural networks, $r = 0.46$ ($t = 6.45$, $p = 1.36 \times 10^{-9}$). FA cases vs. controls differences, $t = 3.5$, $p = 0.00053$, GM cases vs. controls, $t = 2.92$, $p = 0.0041$. Red-yellow represents the FA group differences, blue-green represents the GM group differences. See **Supplementary Figures 5–6** for full axial and sagittal images.

between the loading coefficients identified by the pICA spatial maps as the dependent variables and the subject's age, sex, medication dosage, symptoms (PANSS total scores), and global cognitive score (g) as the covariates, with family-wise error correction to compensate for multiple testing. In a supplemental analysis, a MANCOVA was also used to investigate the relationships of the loading coefficients as the dependent variables and each of the cognitive tests as covariates.

RESULTS

Subject Demographics

A Student's two-sample *t*-test showed there were no significant differences between HC and SZ groups with regards to age ($t = -0.729$, *p*-value = 0.467). A Chi-squared test determined that the two groups, HC and SZ, were balanced for gender as well (X-squared = 0.32614, *p*-value = 0.568). The Mood's median test showed no significant differences between the groups for education level (z = 1.18, *p*-value = 0.24) or for level of occupation (z = 1.26, *p*-value = 0.21) (see **Table 1**).

Parallel Independent Component Analysis

The 12 component model found 6 significantly correlated pairs of FA and GM changes. Of those 6, *t*-tests of the subjects' loading coefficients determined that 3 pairs had significant group differences (cases vs. controls, HC > SZ, Bonferroni-corrected *p*-value < 0.002) in the correlated patterns of FA/GM changes. **Figures 1–3** show the spatial maps of the significantly correlated FA/GM brain regions with significant group differences. **Tables 2A–C** are an abridged list of the brain regions of GM and WM found in the significantly correlated component pairs. See **Supplementary Tables 1–3** for the comprehensive list.

TABLE 2 | Abridged List of Brain Regions—**(A)** Component Pair 1, **(B)** Component Pair 2, **(C)** Component Pair 3.

GM brain region	|Z|	MNI (x, y, z)	FA brain region	|Z|	MNI (x, y, z)
(A)					
Lateral occipital cortex, superior division	11.2	(30, –64, 34)	Thalamus, left	9.6	(–13, –12, 19)
Precunous cortex	9.2	(24, –54, 6)	Caudate, left	8.7	(–15, –15, 21)
Lingual gyrus	8.3	(24, –50, 4)	Thalamus, right	8.3	(16, –21, 19)
Lateral occipital cortex, inferior division	8	(–44, –66, 2)	Planum temporal	6	(–36, –34, 12)
Middle frontal gyrus	7.7	(38, 12, 30)	Retrolenticular part of internal capsule	5.7	(31, –37, 15)
Frontal orbital cortex	7.5	(–24, 30, –18)	Body of corpus callosum	5.7	(5, –11, 28)
Angular gyrus	6.7	(44, –56, 16)	Posterior thalamic radiation, left	5.4	(–31, –39, 11)
Thalamus, right	6.6	(4, –12, 6)	Posterior thalamic radiation, right	5.2	(33, –39, 11)
Thalamus, left	6.4	(–14, 18, 0)	Helschl's gyrus (H1 and H2)	5.1	(–44, –23, 9)
(B)					
Precunous cortex	9.6	(–6, –52, 34)	Middle frontal gyrus	6.3	(36, 17, 31)
Cingulate gyrus, posterior division	9.1	(–8, –48, 34)	Precunous cortex	5.9	(–11, –62, 29)
Angular gyrus	8.8	(–42, –58, 22)	Tapetum, right	5.8	(27, –43, 21)
Postcentral gyrus	7.8	(–52, –24, 36)	Precentral gyrus	5.7	(–15, –16, 62)
Middle frontal gyrus	7.5	(–36, 26, 36)	Supramarginal gyrus, posterior division	5.7	(37, –47, 10)
Superior parietal lobule	6.5	(–30, –50, 44)	Superior frontal gyrus	5.6	(–17, –3, 60)
Middle temporal gyrus	6.3	(50, –36, 4)	Lateral occipital cortex, inferior division	5.3	(33, –80, 9)
Supramarginal gyrus, posterior division	6.2	(50, –40, 8)	Tapetum, left	5.3	(46, –21, 35)
Cingulate gyrus, anterior division	6.2	(–4, 26, 26)	Cingulum (hippocampus), left	5.2	(–15, –80, 30)
(C)					
Frontal pole	9.5	(–26, 56, –2)	Subcallosal cortex	8.8	(–5, 9, –19)
Paracingulate gyrus	7.7	(–2, 32, –14)	Thalamus, right	8.1	(15, –33, 12)
Frontal medial cortex	7.4	(2, 34, –14)	Thalamus, left	7.9	(–8, –28, 16)
Middle frontal gyrus	6.5	(42, 22, 24)	Precentral gyrus	7.3	(–40, –1, 41)
Subcallosal cortex	6.1	(–2, 24, –14)	Middle frontal gyrus	6.7	(34, 26, 30)
Middle temporal gyrus, posterior division	5.7	(–52, –26, –6)	Lateral occipital cortex, superior division	6.7	(37, –67, 30)
Middle temporal gyrus, temporoccipital	5.4	(50, –36, 4)	Cingulum (hippocampus) left	6.5	(–16, –39, –5)
Lateral occipital cortex, inferior division	5.1	(–32, –86, 4)	Cingulum (hippocampus) right	5.9	(17, –39, –4)
Angular gyrus	4.3	(–40, –60, 26)	Lingual gyrus	5.7	(29, –56, 2)

*For each component pair, the top brain regions in the spatial map thresholded above $|Z| > 3.0$ were identified by their Montreal Neurological Institute (MNI) coordinates. See **Supplementary Figures 1–6** for the comprehensive list of regions for each component pair.*

Cognitive Performance

The PCA of the cognitive battery resulted in a primary component that explained 39.9% of the variance. This component was used as a global cognitive measure (g). A Welch's two sample *t*-test of g showed HC performed significantly better on the cognitive tests than SZ ($t = 9.987$, $p < 2.2 \times 10^{-16}$).

Statistical Analysis

The MANCOVA showed the expected significant relationships between the correlated FA/GM brain regions and the subject's age and sex. No significant relationships were found for medication dosage or symptoms, but there was a weak inverse correlation between the Total Negative Score and the global cognitive scores in patients ($r = -0.35$, $p = 0.01$, Bonferroni-corrected for multiple comparisons). Two of the correlated spatial maps showed significant relationships between the GM differences and the global cognitive score (g), shown in **Figures 4**, **5**. See **Supplementary Tables 4–6** for a list the significant relationships found for each of the correlated FA/GM spatial maps. Significant relationships were also found between these same two GM spatial maps and individual cognitive tests, but they did not survive Bonferroni error correction. These were the WTAR Standard Score, the WASI Vocab T-Score, the WASI Verbal T-Score, the WASI Block Design T-Score, the NAB Mazes T-Score, and the Matrics Domain Reasoning and Problem Solving T-Score. See **Supplementary Tables 7**, **8** for full results.

DISCUSSION

This multimodal analysis highlighted three unique structural networks in which patients with schizophrenia had significant differences in their patterns of gray and white matter covariance. Gray matter and white matter are often treated as though they are separate and distinct structures, but they are in fact parts of the same neurons. Studying GM and FA together offers the opportunity to see what patterns of covarying GM and FA differ between HC and SZ and how these relationships present themselves behaviorally across the two different groups. While not networks in the neuroanatomical sense, patterns of structural change/difference are often referred to as structural

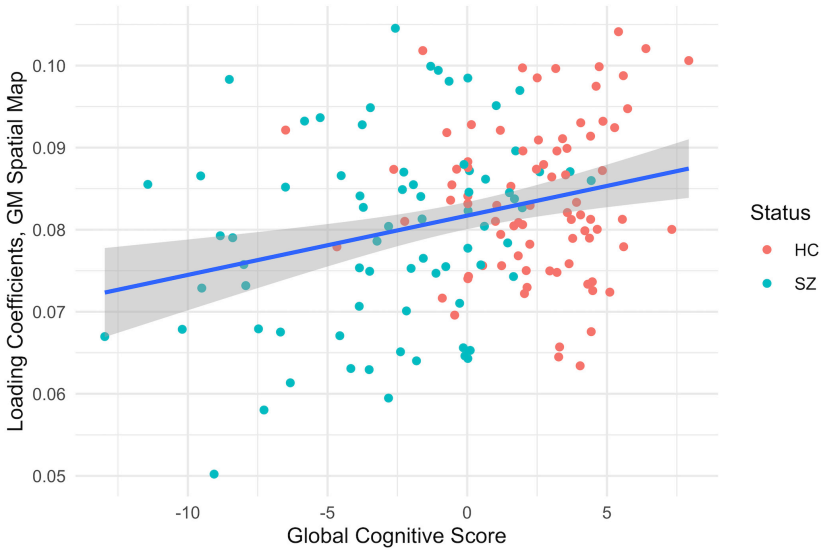

FIGURE 4 | MANCOVA results between the subjects' gray matter loading coefficients from the second component pair (y-axis) and the global cognitive score (x-axis), $F = 12.93$, $p < 0.001$, corrected for multiple comparisons, healthy controls (HC) in red and patients with schizophrenia in teal (SZ). The gray shading indicates a confidence interval of 0.95.

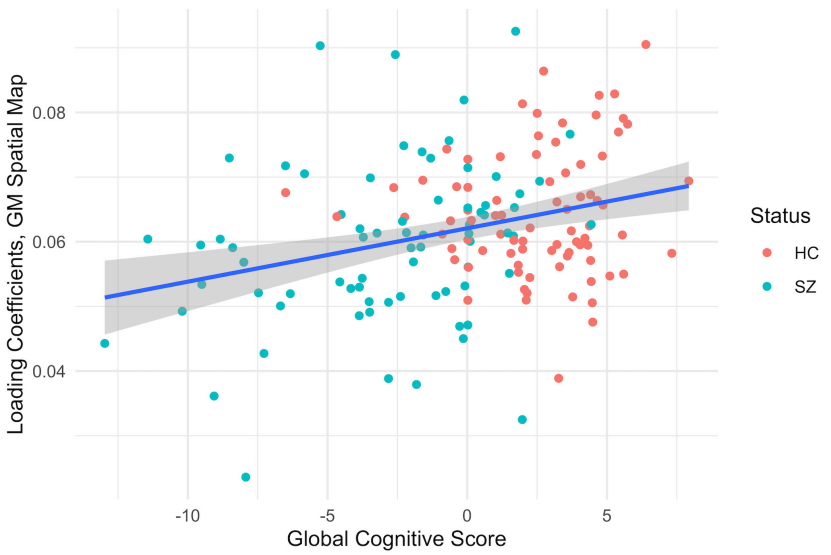

FIGURE 5 | MANCOVA results between the subjects gray matter loading coefficients from the third component pair (y-axis) and the global cognitive score (x-axis), $F = 9.70$, $p < 0.002$, corrected for multiple comparisons, healthy controls (HC) in red and patients with schizophrenia in teal (SZ). The gray shading indicates a confidence interval of 0.95.

networks. The high incidence of co-location of gray and white matter differences in the results of this study lend support to their behaving in a networked manner, as does the similarity between these structural networks and the functional network differences found in unrelated and independent cohorts used in fMRI and rs-fMRI studies of cognition. The brain regions of correlated covariance identified in each of these component pairs had less gray matter volume and reduced white matter integrity than healthy controls. These networks pull from broad regions of the brain, reflecting the ways in schizophrenia globally impacts the brain.

The first component pair found widespread reduction of gray matter volume in areas such as the lateral occipital cortex, the precuneus cortex, the lingual cortex, the thalamus, the frontal pole, the frontal orbital cortex, the angular gyrus, the supra marginal gyrus, the caudate, as well as areas of the middle frontal gyrus and the frontal orbital cortex. The areas of reduced white matter integrity that were correlated to those gray matter

areas were found in the body of the corpus callosum, the thalamus, the caudate, the retrolenticular part of the internal capsule, the posterior thalamic radiations, the superior fronts-occipital fasciculi, as well as the external capsule and subcallosal cortex. The overarching pattern of these regions seems to implicate the front-to-back-to-front connectivity of the brain, approximating control networks seen in functional and resting-state MRI studies (f- and rsfMRI) (Fair et al., 2007; Gratton et al., 2018). These control networks include the fronto-parietal, the cingular-opercular, and the dorsal attentional networks and are responsible for regulating other brain systems such as cognitive control and cognitive flexibility (Cole et al., 2014). Disturbances within this overarching control network would likely cause downstream dysregulation across neural systems (Cole et al., 2014). This particular structural network was the most strongly correlated of the three component pairs, suggesting that the most marked difference between the covarying gray and white matter networks of the two groups lies within this control network.

The second and third component pair showed reduced gray matter volume and less white matter integrity in areas associated with the three subnetworks of the default mode network (DMN) (Andrews-Hanna et al., 2010). The pattern of gray matter volume differences seen in the second component pair resemble those of the midline core subsystem of the DMN. These consist of the precuneus cortex, the posterior cingulate gyrus, the angular gyrus, the postcentral gyrus, the middle frontal gyrus, and the superior and inferior lateral occipital cortex. The correlated covarying white matter areas with reduced integrity were also in tracts and regions that connect these subnetworks, such as the corpus callosum, the middle frontal gyrus, the precuneus cortex, the posterior supramarginal gyrus, and inferior and superior lateral occipital cortex. This midline core subsystem of the DMN is considered a coordinating hub between the other two subsystems (Andrews-Hanna et al., 2010). The third component pair seems to highlight areas of gray matter differences related to the two smaller subsystems of the DMN, the medial temporal lobe subsystem and the dorsal medial prefrontal cortex subsystem, by including the frontal pole, the paracingulate gyrus, the frontal medial cortex, the posterior and temporoccipital middle temporal gyrus, and the inferior lateral occipital cortex. The correlated regions of reduced white matter structural integrity were seen in structures related to the coordination and communication between these subnetworks such as the thalamus, the middle frontal gyrus, the superior lateral occipital cortex, the lingual gyrus, the splenium of the corpus callosum, the posterior cingulate, the temporoccipital middle temporal gyrus as well as the inferior lateral occipital cortex. These two subsystems have been identified as being involved in memory and metacognition, as well as the self-reflective processes typically associated with the DMN (Andrews-Hanna et al., 2010).

In both of these component pairs, the gray matter component loading coefficients were also related to differences in cognitive performance, where loading coefficients of the healthy controls were associated with higher global cognitive scores than patients with schizophrenia. These subnetworks of the DMN have been linked to cognition in several recent studies. In a 2018 fMRI study, researchers established significantly increased activation of subnetworks within the DMN during cognitive task switching and concluded that these networks, normally associated with contextual representation, were recruited when a shift in cognitive focus was required (Smith et al., 2018). Another study in 2020 found that multiple DMN subnetworks were involved in a variety of cognitive tasks (Gordon et al., 2020). A 2021 review of the functional role of the DMN found that it was involved in forms of complex cognition that involve abstract thought and memory (Smallwood et al., 2021). Structural networks map well to functional ones and vice versa (Meier et al., 2016), so deficits in the gray and white matter structural network could be responsible for dysfunction in the functional network, impacting cognitive performance in patients.

Using pICA to look at both of white and gray matter structures simultaneously revealed unique networks of differences in the structural networks in patients with schizophrenia that echo networks found in functional studies. Dysregulation of the functional networks is a common finding within schizophrenia research. Task fMRI studies have found aberrant connectivity both within the frontoparietal network as well as between it and the rest of the brain (Tu et al., 2013) and dysconnectivity with regards to attentional tasks and the frontoparietal network (Roiser et al., 2013). A recent task-based fMRI study found that disrupted frontoparietal control networks as well as dysconnectivity within the DMN were related to metacognitive deficits, demonstrating that global dysfunction of these networks interferes with overarching cognitive processes (Jia et al., 2020). Disrupted DMN network activation/deactivation has been long associated with schizophrenia (Hu et al., 2017), and a dynamic connectivity study has shown that patients have reduced connectivity between the DMN subnetworks (Du et al., 2016).

Many of the brain regions of covarying GM and FA found in the three component pairs of this study are also involved in the cortical-subcortical-cerebellar circuit considered critical for appropriate cognitive performance (Andreasen et al., 1996). Dysregulation of the DMN networks as well as the thalamus, cerebellum, and the inferior frontal gyrus (IFG) in the patients was associated with poor cognitive performance (Matsuo et al., 2013). A 2018 study found that reduced gray matter volume in the insula, inferior parietal cortex, middle temporal cortex, and cerebellum in patients were related to poor cognitive performance (Banaj et al., 2018). A study in first episode patients found that reduced prefrontal and temporo-parietal gray matter volume was significantly correlated with poor cognitive performance (Minatogawa-Chang et al., 2009).

In this study, only the gray matter volume differences were found to be related to poor cognitive performance, differentiating it from the white matter integrity differences. A study done in 2018 investigated gray and white matter volumes independently also found that lower cognitive performance in patients was correlated with brain regions that showed less gray matter volume than healthy controls (Banaj et al., 2018). The affected gray matter regions in that study were similar to the ones found in this study; the inferior parietal cortex, the insula, the middle temporal cortex, and the cerebellum.

The weak inverse correlation between the Total Negative Score and the global cognitive scores in patients is a not

uncommon (although inconsistent) finding and has been linked by several studies to the "difficulty in abstract thinking" question of the PANSS negative sub scale. This measure is thought to have some overlap with cognitive domains, but not enough to consider them collinear (Cruz et al., 2013; Bagney et al., 2015).

Next Steps

To further our understanding of the relationships between the covarying gray and white matter areas identified in the component pairs, a tractography study could shed more light on why these particular patterns are covarying together. A longitudinal study from prodromal through a chronic state could better quantify the causal relationship between the changes within the structural networks and their relationship with the typical decline in cognitive performance.

Limitations

Since this is a cross-sectional study, there are limitations regarding the interpretations of the relationships between the covarying structural networks and cognitive performance.

The decision to create a summary measure of cognitive performance was done to reduce the number of statistical tests required, minimizing the likelihood of Type I errors. This was done with the understanding that schizophrenia is commonly characterized by a pattern of generalized cognitive impairment.

All patients in this study were taking some form of anti-psychotic medication.

CONCLUSION

This multimodal investigation of the correlated patterns of structural network covariance highlighted three unique networks of decreased gray matter volume and reduced white matter integrity in patients with schizophrenia, as well as a relationship between these networks and their diminished cognitive performance consistently across both subject groups. These networks show similarities with frontoparietal control networks and subnetworks of the DMN and could be the structural underpinnings for the well-established disruptions found in schizophrenia. These structural networks are also implicated in the cortical-subcortical-cerebellar circuit, the dysregulation of which is also associated with poor global cognitive performance.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://www.mrn.org/common/cobre-phase-3.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Georgia State University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DJ, EZ, VC, and JT contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Women in Neuromodulation: Innovative Contributions to Stereotactic and Functional Neurosurgery

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Stereotactic neurosurgery emerged in the mid-20th century following the development of a stereotactic frame by Spiegel and Wycis. Historically women were underrepresented in clinical and academic neurosurgery. There is still a significant deficit of female scientists in this field. This article aims to demonstrate the career and scientific work of some of the most important women who contributed to the development of stereotactic and functional neurosurgery. Exceptional women from all over the world, represented in this review, assisted the evolution of modern stereotactic and functional neurosurgery as neurosurgeons, neuropathologists, neurologists, neurophysiologists and occupational therapists. Fortunately, we could conclude that in the last two decades the number of female researchers has increased significantly.

Keywords: functional neurosurgery, neuromodulation, stereotactic surgery, deep brain stimulation, history, women

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INTRODUCTION

Stereotactic and functional and neurosurgery emerged towards the mid-20th century when Spiegel and Wycis first designed a stereotactic frame for use on the human brain (Spiegel et al., 1947). This frame was based on the apparatus developed for animal experiments by Horsley and Clarke (1908). The rationale to develop a human frame was to allow the creation of circumscribed lesions in deep brain structures in psychiatric patients in order to avoid the severe complications seen after the crude frontal lobotomies (Gildenberg, 2004; Rzesnitzek et al., 2019). This method initially labeled stereoencephalotomy and subsequently stereotactic surgery, was eventually implemented also in the treatment of patients with chronic pain, movement disorders, and epilepsy.

Historically and until the 1990s, female scientists were very rare in the fields of neurosurgery and functional neurosurgery, including neuromodulation, and they are still underrepresented in higher academic positions in these fields (Renfrow et al., 2018; Schaller, 2020). Several factors were detected contributing to gender inequality in neurosurgery (Venes and Parent, 2006; Abosch and Rutka, 2018). Women are still commonly identified as primary caretakers of the family; thus, it is difficult to adjust inflexible working hours of neurosurgical training and academic work environment to pregnancy and childcare. Women might also be discouraged by the lack of female leaders and role models in this field.

FIGURE 1 | Marion Smith, reproduced from Duchen (1989).

FIGURE 2 | Natalia Bechtereva, reproduced from Anonymus (2008).

Reports also have shown that women are less likely to have protected research time, an office or laboratory space, or receiving grant support (Venes and Parent, 2006; Abosch and Rutka, 2018). There are, however, few women who did contribute to the evolution of stereotactic and functional neurosurgery (Hariz et al., 2014). The aim of this article is to acknowledge and report the innovative work of female scientists to the development of this field.

Movement Disorders

Marion Smith (1915*–1988†), Neuropathologist

Marion Smith (**Figure 1**) was a neuropathologist and neuroanatomist born in Glasgow in 1915. She graduated in zoology before completing her medical studies at the Western Infirmary in Glasgow. She was trained in the field of neuropathology by Dr. Joseph G. Greenfield at the National Hospital for Nervous Diseases at Queen Square in London, where she continued to work until her retirement at the age of 65. She was a founding member and later president of the British Neuropathological Society (Duchen, 1989).

Her research contributed greatly to the anatomical and functional understanding of the brain and the spinal cord (Nathan and Smith, 1955, 1982; Smith and Deacon, 1984; Nathan et al., 1996). Her studies on post mortem brains of patients who had stereotactic lesioning provided a great insight into the effects and side-effects of the surgery. One of her most important contributions—in an era where there was no brain imaging available—was an analysis of thalamotomies and pallidotomies in 15 patients with Parkinson's disease, hemichorea, or Huntington's disease (Smith, 1962). She described in detail the location and size of the lesions and the involvement of different anatomical structures, and their correlation with the clinical outcome of the patients.

The clinicopathological correlations derived from her post mortem studies provided greatly to optimizing targets for future stereotactic surgeries.

Natalia Petrovna Bechtereva (1924*–2008†), Neurosurgeon

Natalia Bechtereva (**Figure 2**) was a neurophysiologist and neuroscientist born in Leningrad in 1924. She graduated from the Pavlov Institute of Physiology after graduating from the Pavlov First Leningrad Medical Institute and started her research career at the Polenov Institute of Neurosurgery in Leningrad in 1954 (Anonymus, 2008).

From the very beginning of stereotactic surgery, Spiegel and Wycis relied on intraoperative electrical stimulation in order to confirm the correct location of the electrode before performing lesioning (Gildenberg, 2005). Intraoperative acute stimulation continued to be part of the lesional procedures in the following decades (Blomstedt and Hariz, 2010). Natalia Bechtereva was the first to introduce a method for chronic electrical stimulation as a therapy for movement disorders and chronic pain

FIGURE 3 | Thanjavur Santhanakakrishna Kanaka, reproduced from Vilanilam et al. (2016).

(Blomstedt and Hariz, 2010; Hariz et al., 2014). Starting in the 1960s, she implanted gold electrodes for chronic external stimulation of different subcortical targets, including the ventrolateral thalamus and the striatopallidal complex, in some cases targeting multiple structures in the same patient (Bechtereva et al., 1975). She called her method therapeutic electrical stimulation (TES), and she used a current with "high-rate pulses" (Bechtereva et al., 1975), meaning a stimulation with high frequency. The electrodes remained implanted for up to 1.5 years with stimulation sessions conducted once or twice a week through an external stimulator. Her reports showed a significant improvement of the symptoms with few relevant side effects (Bechtereva et al., 1975).

As her first publications were in Russian, her work was not well known in the Western scientific community until her first publications in English appeared in 1975 (Blomstedt and Hariz, 2010; Hariz et al., 2014).

Thanjavur Santhanakrishna Kanaka (1932*–2018), Neurosurgeon

Thanjavur Santhanakakrishna Kanaka (**Figure 3**) was a neurosurgeon born in Chennai, India in 1932. She finished her medical studies in 1954 at the Madras Medical College where she continued specializing in neurosurgery and became the first female neurosurgeon of India in 1968 (Ganapathy and Barreto, 2021).

Under the guidance of Prof. B Ramamurthi she gained her Ph.D. degree researching stereotactic surgery for cerebral palsy and later joined the Madras Institute of Neurology which soon became the center of stereotactic surgery in India (Ozair et al., 2021). Within 15 years Dr. Kanaka and her colleagues performed more than 1,700 surgeries treating involuntary movements, behavioral disorders, psychiatric disorders, epilepsy, and spasticity (Ramesh et al., 2015). She also covered many pathologies in her work as a researcher. She studied thalamotomies and dentatectomies as well as the combination of different targets in patients with cerebral palsy, establishing the basis for symptom-oriented individualized treatment of the patients depending on their symptoms (Balasubramaniam et al., 1974). She also performed studies researching cingulotomy in drug addiction and hypothalamotomy in hyperkinetic behavioral disorders (Balasubramaniam et al., 1973; Kanaka and Balasubramaniam, 1978). Dr. Kanaka was also the first neurosurgeon who performed chronic deep brain stimulation in India (Ozair et al., 2021).

Dr. Kanaka faced great difficulties starting her carrier as a female surgeon in India. As a result of systematic discrimination against women, she required a number of attempts even to qualify for a residency program. During her residency, she was denied training by the chief resident and it also took several tries for her to pass the exit exam. To support female neurosurgeons across Asia she founded the Asian Women's Neurosurgical Association in 1996 together with Yoko Kato, the first professional female neurosurgeon in Japan (Ozair et al., 2021).

Hilda Molina, Neurosurgeon

Hilda Molina (**Figure 4**) was a neurosurgeon born in Cuba in 1943. She graduated from La Havana University in 1974, then specialized in neurosurgery, finishing her training in 1978 as the first female neurosurgeon in Cuba.

She later gained a Ph.D. degree studying multiple saccular aneurysms. She was founder of the Centro Internacional de Restauración Neurológica (CIREN) in Cuba, where she researched neurotransplantation in patients with Parkinson's disease. Studies performed in the 1980s on 1methyl-4-phenyl-1,2,3,6-tetrahydro-pyridine (MPTP) non-human primate models of Parkinson's disease, showed promising results after their treatment with fetal neural grafts (Redmond et al., 1986; Bankiewicz et al., 1990). In 1987, Molina and her colleagues implanted fetal mesencephalic tissue into the caudate nucleus using open surgery techniques (Molina et al., 1992). In 1992, they performed the first CT-guided stereotactic transplantation, implanting neural graft in the caudate nucleus and the putamen (Molina et al., 1994). They even used microelectrode recordings to confirm the right implantation location (Molina et al., 1994). Hilda Molina performed stereotactic thalamotomies in the nucleus ventralis intermedius also in patients who previously received neural grafts but continued to suffer from severe tremors (Quiñones-Molina et al., 1994). Dr. Molina retired in 1994 and lives now in Buenos Aires, Argentina.

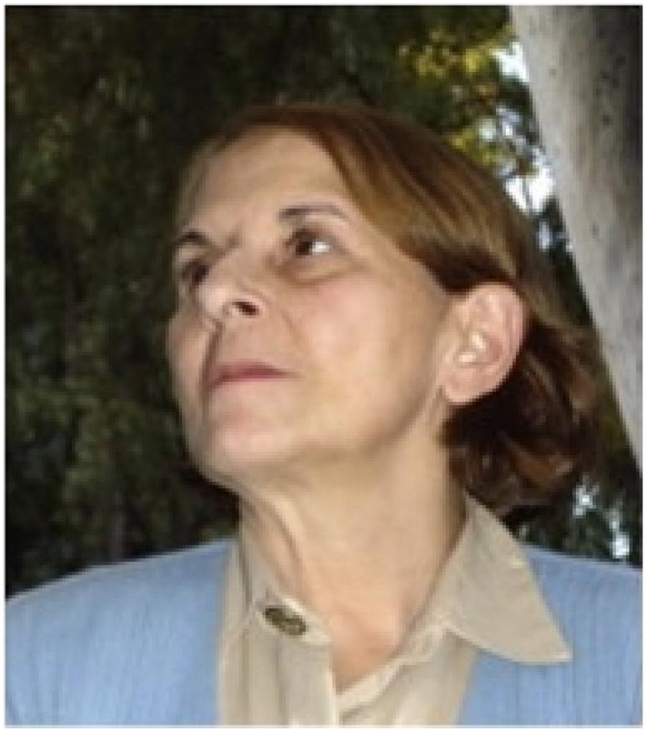

FIGURE 4 | Hilda Molina, reproduced from Hariz et al. (2014).

FIGURE 5 | Gun-Marie Hariz, provided by Gun-Marie Hariz.

Gun-Marie Hariz, Occupational Therapist

Gun-Marie Hariz (**Figure 5**) is an occupational therapist and researcher born in 1954 in Sweden. She obtained her certification in Stockholm in 1979. In 1999 she received a Master of Science and in 2002 a Ph.D. degree at Umeå University where she continued her work as a researcher.

Early on in her research career, she focused on the quality of life of patients with movement disorders, with particular interest in patients treated with DBS (Hariz et al., 1998). Being an occupational therapist, Gun-Marie Hariz has been interested in the concrete challenges met by patients in their activities of daily living, as reported by them. In this respect, she focused on patients' perception of their disease and its treatment (Hariz et al., 2011). For this, she has thoroughly documented the importance of applying qualitative investigations to illustrate the real difficulties met by the patients instead of using solely quantitative methods and scales. The insights obtained from her research have allowed clinicians to remain critical about the use of standardized scales as a unique tool to determine a positive clinical outcome. In addition, she has led the field on investigating social and individual aspects in patients with DBS that have traditionally been underestimated or ignored since the introduction of this therapy. Here, she has focused particularly on gender inequalities in access for persons with Parkinson's disease to surgical treatment (Georgiev et al., 2017; Sperens et al., 2017). Likewise, her research has opened the discussion on topics that are unfamiliar to traditional clinical research, although extremely relevant for patients, such as the perception of living with implanted devices and the experience of managing these systems (Hariz and Hamberg, 2014). These deep analyses of the perception of these patients provide powerful insights into the reality and impact of living with DBS, which should be considered by physicians and researchers at every moment of the treatment. In 2012, she was appointed Associate Professor of Occupational Therapy at the Department of Community Medicine and Rehabilitation at the Umeå University, and a senior researcher at the Department of Clinical Neuroscience, where she currently holds this position.

Patricia Limousin, Neurologist

Patricia Limousin (**Figure 6**) is a neurologist born in 1965. She received a Master of Science degree in 1991 and after completing her specialization in neurology in 1993 at the University of Grenoble she joined the research team of Benabid and Pollak.

In the late 1980s, Alim Louis Benabid and Pierre Pollak in Grenoble, France, initiated the modern era of deep brain stimulation (DBS) using eventually a fully implantable system (Benabid et al., 1987). They started by targeting the ventro-intermediate (VIM) nucleus of the thalamus for patients with Parkinsonian or essential tremor (Benabid et al., 1987, 1991), showing a very good effect on the tremor. However, VIM DBS did not provide relief from other symptoms of advanced Parkinson's disease, such as bradykinesia, dyskinesias, and gait abnormalities (Limousin and Martinez-Torres, 2008).

Based on studies of the role of the subthalamic nucleus (STN) in animal models of Parkinson's disease (Bergman et al., 1990),

FIGURE 6 | Patricia Limousin, provided by Patricia Limousin.

FIGURE 7 | Carine Karachi, provided by Carine Karachi.

Dr. Limousin and her colleagues introduced the STN as a new DBS target in Parkinson's disease showing in a seminal article on bilateral DBS of the STN, published in The Lancet in 1995, that this procedure also improved bradykinesia and rigor significantly (Limousin et al., 1995). In the following years, STN DBS became the most common target used worldwide for DBS in Parkinson's disease (Limousin and Martinez-Torres, 2008).

Dr. Limousin proceeded to obtain her Ph.D. in neuroscience at the University of Lyon, continuing her pioneering work on the study of STN DBS. In the late 1990s, she moved to work at the Institute of Neurology at Queen Square in London where she eventually contributed to the establishment of a Unit of Functional Neurosurgery. Currently, she is a Professor of Clinical Neurology and Consultant Neurologist at the National Hospital for Neurology and Neurosurgery and at University College London.

Carine Karachi, Neurosurgeon

Carine Karachi (**Figure 7**) is a French neurosurgeon and neuroscientist born in 1973. After finishing her medical training in Paris, she defended a Ph.D. thesis on the function and anatomy of the basal ganglia in 2006. She incorporated the insights obtained during laboratory research into the clinical field. This is exemplified by her composite formation, which includes a postdoctoral fellowship in basic sciences at Columbia University in New York and neurosurgical training at the Pitié-Salpêtrière Hospital in Paris.

Her main focus in the field of DBS is on the pathophysiology of gait and balance disorders in patients with movement disorders, in particular on the role of the pedunculopontine nucleus (PPN) and its interaction with other network structures involved in locomotion. Her extensive contribution in this area has increased our understanding of the complex role of cholinergic neurons located in this part of the brainstem and their involvement in posture and locomotion in Parkinson's disease (Karachi et al., 2010; Grabli et al., 2013). Moreover, these insights have contributed to the treatment of these disturbances with DBS of the PPN. Currently, Carine Karachi (who was nominated Professor in 2019) combines her dual expertise working as a neurosurgeon at the Pitié-Salpêtrière Hospital and as a lead researcher at the Institut du Cerveau et de la Moelle Èpinière (Brain and Spine Institute) in Paris.

Laura Cif, Neurologist

Laura Cif (**Figure 8**) is a neurologist born in 1975. She completed her specialist education in 2000 at the University of Montpellier, then pursued a master's degree in neurosciences. In the following years, she continued her education at the University of Pierre and Marie Curie in Paris and at the University of Lille, then returned to Montpellier to complete her Ph.D. degree in 2011. Currently, she is an associate professor and consultant in movement disorders neurologist at the Unit of Functional Neurosurgery at the University Hospital Gui de Chauliac in Montpellier.

FIGURE 8 | Laura Cif, provided by Laura Cif.

FIGURE 9 | Gunvor Kullberg, reproduced from Hariz et al. (2014).

Her main research focus is on DBS in patients with various types of dystonia, especially on pediatric dystonia. DBS of the posteroventral internal globus pallidus (GPi) as a treatment for generalized dystonia was first introduced by Professor Philippe Coubes, a pediatric neurosurgeon in Montpellier (Coubes et al., 2000; Cif and Hariz, 2017). Joining his team, Dr. Cif analyzed the response to DBS in numerous subgroups of isolated and acquired dystonia (Castelnau et al., 2005; Cif et al., 2013, 2014; Cif and Coubes, 2017). The differences in response to DBS observed between individual cohorts contributed immensely to the indications and selection of patients for DBS, and to the various strategies in programming the stimulation, providing thus guidelines towards setting realistic expectations about the treatment.

Neuropsychiatric Surgery

Gunvor Kullberg, Neurosurgeon

Gunvor Kullberg (**Figure 9**) is a Swedish neurosurgeon born in 1927. She started her career in 1955 as a psychiatrist at the University Hospital of Lund in Sweden. Two years later, an opportunity came to work at the Newcastle General Hospital in the UK. Here, after being exposed for the first time to the field of functional neurosurgery, she developed a significant interest in this discipline. As a consequence, she changed her specialization and completed her training as a neurosurgeon under the supervision of George Frederick Rowbotham. In 1960, she returned to the University Hospital of Lund where she conducted a wide array of research projects, involving stereotactic psychosurgery, the role of corticosteroids in postoperative brain edema, and studies using a then relatively new imaging method, the CT scan.

Using the 133Xe inhalation method she studied cerebral blood flow after stereotactic lesioning in psychiatric and Parkinson's patients (Kullberg and Risberg, 1978, 1985). She demonstrated a decrease in cerebral blood flow in the prefrontal cortex following capsulotomy and frontobasal tractotomy and argued that this could indicate a lower prefrontal neural activity as a consequence of disruption in the neural pathways (Kullberg and Risberg, 1985). She also studied the evolution of stereotactic lesions using the CT scan and showed that corticosteroids could reduce the size of the surrounding edema (Kullberg et al., 1980; Cronqvist and Kullberg, 1983). Dr. Kullberg retired in the early 1990s and currently lives in Lund.

Helen S. Mayberg, Neurologist

Helen S. Mayberg (**Figure 10**) is a neurologist born in 1956. She received a Bachelor of Arts degree in psychobiology from the University of California, Los Angeles at the age of 20 and a Medical Doctor degree 5 years later from the University of Southern California. After this, she obtained her certification as a neurologist from Columbia University in New York and a research fellowship in Nuclear Medicine at Johns Hopkins University. Among her multiple honors outstand her election as a member of the National Academy of Medicine of the United States of America, the American Academy of Arts and Sciences, and the National Academy of Inventors of the USA. Currently, she is Director and Professor at The Center of Advanced Circuit Therapeutics at the Mount Sinai Hospital in New York.

Her most influential work comprises the areas of functional neuroimaging and psychiatric disorders, in particular the pathophysiology of major depression. Prof. Mayberg has not only comprehensively examined the functional abnormalities

FIGURE 10 | Helene Mayberg, provided by Helene Mayberg.

of this disorder but also documented the mechanisms of antidepressant treatments in order to predict better clinical outcomes. These contributions led to the development of a novel target for DBS in patients with treatment-resistant depression, Brodmann area 25 also known as Cingulum 25 (Cg25), similar to the original target for subgenual cingulotomy performed by Lauri Laitinen between the 1950s and 1970s in Finland for various psychiatric disorders (Huotarinen et al., 2018). Together with Andres Lozano she pioneered DBS in the subgenual cingulate region in six patients with refractory depression (Mayberg et al., 2005). In this seminal study Mayberg and colleagues reported on changes in local cerebral blood flow in PET scans with reduced activity in the Cg25 and hypothalamus, as well as increased activity in prefrontal cortex and brainstem during DBS. These findings were equivalent to the metabolic changes observed after the administration of antidepressant medication (Mayberg et al., 2000). Four of the six patients in the pilot trial showed a significant reduction of the depressive symptomatology with unchanged medication after 6 months of DBS. Since then, several studies have indicated that Cg25 DBS is an effective and safe treatment for patients with treatment-resistant depression (Puigdemont et al., 2015; Merkl et al., 2018). Nevertheless, this therapy still remains experimental pending enrollment of larger samples of patients. Prof. Mayberg continues her multidisciplinary research in the fields of neurology, psychiatry, neurosurgery, imaging, and neuroscience as acting Director of The Nash Family Center for Advanced Circuit Therapeutics at the Icahn School of Medicine at Mount Sinai.

Veerle Visser-Vandewalle, Neurosurgeon

Veerle Visser-Vandewalle (**Figure 11**) is a neurosurgeon born in 1964. She completed her training in neurosurgery at the AZ St-Jans Hospital, in Bruges and the University Hospital of Ghent in Belgium between 1989 and 1996, where she performed her first DBS procedures in movement disorders.

FIGURE 11 | Veerle Visser-Vandewalle, provided by Veerle Visser-Vandewalle.

In 1999, DBS was introduced in the field of neuropsychiatric disorders by Dr. Visser-Vandewalle and colleagues in Ghent, Belgium (Vandewalle et al., 1999). In this pioneering work, a 42-year old patient with intractable Gilles de la Tourette syndrome (GTS) received thalamic DBS electrodes spanning the centromedian nucleus, the nucleus ventro oralis internus and the substantia periventricular. The targeting procedure was based on the thalamic stereotactic lesions in GTS patients performed by Hassler and Dieckmann in the 70s (Hassler and Dieckmann, 1970). Postoperatively, the patient showed a significant reduction of tics, as well as a decrease in comorbid self-injurious behavior. Since then, numerous studies have replicated these positive findings in GTS patients after stimulation of thalamic targets and of other subcortical areas.

She continued her work in DBS and functional neurosurgery at the Maastricht University Medical Center in the Netherlands for 13 years. During this time, she obtained a Ph.D. degree with a dissertation about her work in DBS and GTS. She was also named full professor in Functional Neurosurgery in 2007. In 2012, she was appointed as Head of the Department of Stereotactic and Functional Neurosurgery at the University Hospital of Cologne in Germany, where she currently holds this position.

FIGURE 12 | (A) Denise Albe-Fessard, reproduced from Hariz et al. (2014). **(B)** Ana Luisa Velasco, provided by Ana Luisa Velasco.

Neurophysiology

Denise Albe-Fessard (1916*–2003†), Neurophysiologist

Densise Albe-Fessard (**Figure 12A**) was a neurophysiologist born in 1916. She graduated from the School of Physics and Chemistry in Paris with an Engineering degree in 1937 (Albe-Fessard, 1998). In 1950 she obtained a Ph.D. degree from the University of Paris. Her first experiments in the field of neurophysiology were performed in Paris and then shortly in Bordeaux during the Second World War.

The use of intracerebral microrecordings is nowadays a worldwide common practice during functional stereotactic procedures. This technique provides precise information about the position of the electrode and ultimately allows to differentiate between different subcortical structures. The pioneering work that led to the development of intraoperative microrecording as we know it was performed by Denise Albe-Fessard.

After the Second World War, she continued her work at the French National Centre for Scientific Research (CNRS) where she studied neurophysiology in electric fish. After learning and perfecting the method to make glass-electrodes, she applied this technique to the large cells of the cat somatomotor cortex. She continued her work in primates after being appointed as faculty at the Sorbonne University Pierre and Marie Curie in Paris in the 1950s, where she mainly focused on the thalamic pathways. These insights allowed her to perform the first microrecordings in humans in the beginning of the 1960s in collaboration with the French neurosurgeon Gerard Guiot at the Hospital Foch in Suresnes near Paris. During these procedures, she documented the presence of rhythmic thalamic activity that was associated with the tremor frequency (Guiot et al., 1962; Albe-Fessard et al., 1967), and she advocated intraoperative stimulation at high frequency to stop the tremor. Her work extended to the delineation of several subcortical networks, in particular, movement disorders and pain pathways through thalamic pain processing. These achievements led her to be elected the first president of the International Association for the Study of Pain (IASP) in 1975. She was the first woman to receive the Spiegel-Wycis Award in 1989 and to be awarded the distinction as Knight of the Legion of Honor and Officer of the Order of Merit by the French government. Her vast legacy in the field of neurophysiology has been continued by her multiple students and colleagues worldwide that currently lead the field of modern neuromodulation.

FIGURE 13 | (A) Zelma Kiss, provided by Zelma Kiss. **(B)** Jocelyn Bloch, provided by Jocelyn Bloch.

Ana Luisa Velasco, Neurologist

Ana Luisa Velasco (**Figure 12B**) is a Mexican neurologist and neurophysiologist born in 1962. She obtained a Medical Doctor degree from the National Autonomous University of Mexico (UNAM) in 1988. After this, she completed her Neurology Training at Mexico General Hospital in 1991. During this time, she started working in the field of neurophysiology and epilepsy and developed a personal interest in these areas.

Her board certification thesis focused on EEG and MRI abnormalities in children with Lennox-Gastaut syndrome and epilepsia partialis continua (Velasco et al., 1993). At this early point of her career, she started working with two of her most important mentors, Marcos Velasco and Francisco Velasco. The former, her father, was himself a neurophysiologist specialized in the study of epilepsy, and the latter, her uncle, a neurosurgeon dedicated to the field of functional neurosurgery. From this singular combination of expertise, she worked with them on electrical stimulation of the thalamus to treat patients with severe refractory seizures. Eventually, she received the prestigious Fogarty International Research Fellowship and spent 2 years at the Brain Research Institute at the University of California, Los Angeles under the tutorship of Jerome Engel Jr. and Charles Wilson. Here, she had the opportunity to study epilepsy from different angles including neuropsychology, neuroimaging, clinical, surgical, and research techniques. Following her return to Mexico, she obtained a Ph.D. degree in biomedical sciences and obtained her certification as a Neurophysiologist. At this point, she founded the Epilepsy Clinic at the Mexico General Hospital, where she worked as Head of the Clinic. During the coming years, she devoted herself to the study of the modulation of the brain through electrical stimulation of different cerebral targets to control refractory seizures. This resulted in diverse seminal publications about the chronic stimulation of the hippocampus and the centromedian thalamic nuclei in the treatment of epilepsy (Velasco et al., 2006, 2007a,b). Among her multiple honors are her election as a member of the Mexican Academy of Surgery in 2010 and the Mexican National Academy of Medicine in 2013. In 2019, she was appointed Head of the Department of Neurology at the Mexico General Hospital in Mexico City, where she still holds this position and continues her clinical and academic activities in the field of neuromodulation.

Neurotechnology

Zelma Kiss, Neurosurgeon

Zelma Kiss (**Figure 13A**) is a neurosurgeon born in 1964. She graduated from the medical faculty of the University of Ottawa,

FIGURE 14 | (A) Mojgan Hodaie, provided by Mojgan Hodaie. **(B)** Andrea Kühn, provided by Andrea Kühn.

Canada at the age of 24. She completed her neurosurgical training at the University of Toronto, where she also received her Ph.D. After winning the Van Wagenen fellowship, she had the opportunity to continue her postdoctoral education under the supervision of Alim Louis Benabid in Grenoble, France. In 2000, she was appointed at the University of Calgary, where she is currently an associate professor of the Department of Clinical Neurosciences and is the Head of the Neuromodulation program of southern Alberta.

The main research interest of Dr. Kiss is the therapeutic mechanism of DBS in different brain areas. Using rodent models, she has thoroughly analyzed the membrane depolarization after thalamic stimulation, concluding that DBS operates through disruption of the rhythmic firing of the tremor cells (Kiss et al., 2002). Likewise, she has been largely involved in the study of the mechanism of action during globus pallidus internus DBS. Particularly, on the question of why pallidal DBS shows an almost immediate effect in Parkinson's patients, while for dystonia it can take weeks or months of stimulation for patients to profit fully from chronic stimulation (Hristova et al., 2000; Coubes et al., 2004). In this respect, she has studied the entopenduncular nucleus in rats during stimulation, a representative for the human globus pallidus, while recording local field potentials in the motor cortex, the striatum, and the ventroanterior thalamus (McCracken and Kiss, 2014). The results from these experiments showed that local field potentials changed over time under continuous DBS, demonstrating how the therapeutic effects of DBS could gradually develop differently after its chronic usage. Her research has immensely contributed to understanding the basic principles of DBS and how this therapy is applied in humans. As a co-editor to "Brain Stimulation" she was the first editor to publish recently an editorial criticizing the lower battery capacity found in new versions of DBS hardware and she drew attention to the need to report this to government approval bodies (Kiss and Hariz, 2019).

Jocelyn Bloch, Neurosurgeon

Jocelyn Bloch (**Figure 13B**) is the Head of the Stereotactic and Functional Neurosurgery team at the University Hospital in Lausanne, Switzerland. She graduated from the medical faculty at the University of Lausanne in 1994 and completed her neurosurgical training in Lausanne and Zurich.

After this, she specialized in functional neurosurgery. From 1997 to 1999, she joined the team of Prof. Patrick Aebischer where she studied gene therapy and translational sciences, which contributed considerably to her later projects. She is vice-president of the European Society for Stereotactic and Functional Neurosurgery.

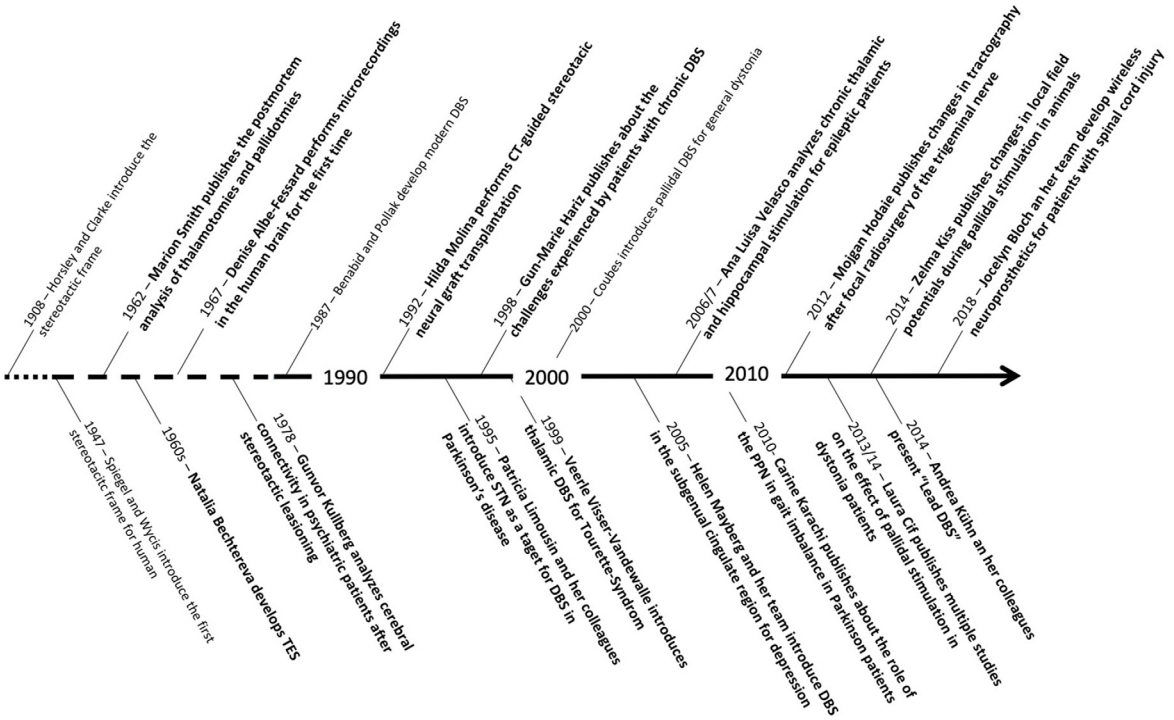

FIGURE 15 | Timeline showing some of the most important scientific contributions of the women mentioned in this review in relation to other major scientific advances in the field of stereotactic and functional neurosurgery.

Together with Jean-Françoise Brunet, she studied the effect of autologous transplantation of adult brain cells in primates after injury to the motor cortex, as well as in monkeys treated with MPTP, modeling Parkinson's disease (Kaeser et al., 2011; Bloch et al., 2014). The promising results obtained from these studies offer a good alternative to other sources of neural transplantation, such as fetal stem cells or genetically modified pluripotent adult somatic cells.

Dr. Bloch has also been actively involved in the field of neuroprosthetics in patients with spinal cord injury. Using microelectrode arrays in the motor cortex and epidural electrical stimulation of the lumbar and the cervical spine, her team conducted ground-breaking research on this topic first in primates (Capogrosse et al., 2016; Barra et al., 2018). Based on the spatiotemporal pattern of motor neuron activation in the lumbar spine during movements and the matching cortical activity in the motor area, they developed a wireless system with a decoder that creates a neural bridge between both areas. After sufficient calibration of the system, they inflicted a lesion in the corticospinal tracts. With the help of brain-controlled sub-lesion stimulation, the monkeys could regain their walking abilities shortly after the surgery (Capogrosse et al., 2016) Based on these studies, in 2018 they developed a wireless, voice-controlled lumbar epidural stimulation system for patients with spinal cord injury (Wagner et al., 2018). With the help of epidural electrical stimulation and months of rehabilitation with the implant, they could significantly improve the patients' ability to walk and stand, and they even showed improvement off stimulation. The innovative work of Dr. Bloch raises hope for patients with spinal cord injury and provides a basis for new applications of functional neurosurgery.

Neuroimaging

Mojgan Hodaie, Neurosurgeon

Mojgan Hodaie (**Figure 14A**) is a neurosurgeon at the Toronto Western Hospital and Professor at the Department of Surgery at the University of Toronto and Institute of Medical Sciences in Canada. She obtained her Medical Doctor degree from Queen's University at Kingston, Canada in 1996. After this, Mojgan Hodaie completed her Neurosurgery Training in 2003 and a Fellowship in Stereotactic and Functional Neurosurgery in 2004 at the University of Toronto. As an established neurosurgeon, she focused on the management of several functional neurological disorders, particularly in the surgical treatment of trigeminal neuralgia and facial pain (Hodaie et al., 2012). Her contributions to this field combine neuroimaging and radiosurgery. She has investigated brain abnormalities in sensory and motor areas using tractography to visualize these microstructural neuroanatomical changes (Hodaie et al., 2010; Desouza et al., 2013). Hodaie and colleagues have observed gray matter abnormalities in patients with trigeminal neuralgia in anatomical structures involved in pain perception and modulation. These morphometric analyses have shown increased volumes in amygdala, sensory nuclei of the thalamus, periaqueductal gray and basal ganglia, as well as higher cortical thickness in the primary somatosensory cortex and frontal lobes

in patients with trigeminal neuralgia when compared to healthy subjects (Desouza et al., 2013). These methods could be used to identify biomarkers in pain syndromes and eventually help to adjust therapies or indicate surgical procedures.

Mojgan Hodaie has devoted herself to distance teaching of functional neurosurgery especially in developing countries (Blankstein et al., 2011). She founded the NEURON (Neurosurgical Education with Universal Reach Online) project, which has implemented online courses in the field of neurosurgery around the world, and she is a member of the Foundation of International Education in Neurological Surgery (FIENS). Her contributions to the academic and scientific fields have resulted in multiple honors and awards, including the Grand Cross of the Legion of Honor of Monisaraphon by the Kingdom of Cambodia. She was also elected as an officer of the Board of Directors of the World Society for Stereotactic and Functional Neurosurgery (WSSFN).

Andrea Kühn, Neurologist

Andrea Kühn (**Figure 14B**) is a neurologist and neuroscientist born in 1970. She studied medicine at the Charité and at the Henri Poincaré University in Nancy, France. After obtaining her medical degree, she worked as a postdoctoral researcher at University College of London under the mentorship of Peter Brown from 2002 till 2007, where her main focus was studying local field potentials in the STN in Parkinson's patients (Doyle et al., 2005; Kühn et al., 2005, 2006; Williams et al., 2005).

In 2010, upon her return to Berlin, she worked as a neurologist at the University Hospital Charité where she became head of the Movement Disorders and Neuromodulation Unit. Her primary research research interest became pathological oscillatory activity in patients with movement disorders under deep brain stimulation (Barrow et al., 2014; Neumann et al., 2017, 2019), which later enabled the development of closed loop stimulation. She also accomplished ground breaking work in the field of neuroimaging. Together with Andreas Horn, she presented "Lead-DBS" in 2014, an open-access toolbox for localization and visualization of DBS electrodes (Horn and Kühn, 2015). With the help of postoperative MRI and CT imaging, this tool allows semiautomated reconstruction of the DBS electrodes and the position of their contacts within the targeted regions. This software can assist clinicians and researchers to generate a virtual reconstruction of the stimulated structures (Horn et al., 2017).

Conclusion

In this review, we summarized the professional achievements of 16 exceptional women working in the field of neuromodulation and functional neurosurgery. Although they are from different backgrounds, including neuropathology, neurophysiology, neurology, and neurosurgery, they all contributed to the development and evolution of stereotactic and functional neurosurgery as we know it today. Fortunately, this summary shows that in the last two decades an increasing number of women have joined these disciplines and many of them are currently in high-ranking academic positions (**Figure 15**). Without a doubt, their scientific and medical contributions will inspire future generations of researchers, both women and men, to participate in the development of stereotactic and functional neurosurgery.

AUTHOR CONTRIBUTIONS

Conceptualization, visualization, and investigation: PH and PA. Methods, writing—original draft, and writing—review and editing: PH, JP, and PA. All authors contributed to the article and approved the submitted version.

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CORRECTION
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Corrigendum: Women in Neuromodulation: Innovative Contributions to Stereotactic and Functional Neurosurgery

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In the original article, there was errors regarding the biographical data of "Helen S. Mayberg, Zelma Kiss and Andrea Kühn."

A correction has been made to the section **Neuropsychiatric Surgery**, **Helen S. Mayberg, Neurologist**, paragraph 1:

Helen S. Mayberg (Figure 10) is a neurologist born in 1956. She received a Bachelor of Arts degree in psychobiology from the University of California, Los Angeles at the age of 20 and a Medical Doctor degree 5 years later from the University of Southern California. After this, she obtained her certification as a neurologist from Columbia University in New York and a research fellowship in Nuclear Medicine at Johns Hopkins University. Among her multiple honors outstand her election as a member of the National Academy of Medicine of the United States of America, the American Academy of Arts and Sciences, and the National Academy of Inventors of the USA. Currently, she is Director and Professor at The Center of Advanced Circuit Therapeutics at the Mount Sinai Hospital in New York.

A correction has been made to the section **Neurotechnology**, **Zelma Kiss, Neurosurgeon**, paragraph 1:

Zelma Kiss (Figure 13A) is a neurosurgeon born in 1964. She graduated from the medical faculty of the University of Ottawa, Canada at the age of 24. She completed her neurosurgical training at the University of Toronto, where she also received her Ph.D. After winning the Van Wagenen fellowship, she had the opportunity to continue her postdoctoral education under the supervision of Alim Louis Benabid in Grenoble, France. In 2000, she was appointed at the University of Calgary, where she is currently an associate professor of the Department of Clinical Neurosciences and is the Head of the Neuromodulation program of southern Alberta.

A correction has been made to the section **Neuroimaging**, **Andrea Kühn, Neurologist**, paragraph 2:

In 2010, upon her return to Berlin, she worked as a neurologist at the University Hospital Charité where she became head of the Movement Disorders and Neuromodulation Unit. Her primary

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research interest became pathological oscillatory activity in patients with movement disorders under deep brain stimulation (Barrow et al., 2014; Neumann et al., 2017, 2019), which later enabled the development of closed loop stimulation. She also accomplished groundbreaking work in the field of neuroimaging. Together with Andreas Horn, she presented "Lead-DBS" in 2014, an open-access toolbox for localization and visualization of DBS electrodes (Horn and Kühn, 2015). With the help of postoperative MRI and CT imaging, this tool allows semiautomated reconstruction of the DBS electrodes and the position of their contacts within the targeted regions. This software can assist clinicians and researchers to generate a virtual reconstruction of the stimulated structures (Horn et al., 2017).

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Why Are We Scientists? Drawing Inspiration From Rita Levi-Montalcini

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In 2007, drawing inspiration from her previous experiments on chick embryos, Rita Levi-Montalcini, at the age of 98, proposed a new project, and a research group, in which I was included, was formed at the European Brain Research Institute (EBRI). Looking back on this experience, I can say that Professor Levi-Montalcini's approach and the relationships she formed with my colleagues and me, contributed to my growth as a researcher. With her welcoming and warm-hearted disposition, she taught me how to consider other people's ideas without prejudice, to reason and not to exclude any hypothesis. I also learned from her how to overcome those difficulties that are so frequent in the research field, always keeping in mind the starting point and looking toward the objective, with a factual optimism. I was just a young researcher and deeply flattered that a Nobel Laureate, with an incredible career and extraordinary life, treated me as her equal. My experience with Professor Levi-Montalcini has also provided me with a reliable path to follow, and when I encounter difficulties and challenges, I ask myself what would she have done. This approach has always helped me to move forward. Indeed, I believe the best way to celebrate Rita Levi-Montalcini as a woman in neuroscience is to recount how her exceptional example is a constant reminder as to why I have chosen to be a scientist. I hope she will always continue to be a source of inspiration for scientists in the future.

Keywords: Rita Levi-Montalcini, NGF, neurotrophin, neuroscience, women in science

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INTRODUCTION

Rita Levi-Montalcini was born in 1909 in Turin and her life was both long and extraordinary.

When I visit schools to tell young students about Rita Levi-Montalcini, I always start by repeating the story described in the first few pages of her autobiography (Levi-Montalcini, 1988) that, in my opinion, represents a defining moment in her life. When Rita played in the park as a child, her friends used to ask her two questions: what does your father do? What is your religion? Rita had no difficulty in answering the first question: her father was an engineer, but, having been raised in a non-religious home, she was not able to respond to the second question. When she asked her father what she should say when her friends inquired about her religion, he replied: "You tell them that you are a free thinker."

Subsequently, Rita Levi-Montalcini remained a lifelong free thinker, something of which she has given us countless examples. As a young girl, she decided to become a doctor following the premature death of her childhood nanny, against the wishes of her family. Following her expulsion from university due to the Fascist racial laws, she set up a small laboratory in her bedroom, so as not to interrupt her research. She was forced to publish through Vatican and Belgian scientific journals

since Jews were not allowed to publish in other journals. Ultimately, she demonstrated that she was a free thinker upon leaving Italy (as a lone woman, in 1946) to continue her research in the United States, and when she subsequently conceived the "theory of neurotrophins," despite it being against the current flow of ideas on nervous system development.

I have listed only a few examples here, since her biography and the story of NGF discovery are well known and described in the books "In Praise of Imperfection," her autobiography (Levi-Montalcini, 1988) and "The Saga of Nerve Growth Factor" (Levi-montalcini, 1997). Another fascinating book, "Cantico Di Una Vita" (Levi-Montalcini, 2000), tells the story of NGF discovery by means of the letters that Rita wrote daily to her mother and twin sister (and latterly to her nephew).

In 1969, Rita Levi-Montalcini returned to Italy to manage the Centre of Neurobiology of the National Research Council (Rome), but continued to "commute" between the United States and Italy in order to monitor her laboratory at Washington University. She was awarded the 1986 Nobel Prize in Physiology or Medicine jointly with Stanley Cohen, and in 2001, she was nominated Senator-for-life by Italian President Carlo Azeglio Ciampi.

However, Rita Levi-Montalcini's story did not simply stop following these and other prestigious awards, nor would her sense of political and social commitment be satisfied by merely monitoring experiments in the laboratory. In September 2001, at the age of 92, she attended to the "Forum Ambrosetti," an annual meeting of industrial leaders, to present the idea of establishing a Brain Research Institute in Italy. Professor Levi-Montalcini obtained the financial support, and in 2002, together with her collaborator Pietro Calissano, she founded EBRI. The Institute became operational in 2005, through the recruitment of Italian and foreign scientists, chosen by the international scientific Committee.

It was common to happen upon Professor Rita Levi-Montalcini, always dressed in the most elegant of outfits, when passing through the halls of the Institute located on the outskirts of Rome. Scientific life at EBRI was very lively, with meetings and seminars, often held by international scientists (**Figure 1**). It was in this period that I won a fellowship at EBRI.

In this article, I will describe Levi-Montalcini's last research project, how she formulated the idea, drawing inspiration from her previous experiments on chick embryos, and how she followed the experiments. Alongside the main project, I also will describe some of her other scientific insights and ideas she conceived about "the vital role of NGF." I will share some significant episodes during her daily working life and describe the relationship she had with her collaborators, her welcoming and warm disposition and her curiosity regarding all aspects of science and research. Moreover, I would like to describe her strong commitment to promoting the role of women in education and science, and her continuous work within science dissemination, in particular with young people and students.

Since much has been written about Rita Levi-Montalcini, and her biography and scientific career are well known to the scientific community, my contribution will be to describe the final part of her life, based on my direct experience. For this reason, I will not mention her other collaborators, who were not involved in the projects I will describe, and with whom I did not interact.

FIGURE 1 | Rita Levi-Montalcini during a seminar at EBRI [Photographer Federico La Regina (EBRI) from EBRI Media Archive].

EMBRYO CHICK PROJECT

In 2007, following the summer holidays, Rita Levi-Montalcini returned to EBRI with a brand new idea. Recalling her previous experiments on chick embryos, she wondered why NGF and its receptors are expressed in embryos many days before the nervous system is actually formed. She was sure that NGF must perform other functions in early chicken embryo development, besides its well-established actions on the developing sympathetic and sensory neurons, because in nature nothing is left to chance.

One might wonder how and when a 98 years old scientist, president of a fledgling research Institute, with an institutional engagement, found the time and energy to dream up a totally new project that was not in line with any research program developed in the institute.

She used to say: "Sleeping is a waste of time at my age. During the night I think" and she did not give up on "sniffing out other truffles" . . .

"I think there are few things in the world as delightful as giving birth to new ideas and nurturing them. (. . .) this is one of the aspects of my work that I find captivating, a bit like a truffle dog searching for truffles, even if they are not to be found, the smell in the air is very exhilarating. I believe I have a very good sense of smell and. . . I hope to sniff out a few more truffles" (Levi-Montalcini, 2000).

She discussed her idea with Antonino Cattaneo and considering that little was known about the actions of NGF during early embryonic stages, they decided to form a new research team. Their strategy was to block the NGF action by means of a well-validated monoclonal antibody [anti-NGF mAb αD11 (Cattaneo et al., 1988)], able to bind mature NGF with high affinity, in an earlier stage of chicken embryo development with respect to that of the nervous system, precisely at HH 11–12,

according to the Hamburger-Hamilton classification (Hamburger and Hamilton, 1992). Indeed, at the embryonic stage HH 20 and HH 33–40, NGF is required for the development and maintenance of a specific population of peripheral sympathetic and sensory neurons (Hamburger and Hamilton, 1992), while NGF mRNA expression was detected initially at HH 3–5, reaching a peak at HH 33–34 (Ebendal and Persson, 1988; Baig and Khan, 1996).

The approach of "destroying" NGF effects by an anti-NGF blocking antibody recalls Rita Levi-Montalcini's seminal immune-sympathectomy experiment: the destruction of sympathetic ganglia in mice by way of an injection of an anti-NGF antiserum (Levi-Montalcini and Booker, 1960). Immunosympathectomy, published in 1960, was recently defined as a sort of knockout *ante litteram* (Cattaneo, 2013).

In 2007, two different NGF knock-out mice were available. The "B6.129S7-Ngftm1Gne/J," also called NGF KO, is a genetic knock-out and exhibits a short life span (about 4 weeks after birth) with delayed development and cell loss in the sympathetic ganglia (Crowley et al., 1994). Another NGF knock-out mouse, named AD11, was developed by Cattaneo's team and was therefore available at EBRI. AD11 is a phenotypic NGF knock-out, since it was achieved by expressing the transgenic anti-NGF antibody αD11. The AD11 mouse is vital, and shows a progressive neurodegenerative phenotype resembling Alzheimer's disease (Capsoni et al., 2000).

On the other hand, there are no knock-out chickens in existence, therefore, in chickens the only way to observe the consequences of NGF deprivation, in order to understand the related NGF function, was to block the NGF action through the use of antibodies. In Rita Levi-Montalcini's opinion, the antibody strategy was not only the means to overcome the absence of knock-out models, but also an occasion to observe the NGF effect in different stages of embryonic development, choosing a precise time-window for the blocking of NGF. Indeed, she argued that, while the transgenic knockout gives global cumulative effects, the antibody interfering approach, that she had pioneered, allows for a much-fixed temporal regulation. A conditional NGF knock-out was indeed not available at the time.

Once the project had been designed, Antonino Cattaneo called upon some of the researchers, already working within the context of EBRI. Annalisa Manca and Anna di Luzio were involved in embryo injections, Simona Capsoni in histology, with the technical help of Domenico Vignone, and Francesca Paoletti and myself in the carrying out of biochemical experiments. As the research progressed, the project had need of further expertise and more people were involved, but we were the initial nucleus of Rita Levi-Montalcini's first group at EBRI (**Figure 2**).

I remember very clearly the first time she spoke to me. I was embarrassed and I felt completely inadequate standing there in front of her. However, those feelings disappeared after a while, when she began to question me, not about the experiments I was carrying out, nor my scientific curriculum, but about my family and where I grew up. When she heard that I came from Puglia, she told me that, as a child, she had lived in Bari due to one of her father's job. I was immediately struck by her sweet smile and the sincere interest she showed in me.

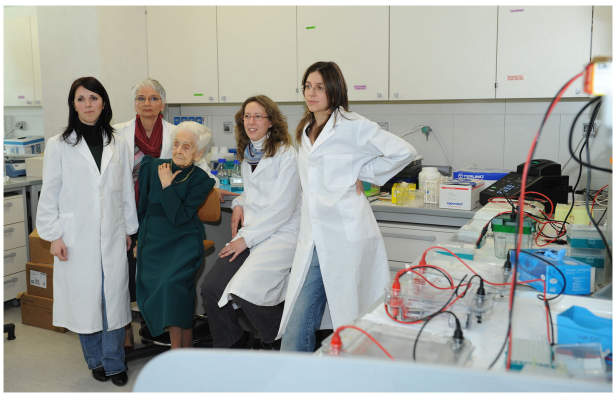

FIGURE 2 | Rita Levi-Montalcini in the laboratory with her group. From left to right: Annalisa Manca, Anna Di Luzio, Francesca Paoletti and Francesca Malerba [Photographer Maurizio Riccardi (Agr Press) from EBRI Media Archive].

FIGURE 3 | Annalisa Manca and Rita Levi-Montalcini close to the microscope with two oculars [Photographer Maurizio Riccardi (Agr Press) from EBRI Media Archive].

My role in the project, together with Francesca Paoletti, was to set up and validate an ELISA to measure NGF, as a protein, in the different stages of chick embryo development. However, beyond our specific roles, my colleagues and I used to participate together in all aspects of the project as a whole. As I will describe in the coming paragraphs, we attended daily meetings with Professor Levi-Montalcini.

It was a particularly touching moment when Professor Levi-Montalcini taught Annalisa Manca how to handle/operate embryos, just as she used to do in the fifties. Despite the fact that she was almost completely blind due to a maculopathy, she came back to the laboratory to assist Annalisa, step by step, during her first injections, by using a microscope with two pairs of oculars (**Figure 3**).

The preliminary results of the experiments unveiled a new NGF function: embryos treated with αD11, and not with the unrelated antibody, exhibited an inversion of the direction of the

FIGURE 4 | Rita Levi-Montalcini during her talk at the International NGF meeting, in Israel, 2008 [Photographer Annalisa Manca (EBRI) from EBRI Media Archive].

axial rotation. The final results were published in 2012 (Manca et al., 2012), and in 2008, Professor Levi-Montalcini presented our preliminary results by giving a 30-min talk and sponsoring a poster at the International NGF meeting, in Israel (**Figure 4**; Bradshaw et al., 2017). She set off on a long journey by air and car, to enthusiastically show the results of her work to the scientific community, as if she were a young postdoc and not a Nobel Laureate. We will never forget her zeal and passion, that drove our research in those years and long into the future.

THE VITAL ROLE OF NGF: OCTOPUS AND FERTILIZATION EXPERIMENTS

While the experiments on chick embryos were ongoing, Professor Levi-Montalcini was intrigued by the evolution of the nervous system and was studying the phylogenetic trees in relation to this.

She used to repeat that vertebrates and in particular mammals were not similar to insects, or in general to invertebrates, which develop entirely on the basis of a fixed genetic program. Conversely, mammals are able to adapt their development in a plastic manner, as she demonstrated in her "neurotrophic theory" (Levi-Montalcini, 1987), which was widely accepted and confirmed. The formation of appropriate numbers of neurons and glia is matched to the needs; plasticity also relates to other important neuronal functions, like learning and memory. Rita Levi-Montalcini always said that epigenetics is as important as genetics in mammals. Her idea of an environmental influence on vertebrate development was indeed the driving force in her discovery of NGF.

"Only insects do not hatch until they are perfect and from that moment, neither a hair nor a cell undergoes further changes. We, vertebrates or rather primates, less perfect and less pretentious, continue to grow, some more and some less, some better and some worse" (Levi-Montalcini, 2000).

It is important to remember that *Caenorhabditis Elegans* has a nervous system, without having NGF. On the other hand, in the nervous system of *Drosophila Melanogaster*, which has additional complexity with respect to *Caenorhabditis Elegans*, homologs of mammalian trophic factors, acting as regulators of neuronal and glial survival, were recently found (Hidalgo et al., 2011; Richardson and Shen, 2019). In particular, a structural NGF homolog, named Spatzle was found, originally discovered related to other functions in embryo development (Mizuguchi et al., 1998; Hoffmann et al., 2008).

However, it was not the fruit flies that attracted Rita Levi-Montalcini's attention. She had known that among invertebrates, the octopus has extraordinary skills in terms of behavior and intelligence.

Indeed, octopi have evolved large and complex nervous systems and sophisticated behaviors, comparable with mammals (Hochner, 2004; Moroz, 2009). Octopi exhibit abilities such as exploring new environments, problem solving and play-like behaviors under stress-free conditions (Hanlon and Messenger, 1996; Kuba et al., 2003). Rita Levi-Montalcini was fascinated by these particular skills and asked herself whether the octopus could have an NGF-like protein, involved in its neurodevelopment. She contacted Professor Graziano Fiorito from the Stazione Zoologica Anton Dohrn of Naples, who enthusiastically agreed to a collaboration. Francesca Paoletti, Simona Capsoni and myself began experiments on different octopus' nervous areas. Our aim was to find an NGF-like immunoreactivity through the means of simple techniques like immunohistochemistry, immunoprecipitation, ELISA and Western Blot, and by using a panel of anti-NGF antibodies. During the years that we were engaged in these experiments, the octopus genome had not yet been sequenced (Albertin et al., 2015).

Furthermore, at that time, Professor Levi-Montalcini was considering a new function for NGF, that she named "the vital role of NGF." She imagined NGF as a sort of "organizer," not only in relation to the nervous system, but also important in pre-embryonic life. The presence of NGF in male genital secretion was known about since the 1980s, when it had been found in the prostates of guinea pigs, rabbits, pigs and bulls, and successively, in bull, rabbit, camel, llama and alpaca seminal plasma (reviewed in Castellini et al., 2020), thus suggesting an important role for NGF in sperm function, as also mentioned by Rita Levi-Montalcini in her Nobel Lecture[1]. The expression of both NGF and its receptor in several parts of the reproductive system and its consequent involvement in sperm function led Rita Levi-Montalcini to postulate a possible involvement of NGF in oocyte fertilization by sperm. Federico La Regina and Simona Capsoni carried out a series of mice sperm and oocyte fusion experiments, both in the presence and absence of the monoclonal anti NGF blocking antibody αD11 (Cattaneo et al., 1988), the same used for the chick embryo experiments.

The preliminary experiments, both relating to octopi and fertilization projects, presented some very interesting results. Unfortunately, the experiments were not continued and the data remained unpublished, due to the fact that these projects were

[1]https://www.nobelprize.org/prizes/medicine/1986/levi-montalcini/lecture

not funded. This was a great pity because we now know that the amazing insights gained into the "vital role of NGF" were confirmed by subsequent studies.

In 2015, the Octopus Genome was published, offering an important input to research (Albertin et al., 2015). In subsequent years, it was demonstrated that the octopus:

1) Has extraordinary sensory organs that intercept signals and integrate them in the central nervous system (Huffard, 2013; Polese et al., 2016; Stubbs and Stubbs, 2016; Di Cosmo and Polese, 2017; Di Cosmo et al., 2018).
2) Has an RNA editing mechanism, probably involved in enhancing its adaptability, in a less risky way than by changing DNA. An example of edited proteins, genetically expanded in the octopus genome, are protocadherins, important in controlling neural circuits and promoting nerve cell excitability (Albertin et al., 2012; Liscovitch-Brauer et al., 2017).
3) Exhibits adult neurogenesis, that is related to its ability to problem solve (Bertapelle et al., 2017).

However, even more surprisingly, Rita Levi-Montalcini's intuition as regards the role of NGF in oocyte and sperm fertilization, was confirmed definitely when the article "The nerve of ovulation-inducing factor in semen" by Ratto et al. (2012) was published in PNAS in September 2012.

A protein factor, called ovulation-inducing factor (OIF), that elicits an ovulatory response in species displaying both induced and spontaneous ovulation, was known to exist in seminal plasma.

In the previously cited article, the authors purified OIF from llama e bull seminal plasma and carried out biochemical analysis to identify and study this protein. Surprisingly, Mass Spectrometry revealed a molecular mass and sequence that was identical to NGF. Moreover, X-ray diffraction data were used to solve the full sequence and structure of OIF, which confirmed the identity of both the sequence and the structure of OIF to NGF. The authors also performed crossed bioassays to test whether NGF was able to induce ovulation and whether OIF provoked neurite outgrowth in PC12 cells. Finally, they concluded that OIF in seminal plasma is indeed NGF, and that it is highly conserved across different species. An endocrine route of action of NGF elucidates a previously unknown pathway for the direct influence of the male on the hypothalamo-pituitary-gonadal axis of the inseminated female.

Undeniably, Professor Levi-Montalcini was a brilliant scientist with a great mind, despite the fact that she often declared that she was merely "reasonably intelligent" and driven mostly by intuition and imagination.

"*Imagination is more important than knowledge*": stated Professor Levi-Montalcini quoting Albert Einstein (Viereck, 1929), and she revealed to us that this sentence had been displayed for years on her little desk in the office at the Istituto Superiore di Sanità. Einstein declared in the same interview "*I am enough of the artist to draw freely upon my imagination. Imagination is more important than knowledge. Knowledge is limited. Imagination encircles the world,*" which is the complete citation (Viereck, 1929). Likewise, Rita Levi-Montalcini often defined her approach to science, as artistic. She asserted that her artistic *modus operandi* in scientific research derived from her genetic inheritance: her twin sister was a painter, her brother an architect. She herself was a very talented illustrator. To further explore the nature of the creative process by which scientists conceive their theories, readers can refer to the bibliography (Holton, 1998; Feinstein, 2006).

Personally, I think that Rita Levi-Montalcini's "artistic" method was the result of certain important qualities: observation and deductive logical reasoning, mixed with a holistic overview. Currently, we may take advantage of a wealth of sophisticated techniques and maybe my generation, more than those previous, tends to delegate the research answers to techniques, missing out some steps of the scientific method. Another erroneous approach of my generation is to lose the "view from above" too frequently, and to focus instead on solving a particular problem, regarding the context of the experiment. Rita Levi-Montalcini taught us to examine the question as a whole, and that each problem or negative result is there to tell us something. Sometimes we cannot understand immediately what these negative results might mean, and therefore we have to change our approach and wait for the answers to come. By way of example, we can consider the so-called "mouse effect" (Cohen et al., 1954). Professor Levi-Montalcini had found that a soluble factor isolated from sarcoma 180 and 37 caused intense proliferation of nervous fibers in chick embryos. While she was developing a bioassay in Rio de Janeiro, Levi-Montalcini found that several normal mouse tissues, used as non-tumoral control, caused a small but significant outgrowth of fibers from the ganglia (Levi-Montalcini et al., 1954).

The mouse effect was a message I was not really capable of taking on board, since I could not help thinking that it diminished – to the extent of annulling – the significance of the induction of the fibrillar halo by S180 and S37 (Levi-Montalcini, 1988).

When she discovered the presence of NGF in mouse salivary glands, the "mystery" regarding the mouse effect was revealed, but in the meantime, Rita Levi-Montalcini had not lost heart and nor interrupted her research.

RITA LEVI-MONTALCINI'S PROJECT AND THE DAILY TEAM MEETINGS

Rita Levi-Montalcini used to come to EBRI almost every day and took the opportunity to have meetings with her team. We would all be seated around her on two large sofas, in her office in the original EBRI headquarters (**Figure 5**). First of all, she would ask to be updated on the progress of the experiments. We each took turns, by changing positions, to sit next to her because she had severe hearing loss and we had to speak close to her ear.

When we informed her of the latest results of our experiments, she used to hold our hands. I always thought that, since she had lost her sight and hearing, maybe she found a sort of compensation through that gentle contact that enabled her to create a stronger connection with us, not only mentally, but also physically. I will never forget that incredibly human gesture,

FIGURE 5 | Rita Levi-Montalcini in the office with her group. Behind the sofa, from left to right: Annalisa Manca, Simona Capsoni, and Anna Di Luzio. On the sofa: Francesca Paoletti and Francesca Malerba [Photographer Maurizio Riccardi (Agr Press) from EBRI Media Archive].

that was simultaneously, meaningful, emotional, moving and the most natural thing ever.

During the first meetings that we ever had with her, she insisted that we call her "Rita" and not "Professor," and to use the familiar form "*tu*" and not "*lei.*" (In contrast to English, in Italian there are two pronouns for "you": the second person form "*tu*" (you) is used when speaking to someone with whom you are intimate or someone younger, and the third person form "*lei*" (he/she) when formality is required). All of us tried to adapt to this more intimate form of speech, but we could not do it! For us, she represented an eminent scientist and woman, with whom we had the fortune to know and work alongside, so it was inconceivable to address her as if she were a friend, despite our deep sense of affection. She was a little disappointed, but incredibly firmly convinced that our refusal was due to her age and not to our being awed by her remarkable personality and value! "I can comprehend" she said, "I am old and my face is full of wrinkles."

After our daily updates on the chick embryo experiments, she was also curious to know about the other projects we were working on. During those years, Francesca Paoletti and I were working on the NGF precursor, proNGF. In particular, we were trying to obtain specific structural information about the pro-peptide, that we had found to be an intrinsically unstructured peptide (Paoletti et al., 2009). To this end, Francesca and I were trying to express proNGF in minimal medium enriched with isotopes to perform NMR spectrum acquisition. It was not a straightforward process due to the fact that proNGF is expressed in *E. coli* inclusion bodies and must be refolded. Professor Levi-Montalcini was extremely interested in our attempts to obtain the recombinant protein enriched for NMR. She was very curious about the new methods and technologies available and asked many questions about structural biology techniques. Despite the fact that she had so much to teach us, she had a constant desire to learn new techniques from us and listen carefully to our little everyday problems in the laboratory.

Science was not the only subject of discussion during meetings with Rita Levi-Montalcini. She used to ask us about our lives, our families, our dreams: Where do you live? Where are you from? Are you in a relationship? What jobs do your relatives/family do? Surprisingly, her questions were driven by a sort of scientific curiosity. My mother and my brother are a mathematician and an engineer, respectively, and my father also deals with financial mathematics. She was intrigued to understand why my entire family was involved in maths and theoretical science, whilst I had decided upon a "soft" and "wet" science, like biology. "It could depend on genetics but also epigenetic reasons, linked to your experiences" she concluded.

Professor Levi-Montalcini was also keen to know if we were satisfied with our job positions and salaries. For almost all of us, the problem was never a question of position or salary but the fact that we only had a fixed term contract, sometimes lasting mere months, since our contracts were and still are linked to the projects that have been granted. As a senator, Professor Levi-Montalcini understood the problem perfectly well and fought to obtain better conditions for Italian researchers, asking for more attention and more funding from the government for Italian research (Clementi et al., 2008).

Another occasional focus of discussion in our meetings was that of books. Professor Levi-Montalcini with the help of her collaborators read a great number of books, and when she was particularly enthusiastic about a text, she bought copies and gave them to us. Some days she gave us two or three different books, novels or essays. To our great amusement, the following day Professor Levi-Montalcini would usually ask us if we had read the books and she remained astonished when we said that we had started reading but not yet finished!

Rita Levi-Montalcini also authored books and she often liked to discuss some of the themes of her publications with us. I would like to recount one significant event in this regard. She had decided to write an essay about the "two brains": the ancient brain, the limbic part, and the new brain, the cortex and neocortex. She said that the limbic brain, fundamental during prehistory for the safety of the Australopithecus during moments of flight from dangerous animals, has been responsible for horrible events in modern history, such as dictatorships, genocides, hate, etc. On the other hand, the neocortex would be the rational part of brain, which distinguishes humanity from beasts that are driven by instinct. She concluded that the "ancient" brain, that saved humanity in the past, would drive men to extinction in the future, if not controlled by the rationality of the "new" brain.

When she illustrated this idea, I expressed my disagreement:

"Professor, I think that instinct has some positive features: parental care, love and empathy are innate, and also important for balanced mental health. We know for example, that parental care is fundamental in avoiding that traumas are transmitted to future generations"

She looked at me with an unconvinced expression.

Some days later, Pina Moliterno, her assistant, called me on the phone:

"The Professor wants to see you"

"OK, let me call the others"

FIGURE 6 | Rita Levi-Montalcini with EBRI researchers after a Christmas party. From left to right: Annalisa Manca, Raffaella Scardigli, Antonino Cattaneo, Francesca Paoletti, Anna Di Luzio, Domenico Vignone, Francesca Malerba, and Simona Capsoni (from EBRI Media Archive).

"No, she wants to see you alone"

When I went to her office and sat next to her, with my hand in hers, she said that she had being having second thoughts and had partially reversed her opinion on the limbic brain. Some instinctual properties are important.

I mentioned to Pina what she had told me, that she had changed her opinion on the basis of my words. I was 30, and a Nobel Laureate had treated me as if I were her equal. I was confused, honored and deeply moved.

These are but a few examples of her humanity, as obviously I have a wealth of stories that deserve to be told. For example, when she gave us Christmas presents, every year she chose a different object that perfectly matched our personal style and often contained our favorite color, despite the fact that we had never declared which one it was. At the same time, we also used to give her a Christmas present, often a flowering plant, as we knew she liked them very much (**Figure 6**). Every year she was very happy to receive our present and always said: I will make sure that I deserve it.

In 2010, Professor Levi-Montalcini suffered a domestic accident and broke her femur. We were all extremely worried, but fortunately, the subsequent operation went well. For months afterward, Professor Levi-Montalcini was not able to move, and therefore could not come to EBRI. Naturally, this did not hold her back; instead, we went to her apartment to hold regular scientific meetings. At the time, we could sense that she was very tired, and we often wondered if our visits were perhaps too stressful for her, but her assistants encouraged us to continue to visit because the Professor wanted to see us, and did not want to interrupt her scientific activities.

PUBLIC LIFE

During the years in which I was involved in Rita Levi-Montalcini's projects, some significant public events took place and once again, she set an important example for me. She tackled everything thrown at her with elegance, assertiveness and irony.

As previously mentioned, in 2001, President Carlo Azeglio Ciampi made her a senator for life, which was a role that she did not take lightly. She never missed a session in the Senate, even though she confessed to us that she found the experience highly stressful. She said that she would prefer to be with us talking about science, but it was her duty to fulfill her institutional commitments.

Rita Levi-Montalcini was a strong advocate of increasing financial support for research. In 2006, she held the deciding vote in the Italian parliament regarding a Financial Act that was backed by the government of Romano Prodi. She threatened to withdraw her support unless the government reversed a last-minute decision to cut science funding.

"*If the Financial Act cuts the funds for research, this country is destroyed and I would not be able to vote for it.*" She declared in an interview. "*Italy has a lot of human capital and if research is not financed, the country will fall apart. We are a country that is poor in raw materials, but very rich in human capital and research is the real engine of a modern country, both in terms of social and economic repercussions.*"

Due to her support of the Prodi government, in 2007 she was the object of shameful attacks by the opposition leader Francesco Storace and his followers. Storace mockingly threatened to send her some crutches, stating that she was too old to vote and therefore represented a "crutch" to an ailing government. She was also denigrated for her Jewish origins by Storace's supporters.

Professor Levi-Montalcini wrote a public letter, reaffirming her institutional role, her duty to participate in political decisions in the Senate, to exercise her right to vote in good conscience, in freedom and with a focus on the common needs of citizens. Moreover, she underlined that, since she had full possession of her faculties and readily continued her scientific and social activities, she did not need any crutches either physically or mentally.

When we met Professor Levi-Montalcini afterward, we expressed our indignation regarding these abhorrent insults, but with her usual calm aplomb, she said that it did not matter, that certain people did not deserve our attention and that we had more important things to attend to together.

However, Professor Levi-Montalcini took full advantage of the opportunity to get her own back on Storace through the use of irony. Shortly after, in a documentary, Watson, Nobel Laureate for DNA discovery, affirmed his previously stated view that black people are intellectually inferior to white people and that that difference is genetic. When Rita Levi-Montalcini was contacted by journalists to express her opinion, she said that "*Races do not exist. Racists do!*" and explained that the brain has the same potential in every human, while it is likely that environmental and living conditions determine the level of intelligence. She then asked:

"Are you sure that Watson expressed this horrible statement, and not Storace?"

Unfortunately, the political hatred directed at Professor Levi-Montalcini was the cause of another scandalous episode. During the 2008 public elections, the scientist went to the polling station.

Since the queue was rather long, some citizens offered to let her pass in front due to her age. Not all of those waiting in line were in agreement and some declared that Professor Levi-Montalcini could wait in line along with everyone else. She was completely unfazed, saying that the people were right and that she must wait. She also refused the chair that the polling station workers had offered her.

As before, she commented to us that these matters were of little importance to her.

WOMEN'S RIGHTS

Rita Levi-Montalcini's life was hard: she was discriminated against for being Jewish and had to overcome many obstacles due to her gender. In her books (Levi-Montalcini, 1988, 2000), she describes having to fight prejudices both within her family and a work setting. As a result of her past, Rita Levi-Montalcini became a strong supporter of women's rights, in particular she said that women and men share the same potential and skills in all jobs, but women are often overshadowed by their partners or colleagues and their names were frequently not remembered. In order to preserve the memory of some important women, she wrote the book "*Le tue antenate*" (Your ancestors), in which she illustrated the biographies of some lesser known women involved in science or social movements (Levi-Montalcini and Tripodi, 2009). The book, addressed to young students, is a great read for a general audience.

In the preface, she wrote:

"This book is dedicated to the next generations, so that they may be aware of the fundamental scientific contributions made by their ancestors, from before the Christian era right up until the twentieth century, a significant period, in which being of the female gender was considered an obstacle to any type of intellectual development. Women were long excluded from important areas of society, on some occasions, the wisest were even accused of witchcraft and burnt at the stake. In many cases, the female contribution has never been fully recognized, attributed to the influence of fathers, brothers or husbands: of figures, always belonging to the male gender. In reality, throughout the ages and up until the present day, women have contributed to scientific development in equal measure to men, while also playing the role of wife and mother."

Rita Levi-Montalcini also established a foundation to assist African girls in studying scientific subjects, in particular medicine or nursing sciences by granting fellowships in European Universities.

Considering that Rita Levi-Montalcini was very active in scientific dissemination, paying particular attention to the younger generations, at EBRI we are committed to continuing her work, by organizing informative scientific meetings with general public and outreach lessons in schools.

DISCUSSION

In the previous paragraphs, I have described Rita Levi-Montalcini's most recent research projects, I have detailed a few of the most relevant episodes of my daily life with her (there are many other stories that remain to be told), described how she used to interact with her collaborators, her commitment to public and political life, and the way in which she faced up to problems. I would like to remind everybody that I met her when she was 98 years old.

What did I learn from her?

1) Enthusiasm and competence in research

"In order to do your job in the best possible way, you need enthusiasm and competence. Only having one of these is insufficient."

Rita Levi-Montalcini also used to cite a Primo Levi quotation from his novel "The Monkey's Wrench" that summarized what her job meant for her.

"If we exclude prodigious and individual moments that fate presents us with, loving our work (which unfortunately is the privilege of few) is the best concrete definition of happiness on earth" (Levi, 2017).

2) Curiosity and open doors

Rita Levi-Montalcini was curious about each and every aspect of life and about the people she interacted with, without any distinction of cultural education and social background, because every person was a potential source of inspiration and personal growth. She always left the door open for everyone. After all, is not being able to listen, observe and show curiosity without prejudice and dogmas, while employing honesty and integrity, part of the basic principles of the scientific method?

3) Learning from negative results

"*Do not fear difficult times, the best results come from there.*" Misleading or negative results are not a waste of time, but the answers to questions that we will eventually comprehend, if not immediately, then later on. In any case, negative results can point us in the right direction, without being discouraged. She taught us to maintain "the vision from above," keeping of the objective in mind, without forgetting the starting point.

4) Intelligence and generosity.

I greatly admired Rita Levi-Montalcini's deep and dynamic intuition and holistic approach to all scientific questions. It was a rare gift that made her a very special person. During our meetings, we were often entranced by her observations and her creative approach that was always centered on the issue in question. On the other hand, Rita Levi-Montalcini took genuine care of us, and was interested in our experiments, opinions and projects in science and in life in general. Often the Professor showed astonishment in the face of our admiration and gratitude. She frequently declared that she was not special, but merely propelled by willpower, hard work and a little bit of good fortune. Sometimes I ask myself if Professor Levi-Montalcini did not fully realize her enormous worth as woman and scientist or, simply, that the people who possess great value somehow ignore this great value themselves. This was yet another lesson imparted by Rita

FIGURE 7 | Rita Levi-Montalcini with her assistant Pina Moliterno, during the workshop "The Brain in Health and Disease," held in the Campidoglio, Rome, on 22nd April 2019, in celebration of her 100th birthday (EBRI Media Archive).

Levi-Montalcini: be wary of arrogant people, since great people always display humility, generosity, attentiveness and availability.

5) Optimism and looking forward

In a video, shot in Israel, during the previously mentioned NGF meeting, in answer to the question "Have you any regrets?" she answered "never!" immediately and decisively. When, during an interview at EBRI in 2007, Moses Chao asked her what was the happiest time in her life, she replied "this one!" (Chao, 2010). In 2009, just before her 100th birthday, while being questioned by Paolo Giordano, an Italian writer and physicist, Rita Levi-Montalcini said that, despite her age, she was continuing to look toward the future with confidence (**Figure 7**).

If I re-read all the points that I have listed, I think that Rita Levi-Montalcini's teachings are not only useful for scientists, but can offer an example to anyone who seeks satisfaction in life by doing something good for themselves and the next person. Being a scientist implies not expressing opinions before having studied the problem deeply, observing, postulating a hypothesis and verifying it through the appropriate checks. We should avoid prejudices and dogmas, instead relying on deduction and induction. Following this reasoning in our everyday lives can help us make important choices, especially in darker moments.

In an interview, a journalist asked me if I had learnt more from Rita Levi-Montalcini the scientist or the woman. My answer was that I could not divide Professor Levi-Montalcini the scientist from the woman.

Rita Levi-Montalcini was a scientist with the grace, delicacy, sensitivity and elegance of a woman. What is more, she was a woman embodying the principles of the scientific method in all aspects of her life. She possessed patience and tenacity, as a consequence of her life experiences, because, as she loved to repeat "*those who are full, gain less satisfaction from food than those who are hungry, and women are hungry because men have kept us out of many aspects of social, political, scientific life for centuries.*"

When I was writing this paper, I undertook some interviews with my colleagues. The question "Why did you decide to become a scientist?" seemingly simple and obvious, was met with some hesitation. For the most part, they needed time and some explanation before attempting to remember why and answer. Their responses were quite varied and interesting, but the intended goal for this "experiment" was to investigate the feelings that accompanied the answers.

I can say that the only ideal I worked for was that of helping others and perhaps this is why research has given me much more than I could have hoped for (From an interview).

Considering in retrospect my long journey, and that of my peers and colleagues and the young recruits who have joined us, I can affirm that in scientific research, neither the degree of intelligence nor the ability to execute and carry out the task at hand perfectly, are essential factors for success and personal satisfaction. In both cases, total dedication and closing our eyes to difficulties are actually more important: in this way, we can face problems that others, who are more critical and more precise, would not face (Levi-Montalcini, 1988).

I wish young people the same luck that led me to lose interest in my own person, but to always pay great attention to everything around me, to everything in the world of science, without neglecting the values of society (From an interview).

Every cell, nerve cell, particularly in the brain, is such a marvelous object to study. (.) So I had all the reasons to want to work in this way, not as a scientist, but in order to see beauty (Chao, 2010).

These are Rita Levi-Montalcini's answers to this question.

In my opinion, scientific research is not simply a job like any other. Being a scientist is also a responsibility, not only with respect to the data we produce and share with the community, but also to those people waiting for answers from science, in particular patients, if we are referring to biomedical research. And research is not a job like any other, because you learn to collect failures and frustrations, and yet to keep going ahead.

In hard times, when we have no funds, conflicting results, many failed attempts and we are feeling frustrated and over focused on particular problems, therefore losing the "vision from above," maybe we should close our eyes and call to mind the reason why we decided to become scientists. While considering the answers of my colleagues I have realized that we often forget this point. During difficult moments, maybe it could be useful to think of Rita Levi-Montalcini's example and then look forward to the future with confidence, as she always did during her life.

I hope Rita Levi-Montalcini will continue to be a source of inspiration for scientists and beyond, and that my testimony in this article can play a small part in this.

"Life does not end with death. What you pass on to others remains. Immortality is not the body, which will one day die. I don't care about dying. That does not matter. . . of importance is the message you leave to others. That is immortality."

AUTHOR CONTRIBUTIONS

FM designed and wrote the manuscript.

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SYSTEMATIC REVIEW
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Clinical and Structural Differences in Delusions Across Diagnoses: A Systematic Review

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Delusions are marked, fixed beliefs that are incongruent with reality. Delusions, with comorbid hallucinations, are a hallmark of certain psychotic disorders (e.g., schizophrenia). Delusions can present transdiagnostically, in neurodegenerative (e.g., Alzheimer's disease and fronto-temporal dementia), nervous system disorders (e.g., Parkinson's disease) and across other psychiatric disorders (e.g., bipolar disorder). The burden of delusions is severe and understanding the heterogeneity of delusions may delineate a more valid nosology of not only psychiatric disorders but also neurodegenerative and nervous system disorders. We systematically reviewed structural neuroimaging studies reporting on delusions in four disorder types [schizophrenia (SZ), bipolar disorder (BP), Alzheimer's disease (AD), and Parkinson's disease (PD)] to provide a comprehensive overview of neural changes and clinical presentations associated with delusions. Twenty-eight eligible studies were identified. This review found delusions were most associated with gray matter reductions in the dorsolateral prefrontal cortex (SZ, BP, and AD), left claustrum (SZ and AD), hippocampus (SZ and AD), insula (SZ, BP, and AD), amygdala (SZ and BP), thalamus (SZ and AD), superior temporal gyrus (SZ, BP, and AD), and middle frontal gyrus (SZ, BP, AD, and PD). However, there was a great deal of variability in the findings of each disorder. There is some support for the current dopaminergic hypothesis of psychosis, but we also propose new hypotheses related to the belief formation network and cognitive biases. We also propose a standardization of assessments to aid future transdiagnostic study approaches. Future studies should explore the neural and biological underpinnings of delusions to hopefully, inform future treatment.

Keywords: delusion, transdiagnostic, structural, schizophrenia, Alzheimer's disease, Parkinson's disease, bipolar disorder

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INTRODUCTION

Delusions are a hallmark of schizophrenia and one of the main diagnostic criteria for the disorder (Maher, 2006; American Psychiatric Association, 2013). Delusions also present transdiagnostically, in neurodegenerative diseases, nervous system disorders, stroke patients, traumatic brain injuries, and other psychiatric disorders. There is even, albeit rare (current prevalence rate of 0.2%), a

standalone diagnosis of delusional disorder that present solely with delusions and can maintain with reduced intensity or remit naturally (American Psychiatric Association, 2013; Opjordsmoen, 2014). Delusions are associated with increased caregiver burden, poorer medication adherence, and overall, worsening prognosis across disorders and can severely impact functioning and independent living (Ismail et al., 2011; Whitehead et al., 2012; Fischer and Sweet, 2016; Altamura et al., 2018; Warren et al., 2018).

Delusion type falls broadly into 12 different categories with some discrepancies: persecutory, jealousy, grandiosity, religious, delusion of reference, erotomania, guilt, somatic, and passive delusions such as, thought withdrawal, thought insertion, thought broadcasting, and the delusion of being controlled. There are additional categories of delusions that are more specific, such as Capgras delusion (believing family members are replaced by an identical imposter) and Othello's syndrome (delusional jealousy about family members) that have been observed across disorders (Moro et al., 2013). Different types of delusions may be associated with co-occurring symptoms (e.g., mood states) and overall clinical presentations that are etiologically heterogeneous. Furthermore, different types of delusions may indicate different underlying psychopathological constructs (e.g., deficits in self-monitoring vs. deficits in source monitoring) (Blakemore et al., 2000; Raveendran and Kumari, 2007; Corlett et al., 2010).

The assessments and measurements of delusional experiences depend largely on the primary diagnosis and delusional symptoms may vary in intensity, persistence, associated distress, and common themes or presentation types. The neurobiology that underlies this heterogeneity in delusions remains limited given obvious challenges in developing animal models of psychosis (Feifel and Shilling, 2010) although a recent mouse model has shown promising support for the involvement of the dopamine circuit in reality testing (Fry et al., 2020). Below, we will describe current theories of delusional development that have not been examined from a transdiagnostic standpoint. With differences in assessments, presentations, associated primary diagnoses, and lack of established animal models, the field would benefit from more clarity to better understand the etiology and neurobiology of delusions.

CURRENT HYPOTHESES ON THE DEVELOPMENT OF DELUSIONS

Delusions: The Dopamine Dysfunction Hypothesis

Psychosis has been shown to be associated with a dysfunction of the dopamine-dependent process of salience attribution (Kapur, 2003; Maia and Frank, 2017). Specifically, an increase in striatal dopamine synthesis capacity is related to psychosis progression (Howes et al., 2011). In schizophrenia, however, the disruption may be further explained by a combination of increased dopaminergic activity for irrelevant stimuli and a decrease in dopaminergic activity in regard to situation relevant stimuli (Maia and Frank, 2017). The mechanism of antipsychotic medications, mostly through dopamine (D2) antagonism (Li et al., 2016), suggests a causal relationship between psychosis and dopaminergic disruptions. However, it should be noted that this dopamine dysregulation model has mostly been developed in the context of psychosis in schizophrenia. Similarly in Alzheimer's disease, an excess of striatal dopamine D2/3 receptors was found to be related to delusion presence (Reeves et al., 2012). Levodopa (L-dopa), a precursor to dopamine, is the gold-standard medication for Parkinson's disease, and may result in the formation of delusions and hallucinations (Ruggieri et al., 1997; Swick and Walling, 2005). However, this association is not specific to delusions, such that there are remaining questions as to specificity of the relationship between dopamine dysregulation and delusions.

The mesolimbic pathway is a collection of dopaminergic neurons beginning at the ventral tegmental area in the midbrain and connecting to the ventral striatum (including the nucleus accumbens) of the basal ganglia in the forebrain. The release of dopamine into the nucleus accumbens regulates motivational salience, influences drive and behavior, and reward-related motor function learning (Kapur, 2003). Disruptions in the dopaminergic system may result in misread salient information, attention to irrelevant stimuli, and ultimately, disruptions to reward-related behavior (Kapur, 2003). Individuals with schizophrenia have been shown to assign overt salience to contextually irrelevant stimuli, potentially because of this disruption of dopamine release (Kapur et al., 2005). This disruption may explain the divergence of belief formation into psychosis formation. Therefore, this framework may begin to explain not only formation of delusions but hallucinations as well.

Delusions: Deficits in Error Monitoring

Recently, delusions have been conceptualized as the result of defects in error monitoring, perhaps related to a disruption in dopaminergic pathways (Corlett et al., 2010; Krummenacher et al., 2010). Deficits in the ability to differentiate information-bearing patterns from noise result in noise taken in as salient information, also referred to as deficits in signal detection. Corlett and colleagues described this deficit as a two factor model (Corlett et al., 2010). First, the prediction error, or a discrepancy between the brain's prediction of a stimulus and the actual perception of that stimulus, occurs, and second, abnormal stimulus information is integrated into previous knowledge (Corlett et al., 2007, 2010). More specifically, the discrepancy between prediction and stimulus perception results in incorrect attention toward potential explanatory cues and subsequently, learning of misrepresentations of the environment, resulting in the formation of a delusion (Corlett et al., 2010; Corlett and Fletcher, 2015). Individuals with schizophrenia have been shown to have impaired error monitoring and importantly, defects in error awareness (Mathalon et al., 2002).

Prediction errors, in an inaccurate inference model for psychosis, have been associated with the right middle/inferior frontal gyrus (Griffiths et al., 2014). Previous structural studies have also implicated the left inferior frontal gyrus (IFG) in error monitoring (Mitchell et al., 2009; Sharot, 2011). As previously

mentioned, the dopaminergic system also plays a role in signaling errors related to reward (or salience) prediction (Schultz and Dickinson, 2000). In functional studies, the anterior insula cortex and the anterior cingulate cortex are activated during errors in performance and error awareness (Klein et al., 2007; Ullsperger et al., 2010; Harsay et al., 2012). Specifically, the insula-cortico-thalamic circuit, including the dorsal and ventral areas of the anterior insula, is responsible for both error awareness and the processing of salience (Harsay et al., 2012). It remains unclear if this theory explains all delusion formation or relates only to delusions in patients with schizophrenia.

Delusions: Cognitive Biases

Additional theories have been postulated about delusions being a form of cognitive bias. This theory states that the maintenance of delusional thinking requires a two-sided approach, or bias, to incoming information. There is a predilection for information supporting the delusion (confirmatory evidence), and an avoidance (or rejection) of evidence not supporting the delusion (non-confirmatory evidence) (Moritz and Woodward, 2006; Woodward et al., 2006). Specifically, cognitive biases such as jumping to conclusions, biases against disconfirming evidence (BADE), and liberal acceptance are more commonly seen in individuals with schizophrenia and delusions than healthy populations without psychosis (Moritz and Woodward, 2006; Veckenstedt et al., 2011).

Functional studies found the jumping-to-conclusion bias was associated with the dopaminergic reward system and the posterior cingulate cortex (Andreou et al., 2018). Bias against disconfirming evidence (BADE) was associated with increased visual network activity and reduced default mode network (DMN) activity when processing confirmatory evidence, and reduced activation in the orbitofrontal cortex, inferior frontal gyrus, and parietal cortex when processing disconfirming evidence in individuals with schizophrenia with delusional ideation (Lavigne et al., 2020).

These cognitive bias theories have all been suggested as separate explanations for the etiology of delusions. Together, the deficits in error monitoring (2.2) and cognitive biases (2.3) theories present the two main components of delusions, formation and maintenance. In other words, the delusion begins with an error in the processing of stimuli (a default) followed by avoiding the contradictory evidence while seeking out confirming evidence (a bias) to maintain the delusion. However, the theories have largely only been examined with individuals with schizophrenia or healthy controls using cognitive-based tasks (e.g., oddball task, antisaccade task) that represent circuits that underlie delusions. The use of these cognitive tasks is largely based on the limitations of examining active delusions in an MRI scanner and the previously mentioned lack of animal models. It remains unclear if these tasks activate all of the networks involved in delusion formation and maintenance. In addition, as the majority of studies examining the neurobiology and neuroanatomy of delusions focus on schizophrenia, it remains unclear if these theories of etiology and related circuitries generalize across the different diagnoses where delusions are present. Our review seeks to examine if these theories can explain the etiology of delusions as a whole or if they are explaining delusions within the context of a single disorder.

RATIONALE FOR REVIEW

For the purposes of this review, we sought to determine what contributions clinical phenotypes and structural neuroimaging have made to our understanding of the heterogeneity of delusions. The presence of delusions across different disorders suggests a potential common mechanism underlying the etiology of the symptom. Previous literature reviews have postulated hypotheses about the neurobiology underlying delusions but have yielded no transdiagnostic findings (Corlett et al., 2010). Previous neuroimaging studies have examined the neural correlates and gray matter changes associated with delusions (Whitford et al., 2009; Cascella et al., 2011; Ismail et al., 2011; Whitehead et al., 2012; Zhu et al., 2016). However, the majority of these neuroimaging studies examined delusions in only one disorder type. Even within a specific disorder, the identified neural substrates of delusions are not consistent across studies and not isolated within a brain region or known neural network. Examining the neural correlates associated with delusions across disorders could allow for identification of previously masked differences. Identification of gray matter atrophy or alterations in specific cortical regions (e.g., mesolimbic dopaminergic pathway) may also aid in supporting one of the above-mentioned models of psychosis and specifically delusions over another.

Studying delusions in their various clinical presentations, as opposed to being organized by the traditional diagnostic categories, might align more closely with the true underlying biology of the symptom. The focus of this paper was to review the current structural imaging research surrounding delusions across four diagnoses (schizophrenia, Alzheimer's disease, Parkinson's disease, and bipolar disorder) to examine to what extent the profile of delusional thought is clinically similar. We examined patient demographics (e.g., age), temporal development, and concurrent symptomatology that relate to the presence of delusions in these disorders to determine if any comorbid factors helped explain the etiology of delusions.

Examining the measurements utilized for delusions across disorders will allow for comparison of the presentation of the symptom itself as well as disorder-specific covariates. A review of disorder specific assessments is also detailed in **Supplementary Appendix 1**. We also reviewed structural brain imaging studies with the symptom of delusion to determine common neural underpinnings across disorders. We then compared our findings to the networks and brain structures previously implicated in the (1) current understanding of belief formation and (2) currently accepted theories on the formation and development of delusions.

There are hundreds of papers using structural imaging to study psychosis, but we will focus on the last decade of structural findings, specifically on delusions in the four identified disorders. The constrain to the four disorders is keeping with the currently available neuroimaging literature although we acknowledge that this review is not all-encompassing of disorders presenting

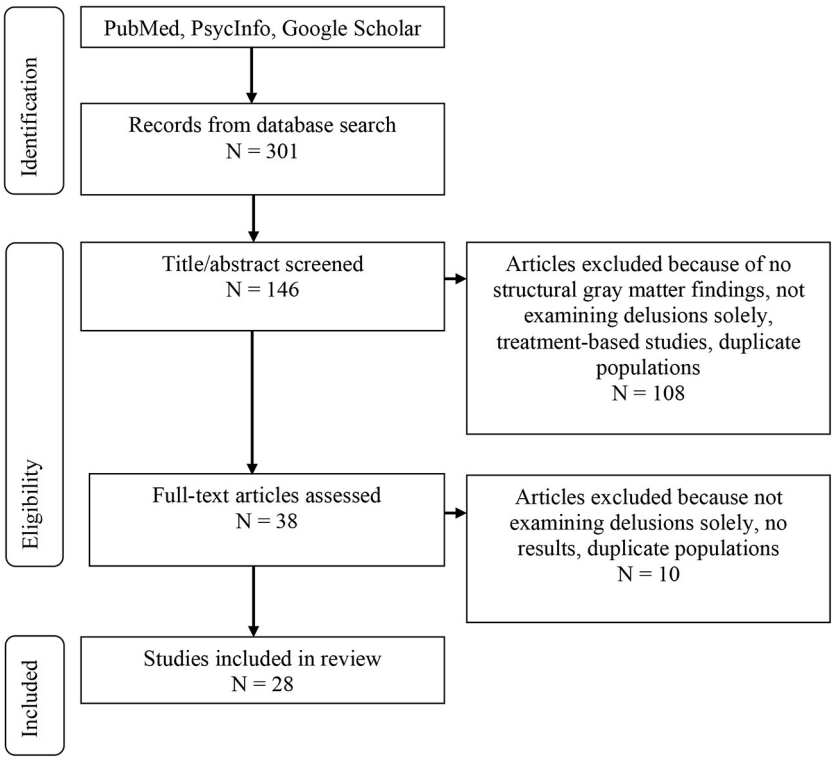

FIGURE 1 | Literature review flow chart.

with delusions. This review sought to add to the current literature from the last extensive review on the neurobiology of delusions (Corlett et al., 2010) and examine what additional brain structures within a multitude of disorders presenting with delusions have been identified in the last 10 years. The domain-level approach of this review may aid in the understanding of the etiology and maintenance of delusions and, ultimately, assist in treatment options for individuals presenting with delusions.

METHODS

Electronic databases including PubMed, PsycInfo, and Google Scholar were searched for primary articles, meta-analyses, and case studies. Searches of the databases were performed using the keywords: ["delusion(s)" or "delusional" or "psychosis" or "psychotic features"] and ["assessment" or "measurement" or "diagnosis" or "structural" or "magnetic resonance imaging (MRI)" or "gray matter"] to find studies specifically assessing and/or reporting on structural neuroimaging of delusions. Abstracts and main texts were assessed with the following including/excluding criteria. The inclusion criteria for the neuroimaging articles were final publication dates from 2009 to 2020. Inclusion criteria for all articles included: examination of delusions in one of the following primary diagnoses such as schizophrenia, Alzheimer's disease, Parkinson's disease, or bipolar disorder; articles in peer-reviewed journals only; English language only; and in studies examining gray matter structural changes using MRI scanning. We allowed for neuroimaging studies that examined either case/control differences and/or dimensional studies (e.g., presence of delusions v. no delusions in the same disorder). Exclusion criteria were the following: publications including letters or brief communications; and studies that did not have results for delusions alone (e.g., examining psychosis as a combination of hallucinations and delusions only). Articles were rejected if it was determined from the title or abstract that the study did not meet the inclusion criteria. This search yielded a total of 301 studies, of which 155 were discarded based on the title/abstract. Of the 146 articles remaining, 118 were removed because of no structural gray matter findings, not examining delusions solely, not examining one of the four mentioned disorders, overlapping patient populations, or treatment-based studies. Twelve studies remained that discussed assessments and cognitive features in delusions and 16 neuroimaging studies remained. See **Figure 1** for more details.

RESULTS

Disorder Specific Presentations of Delusions

Schizophrenia

Schizophrenia (SZ) is a severe mental illness characterized by cognitive, behavioral, and emotional dysfunction (American Psychiatric Association, 2013). First-episode psychotic symptoms

typically present in late teenage years through early to mid-thirties with men having, on average, a younger onset than women (American Psychiatric Association, 2013). Symptom presentation is often gradual in schizophrenia, but delusions are among the first symptoms to present and can diminish over time with some elderly patients reporting reduced or no significant delusions (American Psychiatric Association, 2013). As delusions are part of the diagnostic features in schizophrenia, they are very common, occurring in more than 90% of cases (American Psychiatric Association, 2013; Bebbington and Freeman, 2017). The most prevalent type of delusion is persecutory (more than 70% of first episode patients) but all other previously mentioned types have also been observed in schizophrenia (American Psychiatric Association, 2013; Coid et al., 2013; Picardi et al., 2018).

Bipolar Disorder

Bipolar disorder (BP) is a severe mood disorder characterized by extreme polar mood states, from depression to hypomania to mania (American Psychiatric Association, 2013). There are multiple forms of bipolar disorder defined mainly by the types of mood states; for example, Type I indicates fluctuation from depressed to manic states and Type II indicates fluctuations from depressed to hypomanic states (American Psychiatric Association, 2013). There are common neuroanatomical and genetic features that overlap between bipolar disorder and schizophrenia and even more overlap when further parsing bipolar disorder into bipolar disorder with psychotic features (Potash et al., 2003). Rate of psychosis among individuals with bipolar Type I disorder is 68 and 45% among those with bipolar Type II disorder (American Psychiatric Association, 2013). In terms of comorbid presentations, earlier age of bipolar onset is related to more psychotic features (Schürhoff et al., 2000). Delusions have also been noted in individuals with bipolar disorder independent of hallucinations or cognitive impairment (Tost et al., 2010).

Instances of hallucinations and delusions in bipolar disorder are most often mood congruent, indicating a relation to the current mood state of the individual (i.e., individuals in a depressed state have a higher prevalence of delusions of guilt) (Keck et al., 2003; Goodwin and Jamison, 2007). Delusions of grandiosity are most often related to individuals in a current manic state or mixed state (Keck et al., 2003; Goodwin and Jamison, 2007).

Alzheimer's Disease

Dementia is a neurodegenerative disease categorized by severe cognitive deficits including memory impairment and deficits in executive functioning (American Psychiatric Association, 2013). The most common type of dementia is Alzheimer's disease (AD), marked by memory impairment, difficulty concentrating, and visuo-spatial deficits. It is estimated that psychosis prevalence in Alzheimer's disease ranges from 10 to 73%, with an average of ∼50% (Murray et al., 2014; Fischer and Sweet, 2016). Psychosis presentation in Alzheimer's disease is more common in the middle to later stages of the disease (Sweet et al., 2003). The clinical presentation may be completely free of psychosis for years prior to the first noted delusion (White and Cummings, 1996; Fischer and Sweet, 2016). However, delusions typically present earlier and more frequently than hallucinations in Alzheimer's disease (Cummings and Victoroff, 1990; Lopez et al., 1991).

In terms of comorbid symptoms, psychosis has been related to increased aggression, functional impairment, behavioral symptoms, rapid cognitive decline, mortality, and increased caregiver burden in individuals with Alzheimer's disease (Murray et al., 2014). Common delusions experienced in Alzheimer's disease are paranoid in nature; persecutory and fear driven (e.g., believing they are being stolen from) and delusions of misidentification like the previously mentioned Capgras delusion (Ismail et al., 2012).

Parkinson's Disease

Parkinson's disease (PD) is a neurological disorder that progressively affects motor movement. During the course of Parkinson's disease, neurons gradually break down causing a dopamine reduction that eventually results in abnormal brain activity and motor movements (Ravina et al., 2007; American Psychiatric Association, 2013). The typical age of onset for Parkinson's disease is 60 years old but there are also less common types of early onset and juvenile onset (American Psychiatric Association, 2013). Delusions occur in an estimated 5 to 16% of individuals with Parkinson's disease (Lee and Weintraub, 2012). As previously mentioned, there is also evidence of delusion onset presenting only after initiation of L-dopa treatment (Stefanis et al., 2010; Moroy et al., 2012; Moro et al., 2013).

Delusions in Parkinson's disease are markedly less common than hallucinations and are often assessed and treated together under the diagnosis of Parkinson's disease associated psychosis. A number of comorbid symptoms have been identified in Parkinson's disease associated psychosis; however, when examining only delusions, individuals tend to be younger and less likely to have cognitive impairment (Warren et al., 2018).The most common type of delusion in Parkinson's disease is paranoid in nature, similar to Alzheimer's disease. The themes of paranoid delusions broadly fall into the categories of persecution and jealousy. As individuals age, the most common theme is misidentification or Capgras syndrome.

Neuroimaging Results

Schizophrenia

There were four studies that examined delusions in schizophrenia with a total sample of 198 individuals with schizophrenia and 126 healthy controls. One study examined only first episode psychosis (FEP) (Whitford et al., 2009), one study had a mix of patients with FEP and chronic schizophrenia (Zhu et al., 2016) and two studies examined only patients with chronic schizophrenia (Cascella et al., 2011; Spalletta et al., 2013). Two of the studies stated all individuals with schizophrenia were on at least one antipsychotic at the time of scan (Cascella et al., 2011; Spalletta et al., 2013) and two stated a mix of antipsychotic medication, antidepressant medication, and no medication at the time of scan (Whitford et al., 2009; Zhu et al., 2016). Three of the studies assessed delusions with the Positive and Negative Syndrome Scale (PANSS) (Whitford et al., 2009; Spalletta et al.,

2013; Zhu et al., 2016) and one utilized the SAPS for delusion assessment (Cascella et al., 2011). The presence of delusions in schizophrenia was related to gray matter volume decreases in the left claustrum and right insula (Cascella et al., 2011), and in the dorsomedial prefrontal cortex (cluster = 54 voxels), centered on the medial frontal gyrus (Whitford et al., 2009). However, it was noted in the study on first episode psychosis (Whitford et al., 2009) that severity of delusions was positively correlated with dorsomedial prefrontal cortex volume. More specifically, excessive atrophy in the dorsomedial prefrontal cortex was related to less severe delusional formation (Whitford et al., 2009). Cascella and colleagues found delusion subscale scores were negatively correlated with gray matter volume in the left claustrum (cluster = 795 mm^3) and right insula (cluster = 404 mm^3) (Cascella et al., 2011). There was also reduced gray matter in the left insula (BA 47; cluster = 20 mm^3) when comparing individuals with schizophrenia and delusions to those without delusions (Spalletta et al., 2013). However, Zhu and colleagues found that individuals with delusions showed greater gray matter volume in the right insula, superior temporal gyrus, and thalamus when compared to individuals with schizophrenia and no delusions; they were also not significantly different from healthy controls in these regions (Zhu et al., 2016). See **Table 1** for more details. Three of the four studies examined the severity of delusions (Whitford et al., 2009; Cascella et al., 2011; Zhu et al., 2016) and three of the studies controlled for other positive symptoms that highly correlate with delusions (e.g., hallucinations) indicating that these results show structural changes, although varying across studies, that may be specific to delusions (Cascella et al., 2011; Spalletta et al., 2013; Zhu et al., 2016).

Bipolar Disorder

There were only two studies that examined delusions alone in the context of bipolar disorder. There was a total sample of 115 individuals with delusions compared to 42 healthy controls and 39 individuals with bipolar disorder and no delusions. Both studies listed a total medication load, indicating individuals were prescribed either antipsychotics, antidepressants, mood stabilizers, anti-anxiety medications, a combination of the above, or medication naïve. One study had all individuals on antipsychotic medication at the time of scan and the other study had a mix of some individuals on antipsychotics and some medication naïve individuals. Decreases in gray matter were also found in the inferior frontal gyrus (BA 47; clusters = 141 mm^3 and 17 mm^3), insula (cluster = 83 mm^3), and middle frontal gyrus (BA 9; cluster = 12 mm^3) when compared to non-delusional bipolar individuals (Radaelli et al., 2014) and the inferior temporal lobe (cluster = 264 voxels) (Tost et al., 2010). Specific to persecutory delusions, there was a reduction in gray matter volume in the dorsolateral prefrontal cortex (three clusters with an average size of 5.8 voxels) (Tost et al., 2010). See **Table 1** for further details.

Alzheimer's Disease

There were six studies with a total of 253 individuals diagnosed with Alzheimer's disease and delusions compared to 34 individuals with other dementias and 23 healthy controls included in this review. Across the six studies there was a mix of naïve antipsychotic medication users and individuals on antipsychotic medications (for varying durations). Cognitive status was added to the statistical models in four of the studies to confirm that the structural findings were specific to presence of delusions. Delusions were correlated with less gray matter in the right frontoparietal, left frontal lobe, right hippocampus, and the left claustrum (Bruen et al., 2008; Serra et al., 2010). In a longitudinal study, regional gray matter decreases were found in the insula (left cluster = 350 *k*; right cluster = 1180 *k*), precuneus (cluster = 3011 *k*), cerebellum (left cluster = 252 *k*; right cluster = 90 *k*), superior temporal gyrus (cluster = 902 *k*), right posterior cingulate (cluster = 74 *k*), thalamus (cluster = 200 *k*), and left parahippocampal gyrus (cluster = 633 *k*) in individuals who developed delusions (Fischer et al., 2016). In addition, there was also less gray matter in the sensorimotor area (BA 6; cluster = 8,904 mm^3), left precentral gyrus (BA 6; cluster = 5,912 mm^3), and frontal eye fields (BA 8; cluster = 3,440 mm^3) in individuals with delusions and more accelerated atrophy in the temporal middle gyri (BA 20 and 21; clusters = 5,120 and 3,352 mm^3, respectively) when compared to those without delusions (Qian et al., 2019). Specific to women with Alzheimer's disease and paranoid delusions, there was atrophy in the left lateral and medial orbitofrontal and superior temporal regions (Whitehead et al., 2012). Across multiple dementias (Alzheimer's disease, Lewy Body dementia, and frontotemporal dementia) there were gray matter reductions in the dorsolateral frontal lobes, specifically the superior frontal gyrus and the right posterior lateral temporal lobe (Graff-Radford et al., 2012). See **Table 1** for more details.

Parkinson's Disease

There were a limited number of studies examining brain structures in individuals with Parkinson's disease and delusions. The studies identified in this review were three case reports and two meta-analyses consisting of 23 total case report findings. Two separate meta-analyses found both cases studies presenting with global brain atrophy or frontotemporal atrophy and then other case studies showing no changes related to delusions (Foley et al., 2017; Mitchell et al., 2017). Of note, these case studies had a combination of individuals experiencing both delusions and hallucinations with Capgras syndrome (Mitchell et al., 2017). Other case reports identified in this review found no significant brain abnormalities or changes in individuals with Parkinson's disease and delusions (Moroy et al., 2012; Islam et al., 2015). See **Table 1** for more neuroimaging review results.

DISCUSSION

This paper reviews the current literature on clinical features, phenotypes, and neuroanatomical changes related to delusions across diagnoses where psychosis is common. The results show that the overall definition of delusions across disorders, although varying in prevalence and severity, consists of similar wording and description. Overall, the assessment and

TABLE 1 | Original structural imaging studies and phenotypes associated with delusions.

Diagnosis	Study	Study type	Scan modality	Delusion type	N	Delusion assessment	Direction of findings	Imaging phenotype associated with delusions	Notes
SZ	Cascella et al., 2011	Original	T1	All	SZ (43)	SAPS	↓ GM	L claustrum, R insula	70% D+
	Spalletta et al., 2013	Original	T1	Somatic	SZ (75); HC (75)	SAPS and PANSS	↓ GM	L insula	
	Whitford et al., 2009	Original	T1	All	FEP SZ (31); HC (21)	PANSS	↓ GM	DMPFC centered on medial frontal gyrus	Delusion severity positively correlated with GM
	Zhu et al., 2016	Original	T1	Paranoid (14), Disorganized (1), Undifferentiated (3), Residual (1)	SZ (49); D+ (19), D- (30); HC (30)	PANSS	D+ > D-; D+ < HC	L superior temporal gyrus, R insula, thalamus; amygdala, ACC	
BP	Radaelli et al., 2014	Original	T1	All	BP (73); D+ (34), D- (39)	Clinical notes	D+ < D-	Inferior frontal gyrus, insula, middle frontal gyrus	
	Tost et al., 2010	Original	T1	Persecutory delusions	BP (42); HC (42)	YMRS	Persecutory	L DLPFC, L medial PFC, inferior temporal lobe	
AD	Bruen et al., 2008	Original	T1	Misidentification	AD (31)	NPI	↓ in GM	R inferior frontal gyrus, inferior parietal lobule, inferior and medial frontal gyri, L claustrum	
AD	Fischer et al., 2016	Longitudinal	T1	All	MCI (7); AD (17)	NPI-Q	↓ in GM	L precuneus, insula, cerebellum, L superior temporal gyrus, L parahippocampal, R thalamus, R posterior cingulate	
	Graff-Radford et al., 2012	Original	T1	Othello syndrome	DLBD (5); AD (6); bvFTD (3); D- (14)	UPDRS-TD	D+ < D-	Dorsolateral frontal lobes, superior frontal gyri, R posterior lateral temporal lobe	
	Qian et al., 2019	Original	T1	Not specified	AD (59); D+ (23), D- (36)	NPI-Q	↓ in GM	Precentral and middle frontal gyri, SMA	Significant delusion X time interaction
	Serra et al., 2010	Original	T1	All	AD (27) MCI (19) HC (23)	NPI-12	↓ in GM	R hippocampus	5% D+
	Whitehead et al., 2012	Original	T1	Paranoid	AD (113); D+ (23)	NPI	↓ in GM	L medial orbitofrontal, L superior temporal, L insula	Results only in females
PD	Foley et al., 2017	5 case reports	MRI	Othello syndrome	5 case reports	Clinical notes		Normal brains (4), mild left fronto-temporal atrophy (1)	All delusions appeared following dopamine treatment
	Islam et al., 2015	Case report	MRI	Capgras delusion (animals and inanimate objects)	53 yo F	Clinical notes		Normal brain scan	Scan prior to delusion onset
	Mitchell et al., 2017	Review of case reports and one singular case report		Capgras delusion	15 case reports	None listed		Normal brains (2), mild frontotemporal atrophy (1), nil reported (8), cortical atrophy or microvascular disease (4)	

(Continued)

TABLE 1 | Continued

Diagnosis	Study	Study type	Scan modality	Delusion type	N	Delusion assessment	Direction of findings	Imaging phenotype associated with delusions	Notes
	Moroy et al., 2012	Case report	MRI	Olfactory delusion	59 yo F	Clinical notes		Normal brain scan	
	Sakai et al., 2019	Case report	Autopsy	Delusional jealousy	72 yo M	None listed		Mild frontal lobe atrophy	

SZ, schizophrenia; FEP, first episode psychosis; AD, Alzheimer's disease; MCI, mild cognitive impairment; PD, Parkinson's disease; BP, bipolar disorder; PBD, psychotic bipolar disorder; NPBD, nonpsychotic bipolar disorder; HC, healthy controls; bvFTD, behavioral variant of Frontotemporal dementia; DLBD, dementia with Lewy body disease; GM, gray matter; MRI, magnetic resonance imaging; D+, delusions; D-, no delusions; UPDRS-TD, Unified Parkinson's Disease Rating Scale-Tremor Dominant; NPI, Neuropsychiatric Inventory; NPI-Q, Neuropsychiatric Inventory-Questionnaire; DIGS, Diagnostic Interview for Genetic Studies; MINI, Mini International Neuropsychiatric Interview; YMRS, Young Mania Rating Scale; PANSS, Positive and Negative Syndrome Scale; SAPS, Scale for the Assessment of Positive Symptoms; SCAN, Schedules for Clinical Assessment in Neuropsychiatry; CIDI-SF, World Health Organization Composite International Diagnostic Interview Short Form; L, left; R, right; F, female; M, male; SMA, sensorimotor area; DLPFC, dorsolateral prefrontal cortex; PFC, prefrontal cortex.

measurement of delusions demonstrates related, if not, exact overlap in the clinical definition of delusions across diagnoses; see **Supplementary Appendix 1** for more details. All disorders refer to delusions as fixed, false, idiosyncratic beliefs that are inconsistent with reality and remain intact with contrary evidence presented. Therefore, we conclude that the overall symptom experienced across these disorders is largely the same.

Within each disorder, the symptom of delusions has different presentations. Primary types of delusions vary across diagnoses with persecutory delusions being most prominent in schizophrenia, Alzheimer's disease, and Parkinson's disease (Ismail et al., 2012; American Psychiatric Association, 2013; Picardi et al., 2018); Capgras syndrome and Othello's delusion most common in Alzheimer's disease and Parkinson's disease (Ismail et al., 2012; Foley et al., 2017; Mitchell et al., 2017); delusions of grandiosity more prominent in individuals with bipolar disorder in a manic state (Keck et al., 2003; Goodwin and Jamison, 2007); delusions of guilt more prominent in individuals in a depressed state (Keck et al., 2003; Goodwin and Jamison, 2007); and delusions of misidentification mostly associated with older cohorts regardless of diagnosis (Ismail et al., 2012; American Psychiatric Association, 2013; Foley et al., 2017; Mitchell et al., 2017; Picardi et al., 2018). Although prominent delusion types are noted in the literature, the vast majority of the assessments identified in this review did not contain questions related to the type of delusion. Expansion of the current assessments to include delusion type classification may aid in understanding the heterogeneous presentation of delusions in these disorders as specific delusion types appear to be more prominent in certain disorders, certain levels of cognitive functioning, age groups, and mood states. The findings of this review also strongly suggest that there should be more standardization of the assessment and measurement of delusions as there was little consistency in the assessments utilized across disorders.

In relation to age and clinical presentation, delusions vary inconsistently across disorders. There is no consistent age range for delusional onset in the four disorders. In schizophrenia, bipolar disorder, and some cases of Parkinson's disease, delusions are related to an earlier age of onset of the primary disorder. Delusions present as first or second order symptoms in individuals with schizophrenia (Maher, 2006; Whitford et al., 2009; American Psychiatric Association, 2013). Delusions are also present throughout the lifetime of individuals with schizophrenia with few reports of late life fading of persistent positive symptoms (American Psychiatric Association, 2013). The opposite trajectory is observed for individuals with Alzheimer's disease or Parkinson's disease. For individuals with Alzheimer's disease and Parkinson's disease, the clinical presentation may be completely free of psychosis for years prior to the first noted delusion (White and Cummings, 1996; Forsaa et al., 2010; Fischer and Sweet, 2016). In Alzheimer's disease, delusions are typically seen in the middle to late stages of the disorder. It is important to note that an earlier age of onset for Parkinson's disease is roughly 45–60 years old whereas with the psychiatric disorders, early onset is late teenage years to early twenties (American Psychiatric Association, 2013). Given this variation, delusion onset was also examined in the context of the disease course instead of age. The delusion onset related to the disease course still yields no significant overlap across the disorders as the same differences were seen (delusions in schizophrenia and Parkinson's disease are related to early stage, delusions in Alzheimer's disease are related to late-stage, and in bipolar disorder, delusion onset varied with in stages of the disease).

There are also no consistent comorbid symptoms or clinical presentations across the disorders. For individuals with Parkinson's disease, there is less cognitive impairment in those presenting with delusions (Warren et al., 2018) whereas delusions in Alzheimer's disease and schizophrenia are more likely to relate to cognitive deficits and overall, lower cognitive functioning (Sweet et al., 2003; American Psychiatric Association, 2013). Delusions in schizophrenia most commonly present with hallucinations and cognitive deficits (American Psychiatric Association, 2013). In Parkinson's disease, delusions are most highly associated with impulse control disorders and dopamine dysregulation syndrome (Warren et al., 2018). Delusions in bipolar disorder are most often reported during clinical mood

states and change to be mood congruent (Goodwin and Jamison, 2007; Mahon et al., 2012). These findings are not surprising given the differences in clinical presentation between the disorders. Comorbid symptoms may relate more to the primary diagnosis and may not be biologically related to delusions. However, this hypothesis is limited based on the findings of this review and should be further examined in future studies.

In the neuroimaging studies, across diagnoses, the results showed varying degrees of alteration in the frontal and temporal regions across disorders. Although, there were some inconsistencies in the directionality of the gray matter alterations (Zhu et al., 2016), delusions were most associated with gray matter reductions in the dorsolateral prefrontal cortex (SZ, BP, and AD), left claustrum (SZ and AD), hippocampus (SZ and AD), insula (SZ, BP, and AD), amygdala (SZ and BP), thalamus (SZ and AD), superior temporal gyrus (SZ, BP, and AD), and middle frontal gyrus (SZ, BP, AD, and PD). The association of these additional findings to delusions may be explained when examining the function of these regions individually.

The claustrum has been linked to cognitive control, multi-sensory integration, consciousness, and task switching as well as cortically connected to the insula and the default mode network (Krimmel et al., 2019). The insula is involved with proprioception and the sense of self, self-awareness, more specifically, the posterior part of the insula is related to attention to and processing of salience (Craig, 2002; Harsay et al., 2012). As previously mentioned, the amygdala is connected with multiple regions of the brain and responsible for emotion regulation, emotional responsiveness, salience processing, as well as behavior modulation in connection to salient input and multiple neuromodulatory systems (e.g., dopaminergic) (Costafreda et al., 2008; Fadok et al., 2018). The hippocampus is primarily responsible for both short term and long term memory storage and retrieval (Squire, 1992) declarative memory, recollection of recognition memory, episodic memory, and familiarity (Brown and Aggleton, 2001; Kim, 2014; Bird, 2017) and along with the amygdala, salient information processing (Zheng et al., 2017). The posterior region of the superior temporal gyrus (specifically Wernicke's speech area, BA 22) is associated with auditory processing (Howard et al., 2000), and the caudal region relates to sentence comprehension (Hamilton et al., 2018). The thalamus serves as a relay station between internal and external information as well as is structurally related to the hippocampus, limbic system, and fornix. Specifically, the thalamo-cortical neurons are responsible for receiving external sensory information and relaying it upstream (Torrico and Munakomi, 2019) whereas the cortico-thalamo-cortical loop has been implicated in the maintenance of consciousness and attention to incoming visual stimuli (Trapp et al., 2012). However, these identified regions were not consistently reported across studies, nor across disorders presenting with delusions. In addition, the specificity of structural location (e.g., anterior vs. posterior insula) was not listed in a number of the studies. Since some of the overlap was between schizophrenia and bipolar disorder, those findings may be related to either the neural deterioration commonly seen in psychiatric disorders (DelBello et al., 2004; Lorenzetti et al., 2009; Kempton, 2011; Gupta et al., 2015; Torres et al., 2016), or the genetic and neural overlap amongst schizophrenia and bipolar disorder with psychosis (Tamminga et al., 2016), and less related to delusions specifically. Therefore, we conclude that the reductions in gray matter volumes identified in this review may not fully explain the development of delusional thinking.

The results of this review were then compared to structural regions mentioned in the other psychosis hypotheses (i.e., prediction error model, cognitive biases, and dopamine pathways as mechanisms of stimulus perception, information processing, and reward processing). There is some support for the prediction error model (Corlett and Fletcher, 2015) in the gray matter deficits (SZ, BP, and AD) in the dorsolateral prefrontal cortex (involved in expectation violation), insula (SZ, BP, and AD), and the middle frontal gyrus across all four disorders. These results also indicate some overlap with the mesolimbic dopaminergic pathway as there were structural alterations noted in the limbic system, specifically the amygdala, thalamus, and hippocampus. These areas have previously been implicated in salience prediction and importantly, errors in reward prediction. When taken with our previous hypothesis regarding delusions as negative prediction biases toward the environment, these results offer further support for an error in reward prediction, or aberrant salience, resulting in an observed negative environment. However, this theory is based only on partial results of this review as there are additional findings that do not fit into this prediction error model.

The mesolimbic dopaminergic pathway begins in the ventral tegmental area (VTA) in the midbrain, continues to the ventral striatum of the basal ganglia in the forebrain, and includes the nucleus accumbens. The neurons in the nucleus accumbens receive input from both the dopaminergic neurons of the VTA and the glutamatergic neurons of the hippocampus, amygdala, and the medial prefrontal cortex (mPFC) (Rubenstein and Rakic, 2013). Although gray matter reductions were found in the hippocampus, amygdala, and mPFC in this review, structural alterations were not consistently identified in the mesolimbic dopaminergic pathways. However, we caution that the lack of gray matter alterations in these pathways does not discredit the dopamine dysregulation hypothesis of psychosis. In fact, this review identified a number of studies in the Parkinson's disease literature that found delusions presenting only after treatment of levodopa (L-dopa) was started (Stefanis et al., 2010; Moroy et al., 2012; Moro et al., 2013) and that individuals with delusions were more likely to also have dopamine dysregulation syndrome (Warren et al., 2018). These dysregulations simply may not predict structural alterations.

As previously discussed, disruptions in the dopaminergic system may result in misread salient information or attention to irrelevant stimuli, and ultimately, disruptions in error processing, motivation salience (Kapur, 2003), and cognitive biases (e.g., jumping to conclusions). This dopamine dysregulation may be a downstream result of glutamate dysregulation in the prefrontal cortex (Stahl, 2018). Overactivation from the prefrontal cortex to the ventral tegmental area (VTA) from glutamate signaling may result in excess stimulation of the mesolimbic dopamine pathway. However, the results of this study do not clarify whether

this disruption is an overproduction of dopamine, a combination of increased dopaminergic activity for irrelevant stimuli and a decrease in dopaminergic activity in regards to situation relevant stimuli (Maia and Frank, 2017) or follows an inverted U-shape, where both too much and too little dopamine result in a disruption (Cools and D'Esposito, 2011). The disruption may also be uneven throughout the cortex, with some dopaminergic loss in the striatum and then consequential "overdosing" in the intact structures (e.g., nucleus accumbens) (Cools et al., 2003). Moreover, it is also unclear where the disruption of dopamine occurs (e.g., dopamine synthesis vs. dopamine release). Regardless of the directionality, our results support that there are alterations in areas related to glutamatergic neurons that may be indirectly associated with the dopaminergic pathways. Together with the previous literature, we preliminarily hypothesize that these glutamatergic and related dopaminergic disruptions are the primary mechanisms behind delusions or at least, may explain the divergence of belief formation into psychosis formation. However, the exact relationship between dopamine and delusion formation needs to be examined further.

We then explored our findings in relation to the belief formation network as a potential explanation of delusion development. How individuals form beliefs is integral to understanding how delusions are formed as delusions are defined as fixed, false beliefs (American Psychiatric Association, 2013). Beliefs are formed from integration of previously learned and newly gathered information, and ultimately, guide decisions and actions (Bogousslavsky and Inglin, 2007; Rao et al., 2009). Self-reliant beliefs are most often positively biased and are adjusted to a greater extent when presented with new favorable information than when presented with new unfavorable information (Sharot and Garrett, 2016). This translates to a two-step process of information integration to update and maintain beliefs; (1) an increased tendency to alter beliefs in response to desirable information and (2) a reduced tendency, or an avoidance, to alter beliefs in response to undesirable information (Sharot and Garrett, 2016). These positive biases are most often observed in stress-free environments (Johnson and Fowler, 2011; Sharot and Garrett, 2016). The biases will revert (or reduce in optimism) to a more realistic bias in uncertain settings, stressful environments, or in the presence of harm (Johnson and Fowler, 2011; Sharot and Garrett, 2016). There are even reports of pessimistic and negative judgments in animal models resulting from stressful or negative treatment (e.g., dehorning, separation from mother) but overall, the results are inconsistent (Harding et al., 2004; Bateson and Matheson, 2007; Matheson et al., 2008; Bateson et al., 2011). Whether these beliefs invert completely to a negative bias in extreme stress conditions has yet to be fully examined in humans.

Previous functional imaging literature on belief formation showed incorporating previously held information with new belief formation was negatively correlated with activation in the bilateral inferior frontal gyrus (IFG) (Caton et al., 2020). Additionally, there was strong unilateral white matter connectivity between the left IFG and the multiple left subcortical regions (left amygdala, left hippocampus, left putamen, left pallidum, left thalamus, and insula) during integration of favorable information into new beliefs (Sharot, 2011; Moutsiana et al., 2015) and between the left IFG and the medial frontal cortex when estimating errors in good news (Sharot, 2011). Aligned with information integration, the left IFG [particularly Brodmann's areas (BA) 44, 45, and 47] is involved with speech processing and comprehension (Matchin et al., 2017), memory, attention to stimuli (Eliasova et al., 2014), evaluation of social interactions (Grecucci et al., 2013), and in combination with the temporal parietal junction (TPJ), creates a sensory motor loop for information coding (Downar et al., 2001; Johnson et al., 2019) and regulation of socially-induced emotions (Grecucci et al., 2013). Activation in the right TPJ and precuneus was also related to encoding event improbability based on previously received information (d'Acremont et al., 2013).

Therefore, we compared the identified regions of this review to those implicated in the belief formation network (left IFG, left amygdala, hippocampus, anterior putamen, pallidum, thalamus, and insula), and found some overlap with the regions implicated in schizophrenia, bipolar disorder, and Alzheimer's disease but less so with the regions implicated in Parkinson's disease. Interestingly, the belief formation network has been identified as purely unilateral in the left hemisphere whereas, our results showed structural changes in both hemispheres, but almost consistently unilateral in specific regions. The overlapping regions are associated with emotion and motivation (left amygdala, hippocampus, thalamus, and insula) (Costafreda et al., 2008; Li et al., 2011; Sharot, 2011; Huber et al., 2013) and are consistently reported in psychiatric disorders independent of delusions and therefore, may be more related to the primary disorder than the presence of delusions (DelBello et al., 2004; Lorenzetti et al., 2009; Kempton, 2011; Gupta et al., 2015; Torres et al., 2016).

Based on the findings of this review, we postulate that delusions are formed and maintained similarly to typical beliefs and that both aspects of delusional thinking (formation and maintenance) may be the result of disruptions in the dopaminergic circuit causing misrepresentations of salient and non-salient information, deficits in error processing, and ultimately, cognitive biases. Future research should utilize experimental paradigms in functional studies to isolate activated regions related to belief formation, error processing, and cognitive biases. In addition, future studies would benefit from examining delusions across diagnoses to narrow findings specifically related to delusion formation and not secondary to primary diagnoses.

Limitations

There are some limitations in this review. In completing this review of the literature, there are a number of studies that mentioned the presentation of delusions in healthy cohorts, unipolar depression, right hemisphere stroke victims, following the development of brain lesions, and delusional disorder. This review was constrained to the four disorders because of a limited number of structural studies examining other disorders with delusions. Future studies should examine delusions across additional subsets of patient populations utilizing the NIMH Research Domain Criteria (RDoC) approach (RDoC; www.nimh.nih.gov/research-priorities/rdoc/index.shtml),

especially given the limited studies on some groups (e.g., Parkinson's disease), to fully examine the neural underpinnings associated with delusions more generally. Future studies should also consider longitudinal approaches to parse out if the identified regions found in this review are causing delusions, or merely adding to the severity of presentation.

In addition, the direct comparison of the neuroimaging findings across diagnoses should only be accepted with serious consideration as there are some patient characteristics specific to each diagnosis that could impact how the findings relate to one another. For example, there were significant differences in the median ages of individuals with schizophrenia and bipolar disorder, and those diagnosed with Alzheimer's disease and Parkinson's disease. Although it should be noted that all of the structural findings between individuals with delusions and individuals without delusions (as opposed to those from longitudinal follow-ups) were either age-matched or did not have significantly differing means. Given the breadth of individuals and diagnoses reviewed, the potential confounds of medication (previous and current), length of illnesses, and treatment types (e.g., electroconvulsive therapy) also need to be taken into consideration in future imaging research that cuts across diagnoses. Of note, there were significant variations in the antipsychotic medications taken at the time of the MRI scan in all disorder categories. Some medications, specifically antipsychotic medication, have an effect on gray matter structures in the brain and these effects may be potentially masking or falsifying the relation of gray matter and delusions. Future studies may also consider a medication dosage by delusion interaction when examining structural differences.

There are some limitations within the neuroimaging studies that warrant further examination. A number of Alzheimer's disease and Parkinson's disease studies did not list the activation cluster coordinates and therefore, the regional description was taken at face value. Given the breadth of certain brain areas (e.g., thalamus, insula), the related diverse functions, and connections (e.g., thalamo-cortical vs. thalamo-cerebellar), exact activation coordinates may reveal more nuanced findings. Another limitation is the small sample size for the schizophrenia, Alzheimer's disease, and bipolar disorder studies. Specific to the Parkinson's disease literature, this review only found case reports (with a sample of one) and no sample-based imaging research studies. Future research would benefit from larger sample size and the related increased power to potentially unmask more associated regions. Lastly, this review was limited to structural neuroimaging studies. We limited our search in this way to focus on the anatomical or volumetric foundation in preparation of understanding the functional implications of delusions. Future studies should explore functional or circuitry studies using the structural bases from this review.

There are also a number of neuroimaging studies that consider delusions along a continuum, even finding differences between severe delusions and mild delusions. Although this review found evidence that delusions are not unidimensional, the scaling in most referenced assessments is narrow (see **Supplementary Table 1**) and therefore, the difference between mild and severe may be difficult to interpret. Finally, it should be noted that delusions are not typically a symptom experienced in isolation. Not all the studies in this review corrected for comorbid symptoms (i.e., hallucinations or cognitive deficits) and therefore, these results need to be taken with some consideration as there might be other symptoms contributing to the findings in certain regions. Even with these limitations, this review highlighted the shared symptom of delusions across disorders and the potential underlying biology that is associated with delusions.

Future Directions

The results of this review support the need for a standardized assessment of delusions for all patient populations. Measurement consistency will aid the transdiagnostic approach to studying delusions and hopefully parse out subtle differences in the presentation (e.g., type) that have been mostly understudied in the current literature. As previously suggested, future studies would benefit from examining delusions in a multitude of the disorders they present in. This approach, along with simultaneous fMRI/Positron emission tomography (PET), may aid in measuring and relating the dysregulation of dopamine to brain regions associated with belief formation, cognitive biases, and hopefully, delusion formation.

Conclusions

Delusions are defined globally as fixed, false beliefs that are incongruent with reality and are maintained even when the individual is presented with contrary evidence. There are some overlapping findings in gray matter effects in several brain regions, namely the dorsolateral prefrontal cortex, left claustrum, hippocampus, amygdala, insula, thalamus, superior temporal gyrus, and middle frontal gyrus, but for the most part, the findings showed a great deal of variability between the disorders. The findings of this review suggest that standardization of assessments would aid future transdiagnostic study approaches as there is currently no single instrument designed to be used across all disorders.

The results found that across disorders that present with delusions, alterations in gray matter were found in a variety of cortical areas, most not overlapping across disorders, and therefore, structural alterations are not fully explaining the development of delusions. The leading hypotheses of the neurobiology of delusions, namely the dysfunction of the mesolimbic dopaminergic pathway and aberration in how the brain computes and responses to prediction errors (Corlett et al., 2010), are only partially supported by this subsequent literature. There is not evidence that these entire networks are selectively affected structurally, leading to support for potentially different networks at play. In addition, the directionality of disruptions in dopaminergic pathways, as well as potential negative belief biases, and cognitive biases should be studied transdiagnostically to identify the neural and biological underpinnings of delusions and hopefully, inform future treatment of delusions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

KR-M completed the systematic review and wrote the manuscript. JT and DG reviewed and edited the manuscript and offered insights throughout the process. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnint.2021.726321/full#supplementary-material

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Women in Neuroscience: Four Women's Contributions to Science and Society

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There has been increased cognizance of gender inequity and the importance of an inclusive and diverse approach to scientific research in recent years. However, the innovative work of women scientists is still undervalued based on reports of fewer women in leadership positions, limited citations of research spearheaded by women, reduced federal grant awards, and lack of recognition. Women have been involved in trailblazing work that paved the way for contemporary scientific inquiry. The strides made in current neuroscience include contributions from women who deserve more recognition. In this review, we discuss the work of four women whose groundbreaking scientific work has made ineffaceable marks in the neuroscience field. These women are pioneers of research and innovators and, in addition, contribute to positive change that bolsters the academic community and society. This article celebrates these women scientists, their substantial impacts in neuroscience, and the positive influence of their work on advancing society and culture.

Keywords: women in neuroscience, molecular mechanisms, neuromodulation, addiction, neuroscience, diversity, equity

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INTRODUCTION

Discourses about diversity, equity, and the representation of women have been at the forefront of social and institutional conversations worldwide (Clark and Hurd, 2020; Berryhill and Desrochers, 2021; Cortes, 2021). The year of 2020, accompanied by the COVID-19 pandemic and its restrictions, further highlighted racial and gendered inequalities (Cortes, 2021). The disparities that emerged in career stability for women during the pandemic emphasized the need to create policies in the social and work world to navigate diversity, equity, and inclusion fruitfully (Berryhill and Desrochers, 2021; Cortes, 2021; Machlovi et al., 2021). The scientific field must join in the societal shift toward evaluating cultures and practices to balance structural inequities. One of the ways to bolster these commitments to equity is by acknowledging brilliant women in the scientific arena with the recognition and accolades they deserve (Fairhall and Marder, 2020). Numerous women in neuroscience have made historic contributions to the field, furthering knowledge despite the challenges of gender discrimination and limited acknowledgment in the scientific community (Metitieri and Mele, 2020; Machlovi et al., 2021).

The contributions of countless women have been ignored or minimized, but their accomplishments have inspired younger female scientists (Metitieri and Mele, 2020). Thanks to increasing awareness and a significant cultural shift, there has been substantial advancement toward increasing representation and recognition of women in the field (Haak, 2002). An enormous amount of work needs to be done to level the playing ground in the neurosciences and sciences in general. As the cultural shift continues, women should be cited. Their intellectual contributions should be recognized, and equal opportunities for sharing these contributions with the scientific

community and the public should be provided. Disseminating research findings is vital for advancing any field of scientific inquiry (Schrouff et al., 2019; Fulvio et al., 2021). Having the opportunity to see one's science recognized in conferences and publications is essential for establishing a reputation and securing funding that provides continuity to one's research.

Tremendous scientific progress has been made in neuroscience in the past few decades. Our comprehension of the molecular and cellular mechanisms that drive thoughts and behavior has been exponentially expounded. These advancements have been possible due to the theories and innovations of exceptional neuroscientists, many of whom are women. The scientific work of four of these remarkable women – Drs. Sandhya Koushika, Eve Marder, Mary Jeanne Kreek, Yasmine Hurd – are reviewed here. We choose to highlight the work of these women due to its impact on our scientific journeys and because their cutting-edge work has led to a better understanding of the nervous system and diseases that perturb it.

Dr. Koushika: Novel Techniques for Studying Axonal Transport

Dr. Sandhya Koushika is a neuroscientist at the Tata Institute of Fundamental Research. Her research focuses on the molecular mechanisms that regulate axonal transport in *Caenorhabditis elegans* (*C. Elegans*). Axonal transport is a well-coordinated cellular process that involves the shuttling of substances, including organelles, by motor proteins along the axon. This delivery system is essential for neuronal function and survival (Mondal et al., 2011). Following her doctoral and postdoctoral studies in the United States, Dr. Koushika returned to her home country, India, to establish her laboratory (Sedwick, 2013).

Starting her laboratory allowed her to investigate questions she explored during her postdoctoral work. She was interested in retrograde axon transport and developing a genetic system that would track the exodus of endogenous proteins from the synapse. To investigate this, she needed an experimental setup for live imaging of retrograde transport in the worm. The existing methods at the time, including radiolabeled nerve growth factor (NGF), did not allow for dynamic tracking *in vivo* of specific proteins (Murthy et al., 2011). Dr. Koushika's laboratory developed a tool to exclusively tag retrograde cargo and visualize its transport in neurons *in vivo* (Murthy et al., 2011). Their methodology is based on injecting Alexa Fluor 594-conjugated immunoglobulin G (IgG) anti-GFP antibodies into transgenic *C. Elegans* lines that stably express GFP tagged to the synaptic vesicle protein, synaptobrevin-1 (SNB-1), in neurons. Upon exocytosis, SNB-1:GFP can bind to the Alexa Flour 594 conjugate, allowing retrograde activity to be visualized in both red and green channels under the microscope upon endocytosis of the vesicle. The stability and intensity of the fluorescent antibody enable long-term imaging of retrograde transport of specific transmembrane proteins. Another challenge to studying axonal transport is that model organisms are usually anesthetized, typically slowing or suspending the process. In collaboration with Dr. Venkataraman, her laboratory set up a microfluidic approach to study cellular and sub-cellular events in individual worms. This approach allows for anesthetic-free immobilization of intact genetic model organisms in a membrane-based *polydimethylsiloxane* (PDMS) microfluidic device (Mondal et al., 2011). Utilizing this technique, her group began unraveling the regulation of the various steps involved in axonal transport, such as the fate of the motor protein that carries the cargo, and mitochondria transport (Kumar et al., 2010; Mondal et al., 2011, 2021). This innovative method allows for long-term imaging of events occurring over longer time scales than previously possible as organisms remain viable post-imaging. The setup is not limited to *C. Elegans* and can also be used for imaging Drosophila larvae and zebrafish larvae.

Supervising one of only two worm laboratories in India and the only one at her institute came with its challenges; however, Dr. Koushika has since assisted in expanding the worm community by training new laboratories on *C. Elegans* husbandry and other laboratory techniques (Sedwick, 2013). She is also a mentor dedicated to introducing students to research at a young age, as she did not know of any researchers growing up (Sedwick, 2013). She has provided opportunities for high school students to participate and familiarize themselves with the research environment to raise interest and awareness for scientific work. Dr. Koushika's adventurous and creative character is unmistakable in her scientific work and her group's artistic endeavors, bridging art and science to communicate their findings. She has a page on her website dedicated solely to laboratory art (Koushika Lab, 2016). Dr. Koushika's work contributes to the advancement of basic research in India and worldwide, hence expanding the inclusivity scope of scientific studies and bridging the gap in research across countries.

Dr. Marder: Neuromodulation and Dynamics of Small Neural Networks

Dr. Eve Marder is a University Professor and the Victor and Gwendolyn Beinfield Professor of Neuroscience at Brandeis University. At the start of her career, Dr. Marder questioned a common notion – connections in neural circuits were hard-wired to produce a specific and predictable pattern of output – well-accepted in the field (Nassim, 2018). Dr. Marder's questions led to remarkable novel discoveries. Using the crustacean stomatogastric ganglion circuit (STG) as a model system, she discovered that neural circuits were plastic and could alter their activity in direct response to various neuromodulators. During her doctoral work Dr. Marder aimed to identify all the chemicals neurons used to communicate within the STG circuit. She discovered that the pyloric dilator (PD) neurons, influential in shaping the rhythmic motion of muscles controlling the pylorus, contained choline acetyltransferase activity but lateral pyloric (LP) neurons did not (Marder, 1976). PD used acetylcholine in its excitatory connections to the pyloric dilator muscles (Marder, 1974, 1976). Dr. Marder explored signaling by other neuromodulators, including dopamine, serotonin, and others, and how they interacted with neurotransmitters across synapses (Meyrand et al., 1992; Pulver et al., 2003). Her collaborative work with Dr. Eisen profoundly changed the trajectory of neuroscience research by revealing the flexibility

of neuronal circuits. Using microelectrodes to record voltage and current, they investigated how the electrically coupled PD and anterior burster (AB) neurons modulate postsynaptic neurons and show that PD and AB neurons release different neurotransmitters that generate significantly different outputs (Marder and Eisen, 1984). Subsequent work revealed that these electrically coupled neurons differed in their sensitivity to neurotransmitters and neuromodulators, thereby accounting for the shifts in firing times of the pyloric pattern generator (Eisen and Marder, 1984). Their pioneering work on the STG heralded current knowledge about ion channel and neuromodulator diversity and function.

Dr. Marder's breadth of knowledge and ideas are seemingly endless. Her laboratory is recognized as a leader in computational neuroscience. Dr. Marder's group was among the pioneers who developed the dynamic clamp method, which models artificial electrical conductance that can be injected into target neurons to simulate ionic conductance and synaptic inputs allowing the study of theoretical circuits' output (Sharp et al., 1993). Her group modeled negative feedback homeostatic mechanisms to dissect how neuronal function maintains stability despite perturbation. In collaboration with Dr. Abbot, Dr. Marder's team computationally simulated multicompartment neurons and investigated how the spatial distribution of calcium conductance may alter the physiology of the neuron (Siegel et al., 1994). They observed that local calcium concentration regulates channel density and can produce realistic spatial distributions of channels which shaped realistic non-uniform current distributions that were electrical activity and cell morphology dependent (Sharp et al., 1993; Siegel et al., 1994). They concluded that this activity-dependent modulation is vital to compensate for long-term potentiation and depression destabilizing effects and that intrinsic and synaptic modification creates a plastic yet stable neural network system (Siegel et al., 1994). The biological relevance of this experiment was validated in cultured STG neurons in collaboration with Dr. Turrigiano and Dr. Abbott, where they demonstrated that STG neurons transition from regular tonic firing to bursting activity when isolated in culture (Turrigiano et al., 1994). The tonic firing could be reinstated by hyperpolarizing rhythmic stimulation that mediates an increase in intracellular calcium concentration, indicating that calcium conductance is implicated in maintaining homeostatic and flexible neural systems (Turrigiano et al., 1994).

The general principles from Dr. Marder's work are applicable to neural networks of other organisms, including humans. Her studies open technically and conceptually novel avenues of investigation. Her findings reveal that neural activity necessitates intricate organization and constant fine-tuning, dependent on activity patterns through combinations of channels and receptors, to generate varying outputs and behaviors (Prinz et al., 2004; Bucher et al., 2005; Schulz et al., 2006). The breadth of Dr. Marder's work relies on synergistic use of experimental and theoretical techniques, including electrophysiological, biophysical, computational, anatomical, biochemical, and molecular techniques.

In addition to her scientific achievements, Dr. Marder is well known for her stellar mentorship, fostering a host of trainees who went on to develop successful careers within and outside of academia (Yale University, 2011). Her innovative thinking is renowned in the field, as so many people have been influenced by Dr. Marder's conceptual advances and inspired by her passion for science (Yale University, 2011). Her desire for training future generations of exceptional scientists led her to help establish one of the first undergraduate neuroscience programs in the United States in 1990 (University of Oregon, 2020). In the book "Lessons from the Lobster", a biography of Dr. Marder's life and scientific concepts, Charlotte Nassim describes Dr. Marder as a "supremely gifted scientist", and she hits the nail right on the head (Nassim, 2018).

Dr. Kreek: Innovative Treatment for Drug Addiction

Dr. Mary Jeanne Kreek was a physician-scientist who studied drug abuse and addiction. She was a Professor and Head of the Laboratory of the Biology of Addictive Diseases at The Rockefeller University, and Senior Physician of The Rockefeller University Hospital. Very early in her career, Dr. Kreek successfully developed methadone maintenance therapy to treat heroin addiction (Dole et al., 1966). In the 1960s, there was an opioid crisis due to a surge in heroin abuse; hence Dr. Kreek and her colleagues, Drs. Dole and Nyswander, set out to understand heroin addiction and investigate pharmacological approaches to treatment. A breakthrough contribution of Dr. Kreek's work was identifying heroin addiction as a neurological disease with behavioral consequences (Stimmel and Kreek, 2000). This recognition was a significant shift in ideology in a world where addiction was primarily considered criminal behavior (Chandler et al., 2009).

A seminal work from the efforts of Dr. Kreek and her collaborators showed that a daily oral dose of methadone blocked the euphoric "high" and prevented withdrawal symptoms – general malaise, nausea, tremors (Dole et al., 1966). In this double-blind study, heroin addicts were provided methadone maintenance treatment weeks before intravenous injections of various narcotics, including heroin, and saline. The euphoric effects of heroin and other narcotic drugs were scored and found to be markedly attenuated by the prior administration of methadone (Dole et al., 1966). As a well-rounded scientist, Dr. Kreek initiated longitudinal studies of the physiological effects and safety of long-term methadone use, which led to the development of the earliest laboratory techniques (isotope dilution and gas chromatography) for measuring methadone and other opioids in blood and tissues (Kreek et al., 1972; Dole and Kreek, 1973; Kreek, 1973). This work propelled the U.S. Food and Drug Administration's authorization of methadone maintenance for opiate addiction (Volkow and Koob, 2021).

Dr. Kreek further investigated how drugs of abuse alter gene expression in brain regions, including the nucleus accumbens and ventral tegmental area, and examined how chronic use of such drugs affected behavior (Leri et al., 2006; Yuferov et al., 2010). Utilizing animal models, her laboratory developed administration and behavioral paradigms reflective of drug addicts' use. This approach was instrumental in identifying genes

and biological pathways that confer a predisposition to addictive tendencies. Dr. Kreek and collaborators observed that mu-opioid receptor (MOR) mRNA levels in both the nucleus accumbens and frontal cortex significantly increased in rats exposed to cocaine during place preference (CPP) conditioning – a behavioral paradigm used to study pleasurable and aversive effects of drugs (Leri et al., 2006). However, the upregulation of MOR mRNA levels in the nucleus accumbens was reduced by methadone in a dose-dependent manner (Kreek, 1996; Leri et al., 2006). Her laboratory identified multiple genetic traits, including the first proof of a *cis*-acting polymorphism, a functional haplotype in the *PDYN* gene and substantially higher DNA methylation rate of the *OPRM1* gene in the lymphocytes of heroin addicts, associated with addiction (Stimmel and Kreek, 2000; Yuferov et al., 2010).

Beyond her scientific accomplishments, Dr. Kreek has been described as "an extraordinary role model and mentor, especially for women scientists", which is evident in her trainees' success and in the number of women drawn to science by reading about her accomplishments (Volkow and Koob, 2021). She cared about her patients and refuted the societal stigma against addicts. Methadone maintenance treatment remains a very effective treatment for opiate addiction today (Joudrey et al., 2021). Dr. Kreek recently passed away. The field of addiction neuroscience lost a true intellectual giant, but her work continues to inspire driven and exceptional scientists.

Dr. Hurd: Epigenetic and Cellular Mechanisms Underlying Addiction

Dr. Yasmin Hurd is the Chair of Translational Neuroscience and the Director of the Addiction Institute at Icahn School of Medicine at Mount Sinai. Dr. Hurd's research is grounded in a bidirectional translational research perspective that relies on animal models and human subjects. She employs multidisciplinary approaches to dissect the complex neurobiological systems and mechanisms underlying addiction. Recently, Dr. Hurd's cannabis research has been at the forefront of conversations regarding the legalization of marijuana (Scherer and Barcott, 2015; Caulkins and Kilmer, 2016; Gupta, 2018; Stenson, 2021). Previously, cannabis was classified as an illicit drug due to its addictive properties making it illegal to possess it (Smart and Pacula, 2019). It has been the most abused illicit drug, and Dr. Hurd's research has expanded the limited knowledge on the consequences of cannabis exposure on the human brain (Center for Disease Control and Prevention, 2017). She is a pioneer in work on the transgenerational effect of cannabis on the developing brain (Wang et al., 2004; Szutorisz et al., 2016). Dr. Hurd's investigations indicate that marijuana use during pregnancy could lead to behavioral and cognitive impairment in the offspring (Wang et al., 2004). This research was conducted using *in situ* hybridization histochemistry to visualize mRNA expression of cannabinoid receptor type 1 (CB1) and major dopamine receptor subtypes, D1 and D2, in postmortem human fetal brains from mothers with and without documented evidence of cannabis use during pregnancy (Wang et al., 2004). Dr. Hurd and collaborators observed reduced D2 mRNA expression levels in the amygdala of human fetuses. The reduction was positively correlated with the consumption of marijuana during pregnancy (Wang et al., 2004). These results were groundbreaking because they demonstrated that marijuana does not only affect brain activity in users but can interfere with the development of specific neural circuits in a transgenerational manner.

Dr. Hurd's extensive research on cannabis has highlighted the ying-yang feature of the compound. While most research has focused on tetrahydrocannabinol (THC), the psychoactive component of cannabis, and its adverse effects, Dr. Hurd's laboratory has contributed substantial knowledge on cannabidiol (CBD), a non-psychotomimetic component of cannabis with antipsychotic and anxiolytic properties, to the field. Using a rat drug-seeking and self-administration behavior model, CBD attenuated heroin-seeking behavior reestablished by exposure to a conditioned stimulus and a normalization of the glutamatergic receptor (AMPA GluR1) and CB1 expression, previously disrupted in stimulus cue-induced heroin seeking, in the nucleus accumbens (Ren et al., 2009). A translational clinical study of this effect was conducted in a double-blind, randomized placebo-controlled trial that assessed the effects of CBD administration on drug cue-induced craving and anxiety in drug-abstinent heroin addicts. They observed significantly reduced craving and anxiety induced by the presentation of salient drug cues than neutral cues following CBD administration, corroborating their animal work (Hurd et al., 2019). Her innovative cannabidiol studies are essential to developing potential new treatments for opioid addiction. The push to legalize marijuana has placed her in the spotlight, as evident in her multiple media appearances, including podcasts, PBS and Netflix shows, and CNN documentaries.

Dr. Hurd's outstanding contribution to society does not end in the laboratory, as she utilizes multiple avenues to communicate her research and engage with the public (Scherer and Barcott, 2015; Gupta, 2018; Stenson, 2021). Besides that, she is an avid advocate for diversity and equity, which is conspicuous in the diversity of her laboratory and her participation in programs that foster education and outreach to underserved communities (Clark and Hurd, 2020; Mount Sinai Today, 2020). As one of the few Black women at higher tiers of academia, she shares the implicit and explicit bias she faces in the academy and discusses steps to resolve it in the Nature Human Behavior article "Addressing racism and disparities in biomedical science" (Clark and Hurd, 2020). Dr. Hurd's work inspires many, and she is a role model to many women in the sciences (Mount Sinai Today, 2020).

CONCLUDING REFLECTIONS

Women continue to make meaningful contributions to the field of neuroscience, advancing our understanding of the nervous system and paving paths for modern scientific exploration. The women we highlighted here, Drs. Sandhya Koushika, Eve Marder, Mary Jeanne Kreek and Yasmine Hurd, pioneered new methodologies and proposed conceptual advances that have made indelible marks on the field. Their research in worms, crustaceans, rodents and human subjects has laid a vital

foundation for contemporary brain research. These women, and countless others, are helping change the narrative of science being considered a "male-dominated field" (Aguinis et al., 2018). Their stories and scientific contributions should continue to trend in all cultural spheres to reach aspiring young minds.

It has been repeatedly reported that diversity enhances excellence and innovation due to the variety of thoughts and the breadth and scope of research questions that can arise from different lived experiences (Swartz et al., 2019). Although there has been an increased awareness of the importance of diversity, inclusivity, and equity in science research, the data still indicates that women in scientific fields face persistent challenges and biases as they strive to attain success and take on leadership roles (Schrouff et al., 2019; Swartz et al., 2019; Fulvio et al., 2021). These reported hurdles, in turn, discourage other women from pursuing academic scientific research despite contemporary societal and cultural acceptance. Recognition and visibility of women's contributions in neuroscience are vital to their recruitment and retention (Fairhall and Marder, 2020; Fulvio et al., 2021). With this article, we bring attention to four outstanding women scientists, highlight their impact on neuroscience, and the positive influence of their work on increasing enthusiasm for science in young generations and on advancing society. Our goal is to awaken an interest in learning more about diverse perspectives and instill in the reader the curiosity to explore further the breadth of ideas that contributed to the advancement of the neuroscience field.

AUTHOR CONTRIBUTIONS

PY and AM contributed to this work. Both authors contributed to the article and approved the submitted version.

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frontiers in Integrative Neuroscience

PERSPECTIVE
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The Lonely Brain – Associations Between Social Isolation and (Cerebro-) Vascular Disease From the Perspective of Social Neuroscience

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The current COVID-19 pandemic led to a considerable reduction in in-person social contacts all over the world. In most individuals, reduced social contacts lead to the perception of social isolation causing feelings of loneliness, which are experienced as stressful. Experiencing social distress due to actual or perceived social isolation has been associated with negative health outcomes such as depression, (cerebro-) vascular disease and mortality. Concrete mechanisms behind this association are still a matter of debate. A group of researchers around Hugo Critchley with special contributions of Sarah Garfinkel and Lisa Quadt proposes a framework for the underlying brain-body interactions including elements from models of social homeostasis and interoceptive predictive processing that provides important insights and testable pathways. While in a previous publication, we reviewed literature on the observed association between social isolation and stroke and coronary heart disease, we now extent this review by presenting a comprehensive model to explain underlying pathomechanisms from the perspective of social neuroscience. Further, we discuss how neurodivergent people, e.g. autistic individuals or persons with attention deficit disorders, might differ in these pathomechanisms and why they are especially vulnerable to social isolation. Finally, we discuss clinical implications for the prevention and therapy of (cerebro-) vascular diseases.

Keywords: COVID-19, social isolation, stroke, cardiovascular diseases, interoception, psychological adaptation, physiological adaptation, neurological models

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INTRODUCTION

The current COVID-19 pandemic leads to considerable changes in professional and personal life in people from all over the world. Lockdowns and physical distancing require many people to work from home or work short hours, some even lose their jobs. Reductions in in-person social contacts due to changes in professional life are accompanied by social changes in personal life: in-person

social contacts including meeting family members and friends or engaging in group activities such as sports clubs are restricted to limit spreading of the SARS-CoV-2 virus. Consequently, the COVID-19 pandemic transforms social interactions. While in-person contacts decrease, virtual contacts via video calls, audio calls or text messages increase. Compared to in-person contacts, virtual contacts provide fewer non-verbal cues, greater potential for anonymity, more opportunity to form new social ties and bolster weak ties, and wider dissemination of information (Lieberman and Schroeder, 2020). Especially the restricted potential to deliver and receive non-verbal information such as touch, smell, the exact modulation of one's voice, or movement patterns impairs social interaction, provokes miscommunication and reduces the feeling of connection (Kruger et al., 2005; Hall and Schmid Mast, 2007). Consequently, the restriction of in-person contacts during the COVID-19 pandemic decreases the quality of social interactions which can increase the feeling of loneliness. First analyses from longitudinal population-based studies support this hypothesis. In the German National Cohort, an Austrian older sample, and a biracial American sample for example, an increase in loneliness was observed following the pandemic onset (Berger et al., 2021; Kucharska-Newton et al., 2021; Mayerl et al., 2021), which was related to the perceived COVID-19 related social restrictions (Mayerl et al., 2021). Having a low number of social contacts (social isolation) as well as perceiving a lack of social contacts or a low quality of social contacts (loneliness and lack of social support) has negative influences on mental and physical wellbeing (Quadt et al., 2018, 2020; Berger et al., 2021; Kucharska-Newton et al., 2021; Mayerl et al., 2021) including depression (Erzen and Çikrikci, 2018; Berger et al., 2021; Mayerl et al., 2021; Van As et al., 2021), stroke and myocardial infarction (Gronewold et al., 2020, 2021; Gronewold and Hermann, 2021), and mortality (Vogt et al., 1992; Stringhini et al., 2012; Steptoe et al., 2013; Rico-Uribe et al., 2018; Gronewold et al., 2020; Ward et al., 2021). Performing a comprehensive review and meta-analysis, Valtorta et al. (2016) observed that poor social relationships (including social isolation and loneliness) were associated with a 29% increase in risk of incident CHD and a 32% increase in risk of incident stroke. Three main mechanisms underlying these observations were discussed, that is behavioral (such as physical inactivity, smoking, and alcohol abuse), psychological (such as low self-esteem, limited coping mechanisms, and negative affectivity) and physiological mechanisms (such as disturbed immune functioning and dysregulation of vascular risk factors like blood pressure) (Valtorta et al., 2016). Recently, an innovative framework to decipher pathomechanisms underlying the negative health outcomes of social isolation and loneliness was developed by Quadt et al. (2020) combining the social allostasis model (Matthews and Tye, 2019) with ideas of interoceptive predictive processing (Barrett and Simmons, 2015). Based on our previous studies analyzing the association of social isolation with stroke and coronary heart disease (Gronewold et al., 2020, 2021; Gronewold and Hermann, 2021), we put a specific focus on the outcome of (cerebro-) vascular disease. We also want to highlight the increased vulnerability of neurodivergent individuals.

INFLUENCE OF SOCIAL ISOLATION AND LONELINESS ON (CEREBRO-) VASCULAR DISEASE

According to the framework by Quadt et al. (2020), social isolation increases the risk of (cerebro-) vascular disease by the over-activation of initially adaptive mechanisms. Social contacts represent a basic human need, which developed during evolution because living in a group protected individuals from threats of the environment and improved the chances to get access to water, food and sexual partners. From the evolutionary perspective, the feeling of loneliness evolved as a negative affect, now also called social stress or social distress, in response to isolation to draw an individual back to its group. Thus, the negative affect associated with loneliness is triggered by an adaptive response to perceived social deficits (Cacioppo et al., 2006). Only on rare occasions, social isolation is adaptive. In case of illness, isolating oneself from additional potential sources of infection as part of the sickness behavior is adaptive to foster recovery, and the feeling of loneliness represents the signal to return back to the group after recovery (Dantzer et al., 2008). Consequently, a flexible and context-dependent balance between isolation and connection is needed to ensure survival with usually seeking connection representing the most adaptive response. This balance is also termed social homeostasis.

According to predictive processing models (Barrett and Simmons, 2015), our brain coordinates this balance by comparing incoming sensory signals with predictive models about the likelihood of incoming signals in an efficient way. Before the COVID-19 pandemic, it was highly probably to see and hear large groups of people celebrating on a Friday evening in the downtown area. Going to the downtown area on the first day of a lockdown and only seeing very few people would surprise us because we are used to see large groups of people there and thus expect to see large groups based on our prediction model. In case of such a mismatch between prediction and reality, error signals occur. These error signals help the organism to detect that a specific set point associated with social needs is not met (Matthews and Tye, 2019). Error signals can either update prediction models (we do not expect to see large groups anymore, perceptual interference) or change behavior to change incoming signals (e.g. celebrate with a group of people who are SARS-CoV-2 negative, active inference) (Friston, 2012). Interoceptive predictive processing models put a focus on the sensing of internal bodily signals (Barrett and Simmons, 2015; Quadt et al., 2018). When we go to the downtown area on a Friday evening, we expect to feel a rise in blood pressure, heart rate and oxytocin release as signs of joy. Sensing these bodily sensations feels normal to us because they are within the expected range of our bodily signals. In the present times of lockdowns, where we do not meet a lot of people, we do not feel signs of joy when going to the empty downtown area on a Friday evening, but rather sense headache and tiredness.

Responding to mismatches between predicted and incoming signals, in our case between wanted and perceived social contacts, means effort for the organism. There are different homeostatic responses to perceived social deficits (Matthews and Tye, 2019). An important response is hypervigilance. Short-term increases in vigilance, arousal, and attention represent an adaptive response because it helps the socially isolated and thus more vulnerable individual to recognize and respond to potential threats from the environment. Attention is further directed toward socially relevant stimuli and increasing activity of reward processing systems like the dopaminergic system and the oxytocin system elicits motivation to reconnect. This adaptive short-term response is also known as acute stress response. The acute stress response improves information transmission, provides energy for fight-flight responses, and includes increased pro-inflammatory activity as a preparation for potential physical injury and as a consequence of the decreased risk of contagious viral infections when isolated. If social isolation cannot be resolved actively (e.g. by calling a friend), passive coping mechanisms such as attenuated emotional sensitivity ensure self-protection from emotional distress associated with isolation.

Negative health consequences of social isolation occur if the initially adaptive short-term stress response is prolonged, which occurs when the mismatch between wanted and perceived social contacts cannot be solved. Thus, error signals prevail, the individual continues to feel lonely, causing negative affect and emotional distress, and remains in a hypervigilant state. This results in allostatic overload in the long run which is not adaptive anymore but exhausts and harms the organism (McEwen, 2005). Chronic activation of the endocrine, neural and immunological mediators of the initially adaptive allostatic response aiming to achieve social homeostasis lead to systemic dysregulation of the cardiovascular, metabolic, and immune system. As a response to the dysregulation, subclinical functional deficits occur, finally resulting in clinical system damage manifested as (cerebro-) vascular disease (Peters et al., 2017). The initially adaptive short-term release of the stress hormones adrenalin and cortisol, which improves information processing, can lead to neurodegenerative processes and decreased information processing and memory function when prolonged. Glucocorticoid release secures energy supply in acute energy-demanding situations but leads to insulin resistance and increased risk for cardiovascular disease in cases of chronically increased release. Increases in blood pressure, which represent an adaptive response in situations with an acute need for increased blood flow, stress the vessel walls when repeated over a long time promoting atherosclerosis and damage of the vessel walls, particularly when combined with metabolic factors (McEwen, 2005). Natural immunity as a fast immune response, which includes release of neutrophils, macrophages, proinflammatory cytokines and natural killer cells, is increased during acute stress. This is adaptive because if the individual is harmed during fight-flight behavior, these cells can migrate to the site of injury and fight pathogens to accelerate wound repair and prevent infections. Specific immunity as a less fast but more specific response targeted to concrete stressors is decreased during acute stress to conserve energy. Chronic social stress can both increase risk for diseases associated with decreased immunity, such as infectious and neoplastic disease, and diseases associated with increased immunity, such as allergic and autoimmune disease (Segerstrom and Miller, 2004). Inflammation and infection promotes atherosclerosis, exerts prothrombotic effects, and can thus increase the risk of (cerebro-) vascular disease (Meschia et al., 2014).

DISCUSSION

Multiple factors can influence the vulnerability for social isolation and its (cerebro-) vascular consequences. In our previous review, we discussed the factors age, sex/gender, race/ethnicity, sexual orientation, and depression (Gronewold et al., 2021). Quadt et al. (2020) also suggest an important influence of neurodiversity. Neurodiversity represents an approach to see neurological differences as a result of normal variation in the human genome and as a social category such as gender instead of a pathology and medical disorder that needs to be cured (Armstrong, 2011; Pripas-Kapit, 2020). The neurodiversity approach was first put forward by autistic individuals and subsequently applied to a variety of other neurodevelopmental conditions (e.g. attention deficit disorders) (Jaarsma and Welin, 2012). Both in neurotypical and autistic individuals, loneliness is related to social skills and perceived quality of social contacts (Mazurek, 2014; Ee et al., 2019) and associated with depression (Mazurek, 2014).

According to previous studies, neurodivergent individuals experience loneliness and negative social contact more often than neurotypicals (Ee et al., 2019), despite longing for social contact (Müller et al., 2008). They report many barriers to socializing and that socializing with neurotypicals can be exhausting, challenging, or anxiety provoking (Müller et al., 2008; Ee et al., 2019). Differences in communication style, non-verbal social interactive cue processing and emotional expression, which lead to mutual misunderstanding between autistic and neurotypical individuals (Milton, 2012) foster social distress especially for autistic individuals, which increases their risk of (cerebro-) vascular disease. Yet, models for brain-body interactions underlying the influence of social isolation on (cerebro-) vascular health are based on research on neurotypicals and it remains to be evaluated whether the same mechanisms are involved in neurodivergent individuals (Quadt et al., 2020). Also, the above-mentioned examples are based on neurotypicals. Changes due to lockdowns might have a different influence in different individuals – while some will indeed be distressed by the lack of people in public areas, others will rather enjoy it. Emotional processing, including the sensing of own and other people's emotion, is influenced by interoceptive processes both in autistic and neurotypical individuals (Mulcahy et al., 2019). First studies on interoception in autistic individuals show that they have lower interoceptive accuracy (Garfinkel et al., 2016), which increases the likelihood of prediction errors and allostatic overload, and can finally increase the risk for various stress-related diseases. Concordant with this theory, a large case-control study including 1507 autistic and 15070 neurotypical individuals showed that history of stroke was twice as prevalent in autistic than neurotypical individuals (Croen et al., 2015). Further

research is needed to unravel reasons for increased loneliness in neurodiversity and its association with adverse health conditions such as (cerebro-) vascular disease.

CLINICAL IMPLICATIONS

Perceived social isolation as an important psychosocial stressor, which is increasing due to reduced social contacts during prolonged lockdowns in the COVID-19 pandemic, can represent the starting point of a vicious cycle: if in-person social contacts are not controllable by the individual anymore, social needs and expectations cannot be fulfilled, which leads to a prolonged stress response including negative affect, social withdrawal and negative evaluation of social contacts as sickness behavior to conserve energy and maintain physical health. As a consequence, the brain gets into a "locked-in" state (Barrett et al., 2016), where negative prediction models about social interactions, including also predictions about internal bodily responses toward social stimuli, cannot be corrected due to reduced exposure to potentially corrective stimuli and insensitivity to prediction errors containing corrective information. This in turn causes the social environment to withdraw from the isolated individual which further increases the stress response and progression toward disease states. Thus, disease states are both a cause and consequence of social isolation (Quadt et al., 2020), which puts the prevention and treatment of social isolation and its negative consequences for mental and physical health into focus.

Even though numerous healthcare authorities recommend assessing, documenting and addressing social factors such as social isolation (Gold et al., 2019), evidence demonstrating a concrete improvement in individual and population health and a reduction in health-related costs is still scarce and completely lacking for social isolation (Gottlieb et al., 2017). Support for the validity of the social allostasis and interoception models and its educable therapy suggestions is so far provided for the outcome of depression (Mazurek, 2014). It remains to be established, whether increasing social contacts via interventions in case loneliness is detected in patients during clinical routine and at the population level, decreases the risk of (cerebro-) vascular disease. Additionally, intervention studies which focus on the promotion and quality improvement of social contacts, especially for neurodivergent individuals, are needed.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

JG drafted the manuscript. ME critically revised the manuscript for important intellectual content and took the lead in the associated interview with Sarah Garfinkel and Lisa Quadt. Both authors contributed to the article and approved the submitted version.

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Understanding the Dynamics of the Developing Adolescent Brain Through Team Science

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One of the major goals for research on adolescent development is to identify the optimal conditions for adolescents to grow up in a complex social world and to understand individual differences in these trajectories. Based on influential theoretical and empirical work in this field, achieving this goal requires a detailed understanding of the social context in which neural and behavioral development takes place, along with longitudinal measurements at multiple levels (e.g., genetic, hormonal, neural, behavioral). In this perspectives article, we highlight the promising role of team science in achieving this goal. To illustrate our point, we describe meso (peer relations) and micro (social learning) approaches to understand social development in adolescence as crucial aspects of adolescent mental health. Finally, we provide an overview of how our team has extended our collaborations beyond scientific partners to multiple societal partners for the purpose of informing and including policymakers, education and health professionals, as well as adolescents themselves when conducting and communicating research.

Keywords: adolescence, brain development, social development, mental wellbeing, team science

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INTRODUCTION

Adolescence is a developmental phase between the ages of 10 and 24 years (Sawyer et al., 2018). Adolescence starts with puberty, setting off a cascade of hormonal changes signaling the start of biological maturation (Dahl et al., 2018), and is characterized by major physical, psychological, and social changes (Blakemore and Mills, 2014). Adolescents navigate an increasingly complex social network in which peer relations become more salient and are an important source for social learning (e.g., learning about, with, and from peers to adjust to changing social environments; Westhoff et al., 2020a). Both peer relations and social learning have a great impact on mental well-being (Nelson et al., 2005, 2016; Vitaro et al., 2009). Moreover, adolescence is considered a period of heightened sensitivity to mental health problems, with approximately 75% of adult mental health problems first appearing during adolescence (Kessler et al., 2007; Solmi et al., 2021).

As biological, psychological, and social changes occur concurrently in adolescence, it is crucial to understand how these changes are intertwined and contribute to successful developmental outcomes, such as resilience and mental health, as well as to maladaptive outcomes, such as risky behaviors and psychopathology (Davey et al., 2008; Crone and Dahl, 2012; Güroğlu, 2021). We further argue that understanding adolescence as a developmental phase with risks and opportunities requires incorporating a transactional perspective with measurements at multiple levels (genetic, hormonal, neural, behavioral) and across different social settings (e.g., school, parent relationships, peer relationships; see **Figure 1**). Considering the multitude of factors influencing development and the interlinked complexity of their corresponding measurement levels, we propose that team science is a fruitful approach to understanding the dynamics of adolescent development.

To understand individual differences in optimal conditions for growing up in an increasingly complex social world, we use a variety of neurobiological and behavioral methods. Current influential models of adolescent brain development describe an asynchronous development of the limbic "socio-affective system" and cortical "cognitive control system" during adolescence (Steinberg, 2008; Somerville et al., 2010). These models emphasize that faster maturation of the limbic system compared to the slower maturation of the cortical system underlies heightened reward sensitivity and risk-taking tendencies, leading to risky and impulsive behaviors such as alcohol use (Peters et al., 2017). Recent accounts of adolescent development also include the impact of individual differences in hormonal, genetic, behavioral, and neural influences which are intertwined in a social context (Crone and Dahl, 2012; Pfeifer and Allen, 2021). Specifically, adolescence is seen as a time for heightened goal flexibility, where social goals can influence pathways for development. Hence, the asynchronous development between the limbic and cortical system, together with increasingly complex and influential social experiences, such as peer relations and social learning, makes adolescence a sensitive window for socio-affective development which can lead to multiple pathways, such as risky behaviors and mental illness, or prosocial behavior and mental resilience (Crone and Dahl, 2012; Güroğlu, 2021; see **Figure 1**).

In this article, we highlight the unique position of our highly collaborative multidisciplinary research program and focus on its contributions to the field. We provide an overview of our research focusing on: (1) meso level peer relations; and (2) micro level social learning and next describe their combined influence on mental health. We propose that these two themes are crucial in charting the complexity of the dynamically interlinked biological, psychological, and social changes in adolescence. We provide examples of our research designs with controlled experimental settings at the micro-level and assessments of real-life social relationships at the meso level (see **Figure 1**). Finally, we demonstrate how collaborations can be extended to multiple societal partners to inform and include policy makers, education and health professionals, and adolescents themselves when conducting and communicating research. We conclude that understanding the complex dynamics of adolescent development requires rich measurements and we highlight the promising role of team science in achieving this goal.

Peer Relations

Adolescence is characterized by a significant shift in focus from parents toward peers, also referred to as social reorientation (Nelson et al., 2005). Compared to children, adolescents spend increasingly more time without adult supervision and in the company of their peers, where fitting in the peer group and peers' opinions become vital for adolescents' self-identity development (Laursen and Veenstra, 2021). Social goals, such as acceptance by the peer group and forming and maintaining friendships, are particularly important in the school context, as they are consistently linked with markers of positive social adjustment and academic achievement (Dawes, 2017). Recently, evidence from neuroimaging studies corroborate the significance of the peer context for adolescents by finding that adolescents show heightened neural responses during social decision-making in brain regions related to reward and motivation, such as the ventral striatum, and in social cognition, such as dorsomedial prefrontal cortex (for reviews see Van Hoorn et al., 2019; Andrews et al., 2021). For example, in early- and mid-adolescence, mere peer presence, when being observed by an unfamiliar peer, results in heightened neural activation in the medial prefrontal cortex (mPFC; Somerville et al., 2013), and when being observed by a friend, adolescents show increased risk-taking behavior, with heightened activation of the ventral striatum (Chein et al., 2011). In an Event-Related Potential (ERP) study, we have shown that manipulation of participants' social rank (high vs. low rank) modulated neural responses during social exchanges in mid-adolescents but not in children or adults, signifying that even transient social interactions are particularly salient for mid-adolescents (Zanolie and Crone, 2021). In another study, compared to being alone, the presence of a group of spectators (consisting of adolescent confederate actors) led to increased prosocial behavior which was accompanied by enhanced activation in social brain areas such as mPFC, temporal parietal junction (TPJ), precuneus and superior temporal sulcus (STS; Van Hoorn et al., 2016). Taken together, these studies of peer presence illustrate the importance of capturing the peer context when studying adolescents' social behavior (**Figure 1**, micro level).

Neuroimaging studies aiming to capture real-life peer context (**Figure 1**, meso level), however, face the challenge of bringing peer relationships into the highly controlled experimental laboratory setting, such as in the MRI scanner (see Güroğlu and Veenstra, 2021 for a more extensive review of this research line). In tackling this challenge, sociometric assessments of the peer network provide a useful tool to classify an individual's peer status within a real-life peer group (see **Box 1**). Combinations of sociometric assessments with neuroimaging and/or economic exchange paradigms assessing social decision-making (see **Box 2**) led to insights into how social interactions and their neural underpinnings may depend on peer context (Güroğlu et al., 2014). For example, we found in adults that interactions with familiar peers relate to heightened activation of brain

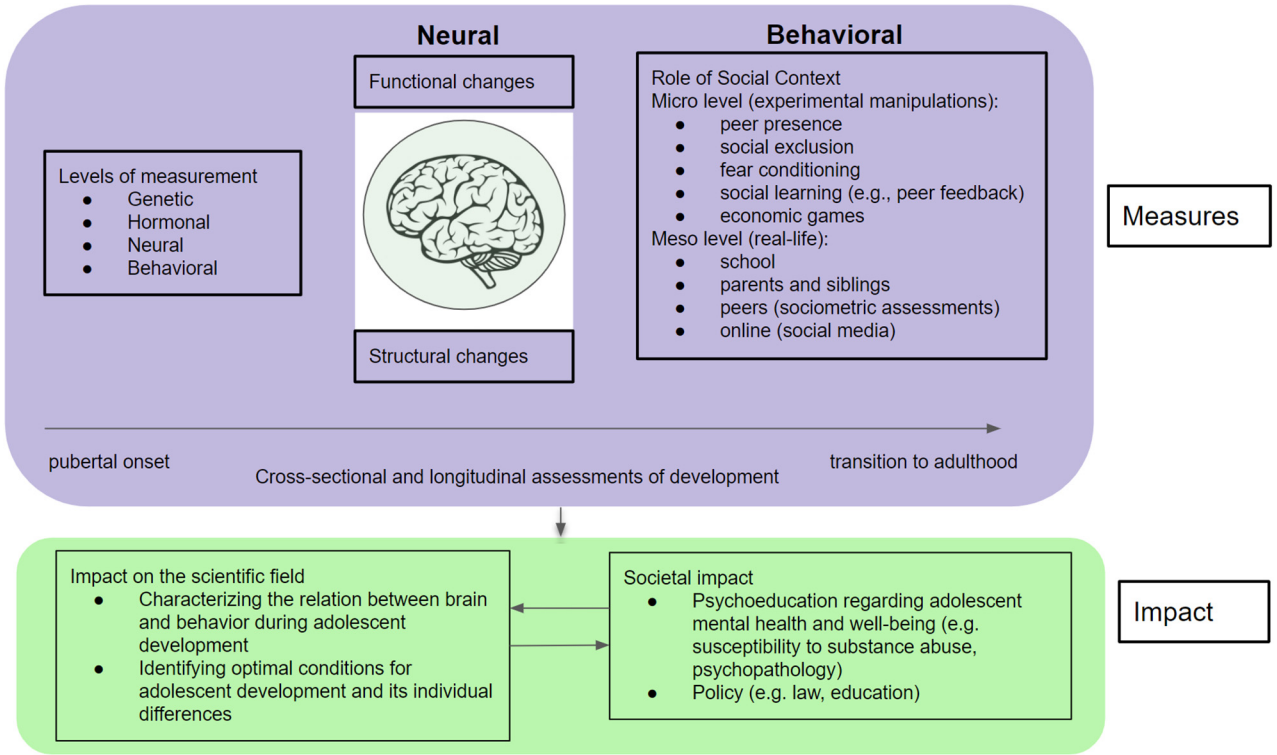

FIGURE 1 | Overview of the measures required to chart the complexity of developmental changes during adolescence and their impact. Note: This figure illustrates the richness of measures needed in studies that aim to capture the complexity of developmental changes during adolescence (purple). Findings from such studies will subsequently have a scientific and societal impact (green). Impact on the scientific field and on society are interlinked, as collaborating and communicating with societal stakeholders (e.g., policymakers, teachers, parents, and adolescents) also informs new research questions.

regions of affect and reward (including the ventral striatum and amygdala) and social cognition (including the mPFC, TPJ, STS, and precuneus; Güroğlu et al., 2008). Recently, we showed that the developmental trajectories of ventral striatum responses to rewards are modulated by friendship stability across a 5-year period (Schreuders et al., 2018) and that ventral striatum responses to winning money are also (negatively) related to acceptance by the peer group (Meuwese et al., 2018). We also showed that in young adults (Schreuders et al., 2018) and mid-adolescents (Schreuders et al., 2019), prosocial decisions toward friends compared to disliked or unfamiliar peers, are related to increased activation of the putamen, part of the reward circuitry, and the posterior temporoparietal regions that are involved in social-cognitive processes. Moreover, neural responses to social rejection depend on the excluder's peer status relative to the adolescent's own status (De Water et al., 2017). Our longitudinal studies further showed that the history of peer experiences across childhood modulates neural responses to social exclusion and during social decision-making in adolescence (Will and Güroğlu, 2016; Will et al., 2016, 2018; Asscheman et al., 2019).

Taken together, increasing evidence shows both current and long-term patterns of social experiences with peers modulate adolescent social behavior and their underlying neural processes (Güroğlu, 2022). In order to understand the developing brain in a social context, future studies need to incorporate measures of social networks with assessments of brain function and structure (Lamblin et al., 2017; Baek et al., 2021). Additionally, the complexity of social dynamics has in recent years only been amplified through the addition of the online social layer where young people can have meaningful connections. Future studies aiming to understand the dynamics of adolescent development need to include assessments of both offline and online connections.

Social Learning

Peer relations interact with individual and social learning. Social learning encompasses learning about, with, and from others. In the peer context, it involves learning about the characteristics and preferences of a peer or a peer group, such as their trustworthiness or cooperativeness (Nelson et al., 2005; Blakemore and Mills, 2014; Sawyer et al., 2018). Adaptive social behavior requires adolescents to learn about these characteristics and adjust their own behavior accordingly, such as learning when to be prosocial and towards whom (Steinberg and Morris, 2001; Van den Bos et al., 2011; Lockwood et al., 2016; Crone and Fuligni, 2020). These social learning processes are crucial for fostering healthy relationships with peers, which are predictive of adolescents' long-term well-being (Paus et al., 2008; Crone and Dahl, 2012; Dahl et al., 2018; Sawyer et al., 2018).

BOX 1 | Using sociometric assessments to study social experiences.

Sociometric assessments based on nominations of classmates on various criteria (e.g., "who do you like?", "who do you dislike?", "who are your friends?") are most valuable for assessing social experiences, and more specifically for assessing peer relationships, in an efficient manner. Crucially, these social assessments require access to a closed network (e.g., classmates, sports team, an orchestra) where the nominations can be made. These nominations can be used to assess social experiences at two levels. At the dyadic level, they reveal reciprocal relationships, such as mutual friendships between two people who nominate each other as a friend (see, e.g., Güroğlu et al., 2008; Schreuders et al., 2019). At the group level, they reveal information on the status of the individual within the peer group, such as accepted or rejected status based on the total number of received nominations from like and dislike nominations (Will et al., 2016; Will and Güroğlu, 2016). Finally, by applying graph theory, these nominations can be used to calculate social network characteristics that can be used to characterize individuals' positions in the network, such as centrality, as well as identifying group level characteristics, such as social cohesion (Van den Bos et al., 2018). An increasing number of studies combine assessments of peer relations with neuroscientific designs to investigate how peer relations modulate brain activity (see for review Güroğlu and Veenstra, 2021).

BOX 2 | Using economic games to study social interactions.

Economic games create social contexts that reveal fundamental aspects about the participant's social preferences in ways that are quantifiable. They have proven highly efficient in assessing various forms of (pro) social behavior both in adults and in development (Camerer, 2011; Will and Güroğlu, 2016). These paradigms are based on an economic exchange between at least two players where the participant's decisions have actual consequences for their own and their interaction partner's payoff. The simplest example is the *Dictator Game* in which the first player is given valued goods (e.g., money, toys, candies) and can share a portion of those goods with a second player. While game theoretical models assume that humans are rational players who aim to maximize their own profit, findings consistently show that people typically share some portion of the goods, thereby revealing other-regarding preferences. In the *Ultimatum game*, the first player (proposer) is a variant where the second player can either accept or reject the share given by the first player. If accepted, the goods are divided as proposed by the first player. If rejected, both players receive nothing. The reward maximizing strategy is to accept any offer greater than zero, but again consistent findings show that offers viewed as unfair are rejected (Fehr and Schmidt, 1999). Offers made by the first player are typically higher in the Ultimatum Game than Dictator Game, revealing strategic considerations to reduce the probability of rejection. Another variant is the *Trust Game*, in which the first player (investor) can again share a portion of goods with the second player (trustee). The portion received by the trustee is multiplied by the experimenters. The trustee then chooses to either share the profit with the investor (reciprocation; both profit from the exchange) or keep all the profit (betrayal; only the trustee profits, the investor loses the entrusted amount; Berg et al., 1995). These paradigms can be presented in a *repeated* fashion, such that participants play multiple rounds of these games with the same partner(s). Feedback received during these repeated interactions facilitates learning about the social preferences of other individuals or groups. Economic exchange paradigms are simple enough to administer to a wide age range (from 3 years old to adults) and enable studying developmental patterns in social behavior (Güroğlu et al., 2009; Meuwese et al., 2015; Zanolie et al., 2015; Ma et al., 2017). Moreover, their structured nature makes them further suitable for neuroimaging research.

Social learning is often studied with repeated behavioral economic paradigms, where participants play multiple rounds of an economic game with the same partner or multiple partners from one experimentally selected group (see **Box 2**). Peer characteristics or peer evaluations are typically experimentally manipulated, allowing participants to learn through positive and negative feedback (Ma et al., 2020; Westhoff et al., 2020b; Zanolie and Crone, 2021). Reinforcement learning models can then be used to characterize individual differences in learning strategies, learning speed, and the underlying cognitive processes that cause age differences in learning about others (Sutton and Barto, 2018; Nussenbaum and Hartley, 2019; Wilson and Collins, 2019). For example, we used an information sampling paradigm in which participants were able to sample information about a peer's history of trustworthiness before deciding to trust or not trust them (Ma et al., 2020). We found that behavioral adaptation to the gathered evidence improved with age, especially from early to mid-adolescence. In other studies, we manipulated the cooperativeness of groups (Westhoff et al., 2020b) and used a probabilistic learning task in which participants could sometimes earn rewards for themselves and sometimes for others (Westhoff et al., 2021). We found that probabilistic learning to benefit others showed age-related improvement across adolescence and was associated with ventromedial prefrontal cortex responses to unexpected outcomes. Learning for the self was stable across adolescence and associated with ventral striatal responses to unexpected outcomes. Together, these findings suggest that adolescents show rapid improvements in behavioral adjustments to the social environment, especially from early to mid-adolescence. These findings are consistent with the idea that learning about the consequences of actions in an interpersonal context is especially salient for adolescents and furthermore highlight early to mid-adolescence as a sensitive period for learning about others (Blakemore and Mills, 2014; Nelson et al., 2016; Sawyer et al., 2018; Andrews et al., 2021).

Considering that learning at school takes place in the peer context (i.e., in classrooms and group assignments), learning about, with, and from peers are also crucial research lines to identify the optimal conditions of learning at school. In ongoing studies, we focus on determining which individuals work well together by combining reinforcement learning or feedback processing paradigms with sociometric assessments. Such studies form the first steps of identifying optimal conditions for learning in the context of peers by informing how peer relationships in classrooms and differences in learning strategies between collaborating students may influence (social) learning.

Social Experiences and Mental Health

It is well-established that social experiences influence mental health and well-being. For example, close friendships during adolescence are a protective factor against mental health problems across adolescence and later in life (Van Harmelen et al., 2017, 2021). However, being rejected by peers is associated with self-harm (Esposito et al., 2019) and depressive symptoms (Platt et al., 2013). Also, epidemiological studies have shown a peak in the emergence of mental health problems across adolescence (Dalsgaard et al., 2020). Showing symptoms of psychopathology in childhood or adolescence are found to

be a key predictor of mental health problems and other adverse outcomes later in life (Zisook et al., 2007; Caspi et al., 2020). These findings indicate that there are developmental processes enhancing or bringing about vulnerabilities to develop mental health problems. Theoretical models propose a complex interplay between brain development, hormonal changes, and social development in interaction with environmental factors that may explain the emergence and maintenance of mental health problems across adolescence (Pfeifer and Allen, 2021). So far, only a few studies have directly linked the relationship between social context, brain development, and mental health outcomes. One study showed that greater subgenual anterior cingulate activity (sgACC) during a social exclusion game was associated with an increase in parent-reported depressive symptoms 1 year later (Masten et al., 2013). Social interactions with friends have also been related to activation of the sgACC and the ventral striatum. These brain regions are associated with the reward circuitry, speculatively providing indirect evidence linking positive peer interactions with mental health (Güroğlu et al., 2008; Schreuders et al., 2021). Research is needed to elucidate the complex interplay between brain development, social context, and mental health (Davey et al., 2008; Pfeifer and Allen, 2021). To better understand mental health and the transition from mental health to mental illness, our ongoing studies aim to contextualize individual differences in relation to social development and genetic factors (e.g., by using twin designs; Crone et al., 2020), and their association with mental health outcomes in a developmental context (Ferschmann et al., 2021).

Crucial in contextualizing individual differences in etiology and maintenance of mental health problems is investigating neurobiological mechanisms of psychopathology from a longitudinal perspective. Specifically, non-linear developmental changes in cortical and subcortical structures may explain why cross-sectional developmental neuroimaging studies may find mixed results depending on the age range of the participants (Wierenga et al., 2014a,b; Mills et al., 2021). With longitudinal designs, we, for example, found that heightened scores on externalizing symptoms were associated with smaller developmental changes in brain structure (Vijayakumar et al., 2014; Oostermeijer et al., 2016; Ambrosino et al., 2017; Bos et al., 2018a; but see Ducharme et al., 2011). Likewise, for internalizing symptoms, such as depression, longitudinal studies revealed associations with aberrant brain development (Whittle et al., 2014; Luby et al., 2016; Bos et al., 2018b). Together, these studies highlight the importance of longitudinal studies for understanding mental health problems and their development.

INTEGRATING SCIENCE AND SOCIETY

Integrating scientific knowledge about adolescent development into society can be achieved in multiple ways. Popular science books such as "The Adolescent Brain" (Het Puberende Brein, Crone, 2018) and "Inventing ourselves" (Blakemore, 2018) help to reach a wider audience, including policymakers ("science for policy"). In our team, we aim to reach adolescents *via* targeted websites designed for youth[1] and scientific articles for children (Westhoff et al., 2020a). We also inform youth professionals by designing educational material for elementary and high schools about the developing brain (e.g., http://www.breinkennisleiden.nl/onderwijs), and through our contributions to science-translation reports such as those on differences between boys and girls in learning (report Dutch Educational Council), and UNESCO's International Science and Evidence-based Education Assessment on the social-emotional learning (Gotlieb et al., 2022).

It is particularly important to include adolescents themselves when forming policies and designing interventions in order to make their participation efforts optimal and to contribute to their sense of autonomy which benefits their mental health (Fuligni, 2019). Especially during mid-adolescence interventions typically tend to fail when they do not align with adolescents' desired feeling to be respected and accorded status (Yeager et al., 2018). Peer-led interventions to generate positive behavioral changes can be powerful when the complexity of peer relations and social networks are taken into account as well as social learning (e.g., imitation, norms, and positive reinforcement; Veenstra and Laninga-Wijnen et al., 2022). The next step toward improving these efforts is setting up projects in which adolescents are involved in co-designing and co-creating research (Whitmore and Mills, 2021). Not only does this enrich the context in which scientific findings can be launched and interpreted, but crucially informs researchers in important ways, helping them to improve their research designs and paradigms. In our current projects, adolescent volunteers are also involved in disseminating knowledge to their peers, thereby increasing the likelihood that the information is relevant and interesting for the target audience (e.g., http://www.instagram.com/breinboost). Finally, in several innovative projects we involve societal stakeholders (e.g., teachers, adolescents, policymakers) in the research consortium from the start of the project and create research and knowledge dissemination projects together throughout the project (see e.g., http://www.neurolab.nl/startimpuls, Vandenbroucke et al., 2021).

CONCLUSIONS

In this perspectives article, we provided an overview of ways of characterizing developmental changes during adolescence and their relation to the developing brain. We highlighted the importance of capturing social contextual factors, as social experiences play a crucial role in shaping many developmental trajectories. We further emphasized the importance of longitudinal approaches in developmental studies in identifying predictors of mental health. The social context is increasingly important given that young people today grow up in a highly socially complex environment. The recent COVID-19 pandemic showed how strong the effects of the changing social context are on adolescents' mental health has been (Orben et al., 2020;

[1] http://www.kijkinjebrein.nl/

Van de Groep et al., 2020; Asscheman et al., 2021; Breaux et al., 2021; Green et al., 2021; Klootwijk et al., 2021). An increased understanding of the effect of social contextual factors on the development and neurobiological mechanisms underlying mental health will inform high stake policy questions, and find their way to daily practice. Throughout this overview, we illustrated the value of collaborative team science to understand adolescent development and the value of integration of science and society to be able to inform policy and practice.

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All authors co-designed the aims and wrote the article. KZ and IM designed the article aims, outline, figure, and integrated individual author contributions. All authors contributed to the article and approved the submitted version.

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Expansion of Clinical and Genetic Spectrum of *DDX3X* Neurodevelopmental Disorder in 23 Chinese Patients

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Aim: *De novo DDX3X* variants account for 1–3% of unexplained intellectual disability cases in females and very rarely in males. Yet, the clinical and genetic features of *DDX3X* neurodevelopmental disorder in the Chinese cohort have not been characterized.

Method: A total of 23 Chinese patients (i.e., 22 female and 1 male) with 22 *de novo DDX3X* deleterious variants were detected among 2,317 probands with unexplained intellectual disability (ID) undertaking whole exome sequencing (WES). The age, sex, genetic data, feeding situation, growth, developmental conditions, and auxiliary examinations of the cohort were collected. The Chinese version of the Gesell Development Diagnosis Scale (GDDS-C) was used to evaluate neurodevelopment of *DDX3X* patients. The Social Communication Questionnaire (SCQ)-Lifetime version was applied as a primary screener to assess risk for autism spectrum disorder (ASD).

Result: A total of 17 *DDX3X* variants were novel and 22 were *de novo*. Missense variants overall were only slightly more common than loss-of-function variants and were mainly located in two functional subdomains. The average age of this cohort was 2.67 (±1.42) years old. The overlapping phenotypic spectrum between this cohort and previously described reports includes intellectual disability (23/23, 100%) with varying degrees of severity, muscle tone abnormalities (17/23, 73.9%), feeding difficulties (13/23, 56.5%), ophthalmologic problems (11/23, 47.8%), and seizures (6/23, 26.1%). A total of 15 individuals had notable brain anatomical disruption (15/23, 65.2%), including lateral ventricle enlargement, corpus callosum abnormalities, and delayed myelination. Furthermore, 9 patients showed abnormal electroencephalogram results (9/23, 39.1%). Hypothyroidism was first noted as a novel clinical feature (6/23, 26.1%). The five primary neurodevelopmental domains of GDDS-C in 21 patients were impaired severely, and 13 individuals were above the "at-risk" threshold for ASD.

Interpretation: Although a certain degree of phenotypic overlap with previously reported cohorts, our study described the phenotypic and variation spectrum of 23 additional individuals carrying *DDX3X* variants in the Chinese population, adding hypothyroidism as a novel finding. We confirmed the importance of *DDX3X* as a pathogenic gene in unexplained intellectual disability, supporting the necessity of the application of WES in patients with unexplained intellectual disability.

Keywords: *DDX3X*, intellectual disability, *DDX3X* syndrome, neuronal development, X-linked intellectual disability

INTRODUCTION

DDX3X (OMIM: 300160) locates in Xp11.4 and encodes a conserved ATP-independent DEAD-box RNA helicase, which is involved in transcription, splicing, RNA transport, and translation (Abdelhaleem, 2005; Garbelli et al., 2011). The DDX3X is composed of 622 amino acid residues containing two functional domains, namely, a helicase ATP-binding domain and a helicase C-terminal domain (Snijders Blok et al., 2015). *De novo DDX3X* variants account for 1–3% of unexplained intellectual disability (ID) or developmental delay (DD) (Deciphering Developmental Disorders Study., 2017; Maulik et al., 2011) and also perform as a highly plausible pathogenic gene for childhood apraxia of speech (CAS) (Hildebrand et al., 2020). Most cases of *DDX3X* variants have been reported in females but very rarely in males, and three previous large cohort studies have described heterogeneous clinical manifestations of *DDX3X* neurodevelopmental disorder, including ID or DD, dystonia, movement disorders, microcephaly, behavioral issues, feeding difficulties in infancy, and seizure (Snijders Blok et al., 2015; Wang et al., 2018; Johnson-Kerner et al., 2020; Lennox et al., 2020). However, the clinical and genetic features of *DDX3X* neurodevelopmental disorder in the Chinese cohort have not been described yet.

In this study, we elaborated on clinical manifestations of pathogenic variants of *DDX3X* in 23 patients (i.e., 22 female and 1 male) in the Chinese cohort and explore the association between genotypes and phenotypes.

MATERIALS AND METHODS

Patients

With the support of the National Key Research and Development Program regarding the birth defect and developmental disorders screening (No. 2019YFC1005100), we collected whole exome sequencing (WES) data on 2,317 patients (1,622 males, 695 females, 5.33 $\pm$ 2.10 years old) with unexplained ID or DD and identified 23 *DDX3X* heterozygous variants in 23 patients by viewing those initial reports of WES. These patients further visited the Xiangya Hospital, Central South University, Hunan Provincial Maternal and Child Health Care Hospital, and Hunan Children's Hospital between March 2018 and December 2020. Basic demographic information and detailed clinical data, including perinatal conditions, gender, date at birth, family history, genetic data, feeding situation, growth, and developmental conditions, were recorded clearly. Electroencephalography (EEG) and brain MRI were re-reviewed and reanalyzed by two experienced neurological physicians, and they were blind to the genetic results.

Assessment

The Chinese version of the Gesell Development Diagnosis Scale (GDDS-C) was applied to assess the neurodevelopment of infants aged 0–6 years, and each participant calculated separate developmental quotient (DQ) of the five sub-domains, namely, adaptability, gross motor, fine motor, language, and social-emotional response. Based on the full-scale DQ results, the development of infants was classified as follows: normal (DQ $\geq$ 85), deficient (DQ $<$ 75), and borderline ($\geq$75 $\sim$ $<$ 85). DQ in any single domain below 75 was considered deficient in this field (You et al., 2019). The GDDS-C was conducted by medical professionals in child health clinics (Yang, 2016).

The Social Communication Questionnaire (SCQ) Lifetime version was a brief, 40-item, parent-report clinical tool, which had been widely used as a primary screener to assess risk for autism spectrum disorder (ASD). It was based on a semi-structured parent interview conducted by a trained clinician or researcher. Each item in SCQ required a dichotomous "yes"/"no" response, and each item received a value of 1 point for abnormal behavior. Complete developmental history was needed to be the reference. The caregivers would indicate whether behaviors of Questions 2–19 had ever been presented and whether behaviors of Questions 20–40 were presented at the age 4 or evaluated these behaviors in the past half a year if the child was aged less than 4. Scores above the cutoff of 12 suggested individuals were above the "at-risk" threshold for ASD, and further extended evaluations should be undertaken (Marvin et al., 2017).

Differences between average scores on 2 scales of this cohort and respective cutoff value were statistically evaluated using a one-sample *t*-test, *p*-values less than 0.05 ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$) were considered significant.

Genetic Analysis

We reanalyzed trio- or single WES data of all probands and their biological parents (19 for trio-WES). Sanger sequencing was conducted to validate whether the variant was *de novo*. The DDX3X transcript was referenced (NM_001193416.2, GRCh37/hg19). Sequenced reads were aligned to GRCh37/hg19 using the Burrows-Wheeler Aligner (BWA) (v.0.7.12) with default parameters. SAMtools (v0.1.12) was used to call the variants and the RefSeq Genomes database. The Genome Analysis Tool Kit (GATK 3.5) was used for local realignment

and base quality score recalibration. Synonymous changes and single-nucleotide polymorphisms with a minor allele frequency greater than 5% were removed.[1] Variant pathogenicity was interpreted based on the American College of Medical Genetics (ACMG) guidelines published in 2015 (Richards et al., 2015). The Genome Aggregation Database (GnomAD) was used to annotate the variants. Pathogenicity of the identified variants was predicted using several *in silico* predictors, including Polymorphism Phenotyping version 2 (Polyphen-2),[2] Protein Variation Effect Analyzer (PROVEAN),[3] Combined Annotation Dependent Depletion (CADD),[4] and Sorting Intolerant From Tolerant (SIFT).[5] Silico analysis data for missense DDX3X variants was shown in Supplementary material. Screening of neonatal genetic and metabolic diseases as a routine procedure was performed on all probands when they were born.

Ethical Issues

This research was approved by the Ethics Committee of XiangYa Hospital, Central South University (Location: Hunan Province, P.R. China, Approval No.: 2019030496). Written consents for inclusion in this study and rights to use portraits of each proband were obtained from parents of all participants.

RESULTS

Genomic Analysis

Among 2,317 individuals studied by WES who had unexplained ID, 23 deleterious variants in *DDX3X* were detected, 22 females were found to carry *de novo* variants in *DDX3X*, and 1 male was identified to carry an inherited variant in *DDX3X* from his asymptomatic mother. Furthermore, 17 were novel variants, and the remaining 6 variants were reported previously [c.1595C > T (Lennox et al., 2020); c.136C > T (Snijders Blok et al., 2015); c.865-1G > A (Wang et al., 2018); c.1703C > T (Snijders Blok et al., 2015); c.1463G > A (Lennox et al., 2020); c.1678_1680del (Snijders Blok et al., 2015)]. Of the 23 identified variants in *DDX3X*, 11 were missense variants, 2 were in-frame deletions, 2 were splice site variants, 5 were frameshift variants, and 3 were nonsense variants (**Figure 1A**). According to the guidelines set out by the ACMG, 22 variants were interpreted as pathogenic or likely pathogenic variants, and 1 variant was of uncertain significance (VUS) (**Table 1**).

All 23 identified variants were likely to cause changes in the DDX3X protein, 6 of which were in the helicase ATP-binding domain (i.e., p.Ala233del, p.P212L, p.R351G, p.R362G, p.A250Gfs*42, and p.C298* while 8 were in the helicase C-terminal domain (i.e., p.R488H, p.T532M, p.Leu560del, p.L560R, p.P568L, p. H527fs*9, p.S543*, and p.F545Ffs*2) (**Figure 1B**).

[1]http://www.ncbi.nlm.nih.gov/projects/SNP

[2]http://genetics.bwh.harvard.edu/pph2/

[3]http://provean.jcvi.org

[4]https://cadd.gs.washington.edu/

[5]http://sift.jcvi.org

Clinical Features

Table 2 shows the clinical features of 23 participants. The average age of this cohort was 2.67 (±1.42) years old. In the cohort of 23 patients with *DDX3X* variants, all of them meet the criteria for ID or DD (23/23, 100%), ranging from mild to severe. Muscle tone abnormalities (17/23, 73.9%), including isolated hypotonia, hypertonia, or mixture of hypertonia and hypotonia, microcephaly (9/23, 39.1%), feeding difficulties, or low weight gain (13/23, 56.5%), associated with ophthalmologic problems (11/23, 47.8%), were the most typical clinical characteristics. Movement disorders (9/23, 39.1%), seizures (6/23, 26.1%), behavior issues (5/23, 21.7%), cardiac abnormalities (3/23, 13.0%), and hearing impairment (2/23, 8.7%) were observed in this cohort. Furthermore, six patients presented with hypothyroidism (6/23, 26.1%).

Intellectual Disability or Developmental Delay

Only 2 patients (i.e., Female 14 and Female 18) could raise their heads at the age of 3 months, and 20 patients (except Female 7, Female 10, and Female 21) could not walk independently before the age of 2 years, all of them had poor motor coordination. All parents complained their children showed poor performance in language or speech function. Nearly 50% could express their simple needs in no more than four words and only say several single words. The two eldest individuals above 5 years (i.e., Female 13 and Female 16) had not developed speech ability but could just follow simple instructions.

Seizures and Electroencephalography Monitoring

All of them underwent scalp EEG monitoring, and 9 of them showed abnormal profiles. Slow background activity was observed in 7 out of 23 patients. Focal epileptiform discharges were detected in two patients, and generalized spike waves and sharp waves were detected in four patients. Hyperarrhythmia, associated with multifocal epileptiform discharges, was prevalent in one patient. In 6 individuals with seizures (i.e., Female 1, Female 9, Female 11, Female 16, Female 17, and Female 19), their age at the onset of seizures ranged from 5 to 14 months. Atonic seizures occurred in 1, absence seizures in 3, focal partial seizures in 1, and infantile spasms in 1. Female 17 was diagnosed with infantile spasms induced by fever at the age of 5 months and recurred at the age of 13 months. They had a good response to antiepileptic drugs and no seizures in 6 months. **Figure 1C** shows the latest EEG of Female 17.

Feeding Difficulties

Among the 13 individuals with feeding difficulties or low weight in our cohort, 8 of their parents reflected that they were intolerant of lactose and allergic to multiple high-protein food sources. Constipation and chewing weakness were common manifestations in 10 cases. Furthermore, 7 of 13 individuals were vitamin B-deficient, but none of them was hyperhomocysteinemia.

Endocrine Abnormalities

Thorough endocrine hormone examinations were conducted in 23 patients because of poor growth and neurocognitive

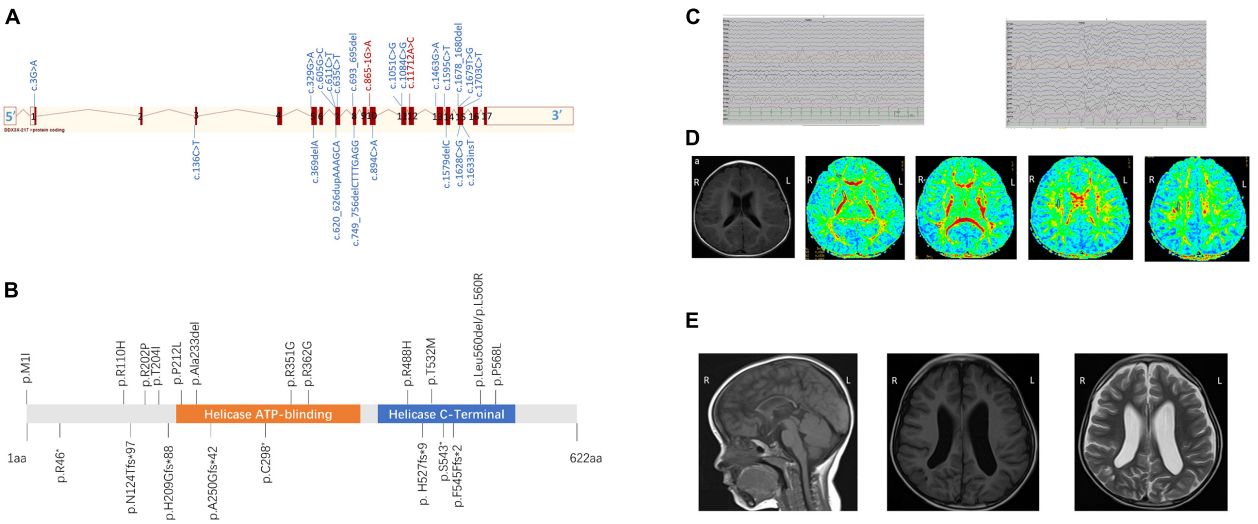

FIGURE 1 | (A) Schematic view of the *DDX3X* exon structure based on NM_001193416. Red blocks represent exons, and the exon number is listed on each exon. cDNA change is listed for each variant. Splice site mutations are shown in red font. **(B)** Location of amino acid substitutions in DDX3X (NM_ 001193416.2). Missense and in-frame deletions (top, 13), frameshift, and non-sense variants (bottom, 8). DDX3X contains two subdomains, a helicase ATP-binding domain (orange bar) and a helicase C-terminal domain (blue bar). **(C)** The latest electroencephalogram (EEG) and of Female 17. Abnormal EEG presentation, multiple slow waves in bilateral occipital lobes. **(D)** Brain magnetic resonance imaging (MRI) of Female 19 at the age of 1 year and 1 month **(A–E)** Axial position T1-weighted images show normal sulci and gyri, Axial position diffusion tensor imaging (DTI) images show delayed of white matter of frontal lobe and centrum semiovale myelination. Numbers 1–8 represent genu of the corpus callosum, white matter of the frontal lobe, anterior limb of the internal capsule, posterior limb of the internal capsule, splenium of the corpus callosum, occipital lobe, and centrum semiovale (7 and 8), respectively. **(E)** Sagittal image shows diffuse thinning of the corpus callosum of Female 13 at the age of 1 year and 3 months **(A)**. MRI **(B)** T1 and **(C)** T2 axial slices showed widened bilateral lateral ventricles of Female 6 at the age of 3 years and 10 months.

development. None of them showed abnormal sex hormone levels. Furthermore, six patients had lower levels of thyroid hormones (THs), and thyroid ultrasound showed normally located thyroid glands. They all received TH supplementation.

Magnetic Resonance Imaging Findings

All patients completed MRI at different ages. A total of 15 individuals had notable brain anatomical disruption in which, 10/15 had a lateral ventricle enlargement, 2/15 had corpus callosum abnormalities, 2/15 had delayed myelination, and 1/15 had white-matter volume reduction. **Figure 1D** shows brain MRI of Female 19, and (**Figure 1E**) shows brain MRI of Female 6 and Female 13.

Assessments

A total of 21 patients have undergone GDDS-C and were counted DQ in five separate domains. The highest scored domain was "gross motor" (56.1 ± 17.2), and the lowest scored domain was "language" (49.1 ± 13.2). The remaining scored domains were "fine motor" (56.1 ± 17.2), "personal-social" (56.0 ± 14.2), and "adaptability" (52.7 ± 12.7) successively. Scores in all 5 domains were significantly below the critical value (75), in which p-value was < 0.01. DQ of each participant is listed in **Figure 2**.

A total of 17 patients completed the SCQ-Lifetime version, and 13 individuals who were above the cutoff value (12) were at the risk of ASD, a comprehensive evaluation for ASD was warranted. Significant deviation was found in the mean score in the SCQ-Lifetime version of 17 participants (16.1 ± 4.4, $p = 0.002$) compared with the cutoff value.

DISCUSSION

In this report, we described a Chinese cohort of patients with *DDX3X* variants (n = 23, 22 confirmed *de novo*), and we are the first study to pinpoint clinical and genetic characteristics in the Chinese population. Among the 23 *DDX3X* variants in our cohort, 17 were novel variants and 14 variants were located in two functional domains of DDX3X (9 were amino acid variants and 5 were truncating variants). Significant differences in sex composition of *DDX3X* neurodevelopmental disorder have been noted (22 female and 1 male). ID was considered as a universal feature of *DDX3X* neurodevelopmental disorder in our study, followed by tone abnormalities, microcephaly, feeding difficulties, and seizures. Major aspects of neural development were assessed and quantified using the GDDS-C, and mean scores of five domains were significantly lower than the critical value of 75 (all p-value < 0.05), and language domain was impaired strikingly. Hypothyroidism was reported in 6 patients with *DDX3X* variants for the first time. Altogether, our study expanded the clinical and genetic spectrum associated with *DDX3X* neurodevelopmental disorder and evaluated the degree of developmental delay by a standardized scale. It highlighted that WES was necessary for those unexplained ID individuals.

DDX3X, an RNA-binding protein of the DEAD-box family encoded by the *DDX3X* gene (Abdelhaleem, 2005), acts as a translational regulator (Lai et al., 2008; Lee et al., 2008), particularly for mRNAs with highly structured 5′ untranslated regions (UTRs) (Chen et al., 2018) and for

TABLE 1 | Clinical interpretation of variants detected in *DDX3X* by the ACMG guideline.

Patient	Genotype	Inheritance	Variant (NM_001193416)	Evidence of pathogenicity based on ACMG guideline				Category
				Very strong	Strong	Moderate	Supporting	
Female 1	Het	*De novo*	c.1084C > G p.(R362G)	/	PS2	PM1 + PM2 + PM5	PP3	Pathogenic
Female 2	Het	*De novo*	c.635C > T; p.(P212L)	/	PS2	PM1 + PM2 + PM5	PP3	Pathogenic
Female 3	Het	*De novo*	c.1579delC p.(H527fs*9)	PVS1	PS2	PM2	/	Pathogenic
Female 4	Het	*De novo*	c.1171-2A > C; ?	PVS1	PS2	PM2	/	Pathogenic
Female 5	Het	*De novo*	c.369delA; p.(N124Tfs*97)	PVS1	PS2	PM2	/	Pathogenic
Female 6	Het	*De novo*	c.1051C > G; p.(R351G)	/	PS2	PM1 + PM2	PP3	Likely pathogenic
Female 7	Het	*De novo*	c.611C > T; p.(T204I)	/	PS2	PM2	PP3	Likely pathogenic
Female 8	Het	*De novo*	c.1595C > T; p.(T532M)	/	PS1 + PS2	PM1 + PM2	PP3	Pathogenic
Female 9	Het	*De novo*	c.749_756del CTTTGAGG; p.(A250Gfs*42)	PVS1	PS2	PM2	/	Pathogenic
Female 10	Het	*De novo*	c.136C > T; p.(R46*)	PVS1	PS2	PM2	/	Pathogenic
Female 11	Het	*De novo*	c.865-1G > A; ?	PVS1	PS2	PM2		Pathogenic
Female 12	Het	*De novo*	c.693_695del p.(Ala233del)	/	PS2	PM2 + PM4	PP3	Likelypathogenic
Female 13	Het	*De novo*	c.894C > A; p.(C298*)	PVS1	PS2	PM2	/	Pathogenic
Female 14	Het	*De novo*	c.1633insT; p.(F545Ffs*2)	PVS1	PS2	PM2	/	Pathogenic
Female 15	Het	*De novo*	c.1678_1680del; p.(L560del)	/	PS2	PM1 + PM2 + PM4	PP3	Pathogenic
Female 16	Het	*De novo*	c.1703C > T; p.(P568L)	/	PS1 + PS2	PM2	PP3	Pathogenic
Female 17	Het	*De novo*	c.1679T > G; p.(L560R)	/	PS2	PM1 + PM2	PP3	Likely pathogenic
Female 18	Het	*De novo*	c.1463G > A; p.(R488H)	/	PS1 + PS2	PM1 + PM2	PP3	Pathogenic
Female 19	Het	*De novo*	c.620_626 dupAAAGCA; p.(His209Glnfs*88)	PVS1	PS2	PM2	/	Pathogenic
Female 20	Het	*De novo*	c.3G > A; p.(M1I)	PVS1	PS2	PM2	/	Pathogenic
Female 21	Het	*De novo*	c.1628C > G; p.(S543*)	PVS1	PS2	PM2	/	Pathogenic
Female 22	Het	*De novo*	c.605G > C p.(R202P)	/	PS2	PM2	PP3	Likely pathogenic
Male 1	Het	Inherited from his mother	c.329G > A; p.(R110H)	/	/	PM2	PP3	Variant of Uncertain Significance

ACMG, American College of Medical Genetics; PVS, pathogenic very strong; PS, pathogenic strong; PM, pathogenic moderate; PP, pathogenic supporting.

repeat-associated non-AUG translation (Cheng et al., 2019; Linsalata et al., 2019). DDX3X is also the key component of ribonucleoprotein (RNP) granules composed of mRNA and protein (Huang et al., 2019), a pathological hallmark of many neurodegenerative diseases (Ramaswami et al., 2013). Dd3x plays an indispensable role in mouse embryogenesis, synaptogenesis, and brain development (Chen et al., 2016; Lennox et al., 2020). Dd3x neural stem cells knockout at embryonic day (E) 9.5 in mice hampered brain growth, accompanied by seizures and ataxia (Patmore et al., 2020). Boitnott et al. (2021) generated a Ddx3x haploinsufficient mouse (Ddx3x$^{\pm}$ female) with construct validity for *DDX3X* loss-of-function mutations. The Ddx3x$^{\pm}$ mice showed global development delay and evolved into behavioral anomalies in adulthood (Boitnott et al., 2021).

Comparing with three previously reported cohort studies of *DDX3X* neurodevelopmental disorder, we found a certain degree of phenotypic overlap but some special points (Snijders Blok et al., 2015; Wang et al., 2018; Lennox et al., 2020; **Table 3**). ID was still a general phenotypic feature of patients with *DDX3X* variants in both our study and previous reports. Similarly, compared with healthy controls, the mean score in GDDS-C of the cohort could reflect global DD. The worst performance in the language subdomain of GDDS-C consolidated language impairments as the most prominent clinical feature of *DDX3X* neurodevelopmental disorder (Johnson-Kerner et al., 2020). Ophthalmologic problems including refractive errors, nystagmus, strabismus, and amblyopia were presented in 11/23 (47.8%) patients. Previous studies have shown that a variety of eye phenotypes including hypoplasia of the eye or absence of

TABLE 2 | Summary of demographic information and phenotypic features in 23 patients with *DDX3X* variants.

Patient	Female 1	Female 2	Female 3	Female 4	Female 5	Female 6	Female 7	Female 8	Female 9	Female 10	Female 11
Current age (years, months)	2y3m	1y7m	1y9m	1y	4y	4y3m	1y	3y5m	3y6m	3y	2y4m
Perinatal conditions	Normal	Normal	Normal	Normal	MLBW	Normal	Normal	Normal	Normal	Normal	Normal
ID/DD	+	+	+	+	+	+	+	+	+	+	+
Weight	−SD	−3SD	−3SD	−2SD	Normal	Normal	−SD	−2SD	−2SD	Normal	−3SD
Height	−2SD	Normal	Normal	Normal	+ SD	Normal	Normal	−SD	Normal	+ SD	−SD
Speech	Single words	Minimally verbal	Single words	Minimally verbal	Single words	Single words	Single words	Minimally verbal	Single words	Single words	Minimally verbal
Age at walking	2y2m (with rollator)	No	No	No	3	1y5m (wide base gait)	No	2y9m (wide base gait)	1y8m	No	No
Tone abnormalities	Hypertonia	Normal	Hypotonia	Mixture	Hypotonia	Mixture	Hypotonia	Hypotonia	Normal	Normal	Hypertonia
Movement disorders	+ ataxia	No	No	No	No	+ abnormal gait	No	+ abnormal gait	+ dystonia	No	+ dyskinesia
Seizures	Absence seizures	No	No	No	No	No	No	No	Atonic seizures	No	Absence seizure
Microcephaly	No	No	No	No	No	No	No	No	+, −2SD	+, −3SD	+, −2SD
Ophthalmologic problems	Refractive errors	No	No	No	Amblyopia	Refractive errors	No	No	Refractive errors	Refractive errors	Refractive errors
Behavior issues	No	No	No	No	ASD	No	Hyperactivity	No	No	No	ASD
SCQ Lifetime	20	NA	NA	13	24	13	NA	22	17	10	21
Others	PFO	Feeding difficulties	Constipation	Constipation, Inability to chew; Hearing impairment	No	No	Feeding difficulties	Feeding difficulties, constipation	Feeding difficulties, constipation	No	Feeding difficulties; constipation
Endocrine abnormalities (TSH level)	Hypothyroidism (8.97 mIU/L)	No (Normal)	No (Normal)	Hypothyroidism (16.53 mIU/L)	No	No	No	No	No	No	Hypothyroidism (9.21 mIU/L)
MRI findings	Ventricular enlargement	Ventricular enlargement	Ventricular enlargement	Normal	Normal	Ventricular enlargement	Ventricular enlargement	White matter volume reduction; subdural effusion	Ventricular enlargement	Normal	Normal

(Continued)

TABLE 2 | (Continued)

Patient	Female 12	Female 13	Female 14	Female 15	Female 16	Female 17	Female 18	Female 19	Female 20	Female 21	Female 22	Male 1
Current age (years, months)	1y4m	6y10m	1y4m	2y	5y	2y10m	3y	3y	1y8m	1y10m	1y10m	1y4m
perinatal conditions	Normal	Normal	Normal	MLBW	MLBW	Normal	Normal	Neonatal jaundice	Normal	Normal	Normal	Neonatal jaundice
ID/DD	+	+	+	+	+	+	+	+	+	+	+	+
Weight	−SD	Normal	−SD	−3SD	−2SD	−3SD	−SD	−SD	−2SD	Normal	Normal	−2SD
Height	Normal	Normal	−SD	Normal	−2SD	−2SD	Normal	Normal	Normal	+ 2SD	−SD	−SD
Speech	Single words	Single words	Single words	Minimally verbal	Minimally verbal	Minimally verbal	Minimally verbal	Single words	Minimally verbal	Single words	Minimally verbal	Single words
Age at walking	No	No	No	No	No	No	No	No	No	1y8m	No	No
Tone abnormalities	Hypertonia	Hypotonia	Normal	Hypotonia	Mixture	Hypotonia	Hypotonia	Normal	Hypotonia	Normal	Hypotonia	Hypotonia
Movement disorders	+ dyskinesia	No	+ ataxia	No	+ dystonia	No	No	No	No	+ dystonia	No	No
Seizures	Absence seizures	No	No	No	Focal partial seizure	Infantile spasms	No	Absence seizures	No	No	No	No
Microcephaly	No	No	+, −2SD	+, −2SD	+, −2SD	+, −2SD	No	+,−2SD	+, −2SD	No	No	No
Ophthalmologic problems	No	No	Nystagmus	No	No	Strabismus	Refractive errors	No	No	No	Amblyopia	Refractive errors
Behavior issues	No	No	No	No	No	No	No	No	Hyperactivity	No	No	ASD
SCQ lifetime	NA	11	NA	13	17	20	18	14	NA	10	12	19
Others	No	Constipation	Constipation	PFO; hearing impairment; feeding difficulties, constipation	Inability to chew	Inability to chew	Normal	Feeding difficulties, constipation	Feeding difficulties, constipation	Feeding difficulties, constipation	Atrial septal defect; inability to chew	Feeding difficulties; constipation
Endocrine abnormalities (TSH level)	No	No	No	Hypothyroidism (7.74 mIU/L)	Hypothyroidism (5.8 mIU/L)	No	No	Hypothyroidism (8.35 mIU/L)	No	No	No	No
MRI findings	Delayed myelination	Corpus callosum abnormalities	Normal	Ventricular enlargement	Corpus callosum abnormalities	Ventricular enlargement	Ventricular enlargement	Delayed myelination	Normal	Normal	Normal	Ventricular enlargement

ID, intellectual disability; DD, developmental disability; MLBW, mature low birth weight; Mixture, Mixed hypo and hypertonia; PFO, patent foramen ovale; ASD, atrial septal defect; SD, standard deviation; + , positive; NO, negative; NA, not available; TSH, thyroid-stimulating hormone.

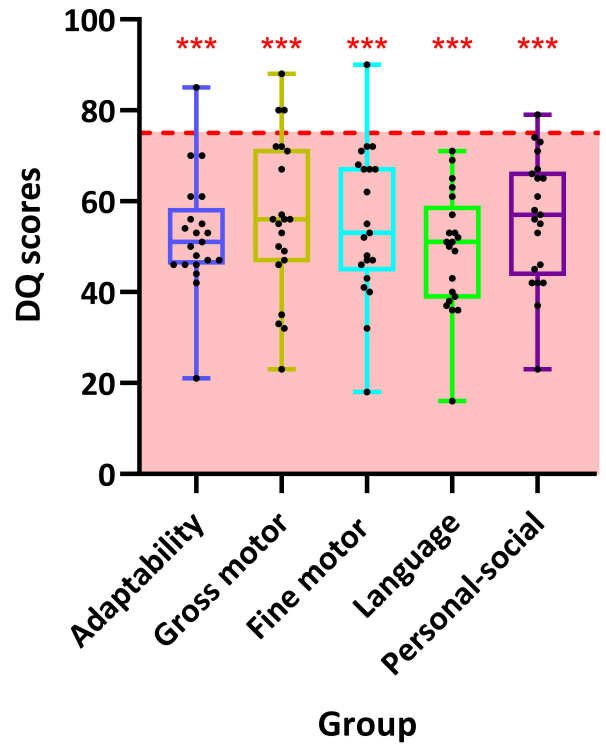

FIGURE 2 | Distribution of developmental quotient (DQ) scores of 19 individuals in the five sub-domains of the Chinese version of Gesell Development Diagnosis Scale (GDDS-C). The interquartile ranges are shown as a box-and-whisker plot. For one-sample *t*-test, the critical value is 75 (*** represents $p < 0.001$).

one or both eyes in functional studies of ddx3x knockdown in zebrafish, suggesting deleterious variants in *DDX3X,* may hamper eye function (Snijders Blok et al., 2015; Kellaris et al., 2018). The SCQ-Lifetime version scores indicated an increased risk for ASD in *DDX3X* neurodevelopmental disorder, suggesting the necessity of screening for behavior problems by trained behavioral pediatricians.

Furthermore, we noticed hypothyroidism in 6 patients. This novel or rare clinical feature was not previously reported in the original description of *DDX3X* neurodevelopmental disorder. Poor nutrient absorption and feeding difficulty could make them at increased risk of iodine deficiency, which could be an extrinsic factor causing hypothyroidism in these younger children (Bauer and Wassner, 2019). THs play an essential role in the growth and metabolic homeostasis in humans as well as in animals (Prezioso et al., 2018). Triiodothyronine (T3), the active form of thyroid hormone, acts on its nuclear receptor and modulates target gene transcription (Kumar et al., 2015). Even a 25% reduction in *DDX3X* levels strongly perturbs neurogenesis, suggesting the high dose-dependency of embryonic cortical development to DDX3X (Lennox et al., 2020). Defective RNA metabolism was considered as the potential mechanism through which *DDX3X* missense variants hamper fetal brain cortical development (Kumar et al., 2015; Lennox et al., 2020). We speculated that TH deficiency may intensify the adverse effect on RNA metabolism caused by *DDX3X* missense variants. Appropriate TH supplementation in *DDX3X* patients with hypothyroidism could be worth trying, but overtreatment or prophylactic hormonal therapy should be avoided because the higher dose of TH supplementation could worsen the outcome (Tuhan et al., 2016). Therefore, it is crucial to have a close follow-up in *DDX3X* patients with hypothyroidism. Hypothyroidism has not been verified in animal models with *in vivo* depletion of Ddx3x.

Many study reports have tried to establish the connection between the severity of clinical phenotypes and the location and type of variants and obtained two main findings (Lennox et al., 2020). First, the same recurrent *de novo* variants were more likely to have similar phenotypes. Recurrent amino acids changes, including R326, I415, and T532, all could cause polymicrogyria (PMG) (Abdelhaleem, 2005; Tuhan et al., 2016; Lennox et al., 2020). Besides the 11 individuals with PMG, 10 were missense variants and one was in-frame deletion, underscoring a striking association between missense variants and severe cerebral anatomical phenotypes, like PMG or dysgyria (Lennox et al., 2020).

Regrettably, no PMG or dysgyria was observed in our cohort, nor the previously reported *DDX3X* variants relating with PMG. However, we found that females with missense or in-frame deletion *DDX3X* variants (10/11, 90.9%) were more likely to have abnormal brain structural MRI compared with those with LOF variants (4/11, 36.3%). This could be interpreted by different pathogenic mechanisms. Many aberrant truncating mRNAs (i.e., frameshift or non-sense variants) might undergo non-sense-mediated RNA decay (NMD) and resulted in a haploinsufficiency effect, while a subset of missense variants could function in a dominant-negative manner (Chen et al., 2020; Lennox et al., 2020). Further investigations about gain-of-function mechanism behind certain missense mutations will be carried out by modeling missense mutations in mice. Multiple malignancies have a solid association with somatic *DDX3X* variants, like malignant melanoma and medulloblastoma (Phung et al., 2019; Patmore et al., 2020). Even though no malignancy was reported in our cohort yet, regular cancer screening is still quite necessary. Finally, a small sample size and a relatively small number of novel phenotypes, such as hypothyroidism, were the main limitations of this study.

In summary, we identified 23 unrelated Chinese patients with causal variants in *DDX3X* and expanded the knowledge of these increasingly recognized ID disorders. Our study delineated many clinical characteristics of the Chinese cohort with *DDX3X* variants, largely overlapping with phenotypic spectrum in previously reported studies, but hypothyroidism was first noted as a novel clinical feature. Overall, missense variants were only slightly more common than loss-of-function variants and were mainly located in two functional subdomains. The *DDX3X* missense variants may have a certain association with abnormal brain anatomical structures. Given the heterogeneous clinical

TABLE 3 | Comparison of clinical characteristics in our cohort and three previously published cohorts.

Clinical findings	Numbers (percentage,%)[our study]	Numbers (percentage,%) (Snijders Blok et al., 2015)	Numbers (percentage,%) (Wang et al., 2018)	Numbers (percentage,%) (Lennox et al., 2020)
Neurological				
Intellectual disability (ID) or developmental delay (DD)	23/23 (100%)	38/38 (100%)	28/28 (100%)	106/106 (100%
Hypotonia	11/23 (47.8%)	29/38 (76%)	19/28 (68%)	54/93 (58%)
Hypertonia	3/23 (10.7%)	N/A	2/12 (17%)	5/93 (5%)
Mixed hypo and hypertonia	3/23 (10.7%)	N/A[a]	N/A	31/93 (33%)
Movement disorders	9/23 (39.1%)	17/38 (45%)	17/28 (61%)	18/83 (22%)
Seizures	6/23 (26.1%)	6/38 (16%)	N/A	17/93 (18%)
Ophthalmologic problems	11/23 (47.8%)	13/38 (34%)	9/28 (32%)	29/92 (31.5%)
Microcephaly	9/23 (39.1%)	12/38 (32%)	7/28 (25%)	34/90 (38%)
Behavior issues	5/23 (21.7%)	20/38 (53%)	6/28 (21%)	N/A
Others				
Cardiac abnormalities	3/23 (13.0%)	N/A	5/27 (71%)	13/90 (14%)
Feeding difficulties or low weight	13/23 (56.5%)	12/38 (32%)	N/A	N/A
Hearing impairment	2/23 (8.7%)	3/38 (3%)	N/A	4/78 (5%)
Imaging findings				
Corpus callosum abnormalities	2/23 (8.7%)	13/37 (35%)	18/20 (90%)[b]	77/89 (87%)
Ventricular enlargement	10/23 (43.5%)	13/37 (35%)		61/89 (68%)
Cortical malformation	3/23 (13.1%)	4/37 (11%)		50/89 (56%)
Delayed myelination	2/23 (8.7%)	N/A		N/A

[a] *In Snijders Blok et al. (2015), movement disorders include spasticity.*
[b] *In Wang et al. (2018), imaging findings refer to structural brain abnormalities.*

manifestations and involvement of the nervous system and non-nervous systems, unexplained ID in both males and females should take the use of multigene panels that include *DDX3X* or WES into consideration.

DATA AVAILABILITY STATEMENT

Sequencing data involved in the study are available through the Genbank repository (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA795095). There are restrictions to the full availability of sequencing data of the research participants due to privacy and ethical/legal issues. The data that support the findings of this study are available from the corresponding author, upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of XiangYa Hospital, Central South University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YD, ZY, and LL: study design, analysis and revision of the manuscript. YD, ZY, and JGu: follow-up of patient's information. HL, JGo, and YX: reanalysis of WES data and original draft preparation. YD, BX, HW, and LL: collection of clinical and WES data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2022.793001/full#supplementary-material

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Enhanced Performance by Interpretable Low-Frequency Electroencephalogram Oscillations in the Machine Learning-Based Diagnosis of Post-traumatic Stress Disorder

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Electroencephalography (EEG)-based diagnosis of psychiatric diseases using machine-learning approaches has made possible the objective diagnosis of various psychiatric diseases. The objective of this study was to improve the performance of a resting-state EEG-based computer-aided diagnosis (CAD) system to diagnose post-traumatic stress disorder (PTSD), by optimizing the frequency bands used to extract EEG features. We used eyes-closed resting-state EEG data recorded from 77 PTSD patients and 58 healthy controls (HC). Source-level power spectrum densities (PSDs) of the resting-state EEG data were extracted from 6 frequency bands (delta, theta, alpha, low-beta, high-beta, and gamma), and the PSD features of each frequency band and their combinations were independently used to discriminate PTSD and HC. The classification performance was evaluated using support vector machine with leave-one-out cross validation. The PSD features extracted from slower-frequency bands (delta and theta) showed significantly higher classification performance than those of relatively higher-frequency bands. The best classification performance was achieved when using delta PSD features (86.61%), which was significantly higher than that reported in a recent study by about 13%. The PSD features selected to obtain better classification performances could be explained from a neurophysiological point of view, demonstrating the promising potential to develop a clinically reliable EEG-based CAD system for PTSD diagnosis.

Keywords: machine-learning technique, classification, computer-aided diagnosis, resting-state electroencephalogram (EEG), slow-frequency EEG oscillation, post-traumatic stress disorder (PTSD)

INTRODUCTION

Post-traumatic stress disorder (PTSD) is a psychiatric disorder caused by experiencing or witnessing traumatic events (Yehuda, 2002), and PTSD patients are diagnosed through interview with clinical experts based on the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5, note that all abbreviations were summarized in **Supplementary Table 1**) (Friedman et al., 2011). Post-traumatic stress disorder patients generally show a high rate of comorbidity with other mental illnesses, which leads to confusion of diagnosis (Ginzburg et al., 2010; Elhai et al., 2011; Gros et al., 2012). According to a previous study, 73.3% of veteran PTSD patients had comorbid anxiety disorders (e.g., general, panic, and social anxiety disorder) (Magruder et al., 2005). Another study reported that 68% of veteran PTSD patients met the criteria for major depressive disorder (MDD) (Grubaugh et al., 2010). These results imply that when interviewing with clinical experts, if PTSD patients hide their traumatic histories or symptoms, they could be misdiagnosed as other mental diseases with high probability. Thus, it is necessary to introduce a PTSD diagnosis tool to compliment the diagnostic failure rate of traditional diagnosis (Sumpter and McMillan, 2005).

In recent years, the machine-learning-based computer-aided diagnosis (CAD) system has received increased attention, due to its ability to predict the state of neuropsychiatric diseases using objective neurophysiological biomarkers (McBride et al., 2011; Mitra et al., 2016; Vergara et al., 2017, 2018). Early CAD systems developed for those suffering from traumatic events have used neuroimaging-based features for its diagnosis, but they have focused on patients with traumatic brain injury (TBI), rather than PTSD (McBride et al., 2011; Mitra et al., 2016; Vergara et al., 2017, 2018). For example, Mitra et al., achieved 68% classification accuracy when differentiating TBI from healthy controls (HC) using diffusion tensor imaging (DTI) features (Mitra et al., 2016), meanwhile, Vergara et al., reported a classification accuracy of 84% when differentiating TBI patients from HC using DTI and functional magnetic resonance imaging (fMRI)-based features (Vergara et al., 2017). However, although both TBI and PTSD are developed by traumatic events, TBI and PTSD should be studied independently because the cause and characteristics of TBI and PTSD are totally different, in that TBI patients are troubled with physical brain damage, while PTSD patients suffer from mental problems (Bryant, 2011).

A few studies have attempted to differentiate PTSD patients from HC using neuroimaging-based features. Rangaprakash et al. (2017) achieved 81% classification accuracy when classifying PTSD patients and HC using effective connectivity network features extracted from resting-state fMRI data. In addition, Zhang et al., introduced a classification model for PTSD diagnosis using magnetoencephalographic (MEG) connectomes, and reported the performance of an area-under-the-curve (AUC) value of 0.9 (Zhang et al., 2020). However, while fMRI and MEG have shown the potential to differentiate PTSD patients from HC, due to their high cost and low portability, they are not usable in practice for both clinicians and patients (Wienbruch et al., 2006). To overcome the mentioned limitations, electroencephalogram (EEG) could be an adequate alternative neuroimaging tool for the diagnosis of PTSD patients (Chen, 2001).

Electroencephalogram (EEG) is more portable than other neuroimaging tools, such as fMRI and MEG (Chen, 2001), and is also suitable to investigate dynamic neuronal changes due to its high-temporal resolution (Saletu et al., 1991; Burle et al., 2015). In particular, since abnormal neuronal changes in psychiatric patients reflect their pathophysiology (Newson and Thiagarajan, 2019), they could be used for the diagnosis of various psychiatric diseases (Orru et al., 2012). Therefore, many researchers have introduced EEG-based CAD systems for the diagnosis of various psychiatric disorders, and achieved promising classification accuracies when differentiating psychiatric disorders from HC (Orru et al., 2012). Recently, we introduced 2 EEG-based CAD systems to assist the accurate diagnosis of PTSD patients (Shim et al., 2019; Kim Y. W. et al., 2020).

In our previous study, we attempted to classify PTSD patients and HC using P300 event-related potential (ERP) based on machine-learning technique (Shim et al., 2019), and obtained a classification accuracy of 80% in differentiating 2 groups. Although we achieved acceptable classification accuracy, the previous study was limited in terms of its usability; PTSD patients were required to perform an auditory attention task to evoke P300 activation even though they generally have difficulty in concentrating on an attention-related task. To overcome this limitation, resting-state EEG could be an appropriate alternative because no effort is required to record resting-state EEG from PTSD patients; also, resting-state EEG reflects the pathophysiological traits of PTSD patients (McFarlane et al., 2005; Veltmeyer et al., 2006). Recently, we investigated the possibility of using resting-state EEG to distinguish PTSD patients from HC, where we employed 2 types of source-level features: (i) power spectrum densities (PSDs) and (ii) network indices based on graph theory (Kim Y. W. et al., 2020). We confirmed that PSD features showed significantly higher classification accuracy than network features, and obtained a maximum classification accuracy of 73.09%.

The objective of this study was to enhance the performance of classifying PTSD patients from HC by optimizing the frequency band used for extracting PSD features from resting-state EEG. Our previous study (Kim Y. W. et al., 2020) did not consider a delta-frequency band of 1–4 Hz even though slow EEG waves were closely related to the typical endophenotypes of PTSD patients (McFarlane et al., 2005; Veltmeyer et al., 2006; Newson and Thiagarajan, 2019), and an optimization of frequency bands was also not performed using traditional 6 frequency bands (delta, theta, alpha, low-beta, high-beta and gamma) in terms of classification performance. Therefore, in this study, we aimed to optimize PSD frequency bands by introducing the delta band and investigating all possible combinations of the 6 frequency bands in order to increase the classification performance between PTSD patients and HC. Furthermore, we investigated the neurophysiological meanings of most discriminable features selected to obtain the best classification performance, thereby contributing to the development of a reliable CAD system to

assist the diagnosis of PTSD patients. The main highlights of our contributions are briefly listed below:

i) Optimization of resting-state EEG-based features for assisting diagnosis of PTSD patients.
ii) Significant improvement of classification performance as compared to a recent study by about 13%.
iii) Interpretation of useful features selected to attain the best classification performance from a neurophysiological point of view, thereby providing the basis to develop a reliable CAD system for PTSD diagnosis.

MATERIALS AND METHODS

Participants

Seventy-seven PTSD patients aged between 20 and 60 years and 58 HC aged between 23 and 60 years were recruited for this study from the Psychiatry Department of Inje University Ilsan Paik Hospital (see **Table 1** for detailed demographic data). The patients' diagnosis by a board-certified psychiatrist was based on the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) Axis I Psychiatric Disorders. Patients were excluded if they accorded with the following criteria: (1) abnormality of the central nervous system, (2) medical histories of alcohol or drug abuse, (3) intellectual disability, (4) history of head injuries with loss of consciousness and experience with electrical therapy (e.g., electroconvulsive therapy, ECT), and (5) psychotic symptoms lasting for at least 24 h. HC was recruited from the local community through local newspapers and posters. Individuals without any psychiatric medical history were recruited for HC. If the HC was taking or had taken any kinds of psychotropic medication, they were excluded from the study. The study protocol was approved by the Institutional Review Board of Inje University Ilsan Paik Hospital (2015-09-018), and all participants provided written informed consent.

Three psychiatric symptoms of PTSD patients were evaluated by clinical experts. Impact of Event Scale-Revised (IES-R) (Weiss, 2007) was used to determine whether the patients had PTSD or not by evaluating the response severity of traumatic events. Beck Anxiety Inventory (BAI) (Beck and Steer, 1990) and Beck Depression Inventory (BDI) (Beck et al., 1996) were used to check anxiety symptom and depression symptom, respectively (**Table 1**). Three types of psychotropic medications were prescribed to the patients based on patients' clinical symptoms examined by clinical experts: antidepressants for depressive symptoms [selective serotonin reuptake inhibitors (n = 67), venlafaxine (n = 10)], antipsychotics for psychotic symptoms [aripiprazole (n = 5), quetiapine (n = 17)], and sedative-hypnotics for anxiety symptoms [lorazepam (n = 37), clonazepam (n = 27), diazepam (n = 15), and alprazolam (n = 35)]. All patients received combined psychotropic medications as follows: (1) antidepressant, antipsychotics, and sedative-hypnotics (n = 32); (2) antidepressant and antipsychotics (n = 12); (3) antidepressant and sedative-hypnotics (n = 33).

TABLE 1 | Demographic data of post-traumatic stress disorder (PTSD) patients and healthy controls (HC). The *p*-values represent significant differences between the two groups.

	PTSD	HC	*p*-value
Cases (*N*)	77	58	
Gender (male/female)	28/49	30/28	0.082
Age (years) Range	40.92 ± 11.93 20 – 60	39.98 ± 11.63 23 – 60	0.646
Education	13.51 ± 2.80	14.45 ± 3.37	0.120
IES-R	51.34 ± 21.71		
BDI	26.99 ± 13.13		
BAI	29.48 ± 15.44		

IES-R, Impact of Event Scale-Revised; BAI, Beck Anxiety Inventory; BDI, Beck Depression Inventory. The p-values are obtained using an independent t-test for age and education, and chi-squared test for gender.

Electroencephalogram Recording and Preprocessing

Resting-state EEG data used in this study were the same as presented in our previous study (Shim et al., 2017), where we only investigated disrupted brain networks and the relationships between network indices and symptoms of PTSD patients. Resting-state EEG data were recorded with a band-pass filter of 1–100 Hz for 5 min with eyes closed (sampling rate: 1,000 Hz), for which 64 Ag/AgCl scalp electrodes were evenly mounted on the scalp according to the extended international 10-20 system [NeuroScan SynAmps2 (Compumedics United States, El Paso, TX, United States); references: M1 and M2]. To reduce both external and internal artifacts of EEG data, a series of pre-processing approaches were applied to raw EEG data. Firstly, external artifacts, such as electrocardiography (ECG) and eye-related artifacts (e.g., eyes blinks and horizontal movements), were removed using established mathematical procedures based on regression approach (Semlitsch et al., 1986), and other gross artifacts (e.g., if electrodes showed amplitudes higher than 200 μV) were rejected by visual inspection. To correct the baseline, the DC offset of EEG channels was removed by subtracting the average values of EEG data from each time point for each channel. The baseline corrected EEG data were then band-pass filtered between 1 and 55 Hz using a third order Butterworth IIR filter to remove high-frequency external artifacts, and they were epoched with a length of 4.096 s. Epochs were rejected if they contained significant physiological artifacts ($\pm$ 100 μV) at any electrode (Kim et al., 2019), and 10 artifact-free epochs extracted for each subject were used for further analysis, as in previous studies (Gudmundsson et al., 2007; Shim et al., 2017). Since a previous study reported that a total of 40 s epoch is sufficient to obtain reliable results of quantifying resting-state EEG data, we used 10 epochs (40.96 s) to extract classification features from resting-state EEG (Gudmundsson et al., 2007).

Source-Level Power Spectrum Densities Feature Extraction

Because related studies reported the superiority of source-level features as compared to sensor-level features (Shim et al., 2016, 2019; Kim J. et al., 2020) as well as of PSD features as compared

to network features in terms of classification performance (Kim Y. W. et al., 2020), we used source-level PSD features for the classification of PTSD patients from HC. To estimate the source-level time series, a lead-field matrix was computed using a three-layer (inner skull, outer skull, and scalp) boundary element method (BEM) model, which was constructed from the standard head model (Colin 27) using the OpenMEEG toolbox (Gramfort et al., 2010). An inverse operator was created using a weighted minimum-norm estimation (wMNE) algorithm implemented in Brainstorm toolbox (Tadel et al., 2011). A time-series of source activities at 15,000 cortical vertices was estimated for every time point using the EEG data created by concatenating 10 artifact-free EEG epochs to improve computational efficacy (Kang et al., 2018). After computing current source densities, representative source signals at 68 regions of interests (ROIs) based on the Desikan–Killiany atlas were estimated using the 1^{st} component of principal component analysis (PCA) (Dimitriadis et al., 2018). We excluded 18 ROIs that showed statistical difference between 2 groups (PTSD vs. HC) in terms of the variance explained by the 1^{st} PC because in this case the 1^{st} PCs of 2 groups did not similarly explain the variance of original data (independent *t*-test; Bonferroni corrected $p < 0.05$). Therefore, the source signals at the remaining 50 ROIs were used for further data analysis, and the source signals of each ROI was epoched into 4.069 s. **Supplementary Table 2** provides the name of all 68 ROIs, their corresponding variances explained by the 1^{st} PCs, and statistical test results, respectively. Time-varying source-level PSDs were then estimated by a complex Morlet-Wavelet method using "ft_freqanalysis" Matlab function implemented in Fieldtrip toolbox (Oostenveld et al., 2011). A crucial input parameter "cgf.width" in "ft_freqanalysis" was set as 3 to appropriately determine a wavelet width (= cfg.width/frequency/pi) according to the recommendation of the Fieldtrip guideline, thereby guaranteeing the accurate estimation of PSDs for all frequencies with the EEG epoch of 4.096 s. The Morlet-wavelet transform with a sinusoidal wave modified by a Gaussian shape was applied to the source-level time series of each ROI. Source-level PSDs of each ROI were independently quantified by averaging time-varying PSDs in 6 frequency bands, i.e., delta [1–4 Hz], theta [4–8 Hz], alpha [8–12 Hz], low-beta [12–22 Hz], high-beta [22–30 Hz], and gamma [30–55Hz]. Note that the beta-frequency band was divided into 2 sub-bands: low-beta [12–22 Hz] and high-beta [22–30 Hz] bands (Kim et al., 2018; Shim et al., 2018). 30 PSD feature sets were finally constructed by integrating the PSD features of different frequency bands (**Table 2**).

TABLE 2 | Thirty power spectrum density (PSD) feature sets constructed by combining different frequency bands and the number of features for each feature set. Fifty features were extracted for each frequency band.

Frequency bands	The number of features
D, T, A, LB, HB, G	50 ROIs × 1 frequency band = 50
D + T, D + A, D + LB, D + HB, D + G, T + A, T + LB, T + HB, T + G, A + LB, A + HB, A + G, LB + HB, LB + G, HB + G	50 ROIs × 2 frequency bands = 100
D + T + A, T + A + LB, A + LB + HB, LB + HB + G	50 ROIs × 3 frequency bands = 150
D + T + A + LB, T + A + LB + HB, A + LB + HB + G	50 ROIs × 4 frequency bands = 200
D + T + A + LB + HB	50 ROIs × 5 frequency bands = 250
D + T + A + LB + HB + G	50 ROIs × 6 frequency bands = 300

D, delta; T, theta; A, alpha; LB, low-beta; HB, high-beta; G, gamma.

Classification

30 PSD feature sets were independently tested to evaluate the performance of classifying PTSD patients and HC, thereby finding an optimal combination of PSD frequency bands with respect to classification performance. To this end, classification performances were evaluated using the features by sequentially eliminating recursive features from all features for each feature set using sequential backward selection (SBS) method (García-Laencina et al., 2014). The classification accuracy was evaluated using a linear support vector machine (SVM) classifier (Orru et al., 2012; Alimardani et al., 2018) with a 10-fold cross validation method to prevent overestimate classification performance and improve computation efficacy for each of the 30 feature sets (Kim Y. W. et al., 2020). Note that we also tested 2 other machine-learning classifiers, Random Forest and AdaBoost, as well as 3 deep learning classifiers based on convolutional neural network (CNN), shallow ConvNet (Schirrmeister et al., 2017), EEGNet (Lawhern et al., 2018), and a 13 layers-based deep CNN (Acharya et al., 2018), but we only report SVM results due to its superior classification performance as compared to the other classifiers. In this study, the number of subjects (sample size) in each group was imbalanced (PTSD – 77 and HC – 58), which could lead to biased classification performances. To complement the effect of the imbalance sample size on classification, we employed 2 strategies: (1) a cost-sensitive SVM classifier that modifies the weight of margin penalty proportional to sample size and (2) a balanced classification accuracy [sensitivity + specificity)/2]. Moreover, we evaluated the receiver operating characteristics (ROC) curve and the area under the ROC (AUC) as another performance measure (Long et al., 2017). In addition, to investigate what features (ROIs) were most importantly used for classification, SVM coefficients were evaluated for each ROI (Bosilj et al., 2018) when maximum classification accuracies were obtained for 2 best feature sets extracted from delta and theta frequency bands. Since different features were selected within each cross-validation loop, the absolute values of SVM coefficients were averaged for each of the ROIs selected across cross validation, and they were normalized between 0 and 1. Then, we visualized the ROIs with different sizes and colors along with PSD features where the size of ROIs was proportional to SVM coefficients. A higher SVM coefficient (lager circle) means higher importance in terms of classification based on which neurophysiological interpretation is possible (Bosilj et al., 2018). **Figure 1** represents the flowchart of this study.

RESULTS

Classification Accuracy

Figures 2A,B show the maximum classification accuracies of each frequency band and the corresponding ROC curves with AUC values, respectively: delta: 86.61% with 0.93 (AUC); theta:

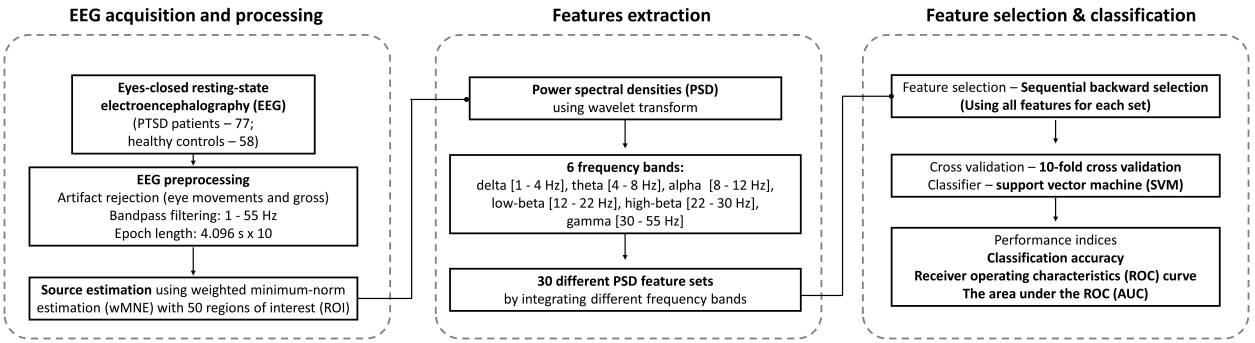

FIGURE 1 | Flowchart of machine-learning-based classification approach.

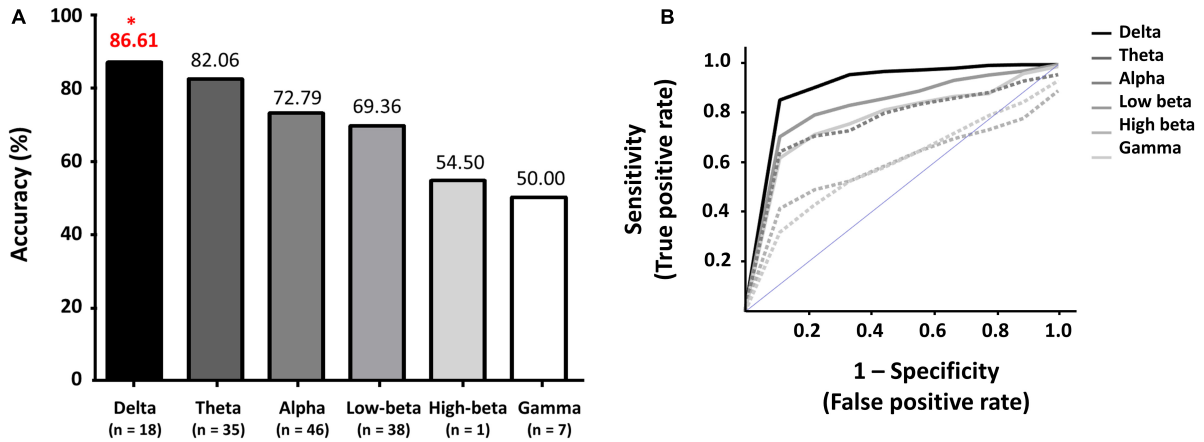

FIGURE 2 | (A) Maximum classification accuracies of each frequency band, and **(B)** Receiver operating characteristic (ROC) curves with corresponding areas under the curves (AUC) for each frequency band. *indicates a maximum classification accuracy.

82.06% with 0.86; alpha: 72.79% with 0.80; low-beta: 69.36% with 0.79; high-beta: 54.50% with 0.56; and gamma: 50.00% with 0.56 (see **Supplementary Table 3** for the balanced classification accuracies of all feature sets). The delta and theta feature sets only showed acceptable classification performance for a practical binary classification system (> 70%) (Perelmouter and Birbaumer, 2000; Hwang et al., 2014). The combinatory feature set of delta and theta features showed almost same classification performance with the delta feature set (86.39%). Other combinatory feature sets, including the delta feature set, also showed comparable classification performance with the delta feature set, e.g., 86.17% for delta + alpha, delta + low-beta, and delta + high-beta, and 85.96% for delta + theta + alpha + low-beta. Note that no feature sets outperformed the delta feature set in terms of classification performance (**Supplementary Table 3**).

Spatial Power Spectrum Density Distribution and Important Features

Figure 3 presents the spatial PSD distributions of delta and theta bands, respectively, and ROIs selected when achieving the maximum classification accuracies. Overall, the PSDs of PTSD patients were considerably reduced, as compared to those of HC (first and second columns of **Figure 3**). Red circles represent the important ROIs that have SVM coefficients over the upper bound of 95% confidence interval (mean + 2 standard deviation) and blue circles indicates the other selected ROIs. Most of the selected ROIs were overlapped between the 2 frequency bands and they were located in the fronto-temporal area.

DISCUSSION

In the present study, we investigated the optimal PSD frequency bands to improve the performance of a resting-state EEG-based CAD system to assist the diagnosis of PTSD patients using machine-learning technique. The classification accuracies of the lower frequency PSD feature sets (delta and theta) were significantly higher than those of the relatively higher frequency PSD feature sets (alpha, low-beta, high-beta, and gamma). The best classification performance was obtained when using delta PSDs (86.61% and AUC – 0.93). The features that were selected to attain the best classification accuracy were closely related to the neurophysiological characteristics of PTSD patients, which will be discussed in detail.

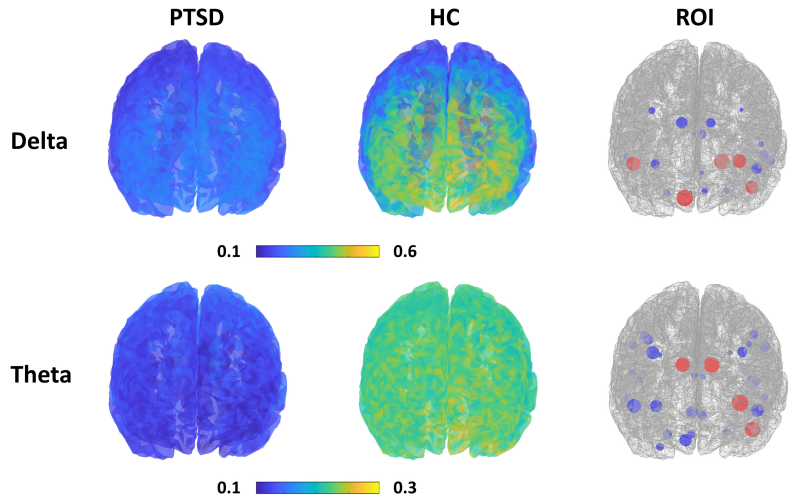

FIGURE 3 | Spatial PSD distributions for each group (first and second column) with respect to the frequency band, and the ROIs selected when achieving the maximum classification accuracy for each frequency band (third column). Red circles represent the important ROIs that have SVM coefficients over the upper bound of 95% confidence interval and blue circles indicate the other selected ROIs. The size of circles is proportional to SVM coefficients.

Source-Level Power Spectrum Density Features for the Classification of Post-traumatic Stress Disorder Patients

It has been well documented that the results of sensor-level analysis would be distorted and smeared by volume conduction effects due to different tissue conductivities (van den Broek et al., 1998), and thereby sensor-level analysis could not be used to accurately extract neuronal information (Babiloni et al., 1997). The introduction of source-level analysis could redeem the weaknesses of sensor-level analysis (Michel et al., 2004). In fact, many researchers have utilized source-level features to develop CAD systems to assist the diagnosis of psychiatric disorders. For example, previous EEG and MEG studies showed higher classification accuracies when using source-level features than sensor-level features to differentiate schizophrenia patients from HC (Shim et al., 2016; Kim J. et al., 2020), PTSD patients from HC (Shim et al., 2019; Kim Y. W. et al., 2020), and PTSD patients from major depressive disorder patients (Shim et al., 2019). Therefore, in this study, we only considered source-level features to classify PTSD patients from HC rather than using sensor-level analysis, and achieved reasonable classification performance.

Power spectrum densities (PSDs) have been mainly used as a classification feature type to develop a CAD system to assist in the diagnosis of psychiatric patients (Cassani et al., 2018; Radenković and Lopez, 2019) because distinct and abnormal PSD patterns were shown in psychiatric disorders, as compared to HC (Newson and Thiagarajan, 2019); these abnormal PSD patterns were also related to genetic traits of psychiatric disorders (Venables et al., 2009). PTSD patients also showed altered PSD patterns compared to HC; PTSD patients revealed significantly diminished PSDs in delta and theta bands (McFarlane et al., 2005; Veltmeyer et al., 2006; Newson and Thiagarajan, 2019). Moreover, the diminished PSDs in slow-frequency bands significantly correlated with patients' symptom scores (Clinician-Administered PTSD scale, CAPS) (Veltmeyer et al., 2006), indicating that the altered PSD patterns would reflect the pathophysiology of PTSD patients, such as re-experiencing and arousal (Veltmeyer et al., 2006). Abnormal PSD patterns of PTSD patients were also clearly observed in this study (**Figure 3**), and they played an important role in classifying PTSD patients from HC. However, unfortunately, we did not find any significant relationship between the PSD features used to obtain a relatively high performance (i.e., 18 features for delta band and 35 features for theta band) and symptom scores (i.e., IES-R, BAI, and BDI) in this study. This would be because these features were selected from a machine learning perspective for better classification; low frequency PSD features for each group (PTSD and HC) were separately grouped with a discriminable distance (i.e., relatively high PSD values for HC vs. low PSD values for PTSD, as shown in **Figure 3**), but which does not guarantee that the low frequency PSD features of PTSD patients are necessarily correlated with neuropsychological estimates (e.g., symptom scores). Note that the discriminable low frequency PSD features were selected by comparing those of PTSD patients and HC, but symptom scores were acquired only from PTSD patients. Because of the mentioned reason, the low frequency PSD features of PTSD patients did not seem to be proportional to symptom scores even though the low frequency PSD features can be used for the discrimination of PTSD patients from HC. In order to find a certain feature set that is directly correlated with neuropsychological estimates, regression should be used instead of classification by focusing on only PTSD data, but which would be beyond the scope of this study, focusing on the discrimination of 2 groups. Nevertheless, it is important to investigate neuropsychological traits using objective neurophysiological biomarkers in order to improve the understanding of neural mechanism in psychiatric disorders. Therefore, we will keep going to develop a new feature-based CAD system that can provide both the accurate

diagnosis of psychiatric patients in terms of machine learning and the understanding of neurophysiological traits in terms of neuroscience by adopting other EEG-based metrics, such as effective connectivity and complexity measures.

Crucial Role of Slow Brain Waves

In the present study, the best classification performance was obtained when using delta PSD features. The best classification performance obtained in this study (86.61%) was considerably higher than that of our previous PTSD study by approximately 13% (Kim Y. W. et al., 2020). Unlike the previous study, we used delta PSD features when differentiating PTSD patients and HC, which mainly led to the relatively higher classification accuracy. Moreover, when using theta PSD features, a reasonable classification accuracy of 82.06% was also achieved, which was also higher than that of the previous study (Kim Y. W. et al., 2020). The performance improvement would be caused by the distinct characteristics of slow EEG waves of PTSD patients in the delta and theta frequency bands. Many previous studies reported that slow EEG waves were a typical endophenotype of PTSD patients (Franke et al., 2016; Sheerin et al., 2018; Newson and Thiagarajan, 2019). As mentioned above, PTSD patients showed significantly decreased delta and theta PSDs compared to HC, and the decreased slow-frequency PSDs were closely associated with their altered symptoms, such as arousal and numbing (Franke et al., 2016; Sheerin et al., 2018). That is, due to the distinct pathological traits of slow EEG waves in PTSD patients, the present study could significantly improve the classification performance for the diagnosis of PTSD patients, compared to that of our previous study (Kim Y. W. et al., 2020).

Spatial Distribution of Power Spectrum Density Features

All PSD features selected when attaining maximum classification accuracies for delta and theta bands were extracted from fronto-temporal areas, such as frontal pole, opercular, anterior cingulate gyrus, superior temporal gyrus, and temporal pole (third column of **Figure 3**). The mean PSD values of each ROI of the mentioned fronto-temporal areas were significantly smaller in PTSD patients than those in HC. According to previous studies, the frontal regions, including the frontal pole and opercular part of the inferior frontal area, are involved in emotion regulation processing (Grecucci et al., 2013; Bramson et al., 2019) and temporal areas are significantly related to rumination symptom of PTSD patients (Ferdek et al., 2016); PTSD patients showed significantly reduced brain activation in both brain areas in resting-state (Rabe et al., 2006), as shown in this study. Therefore, it is neurophysiologically plausible to obtain better classification performance for PTSD diagnosis when using PSD features extracted from fronto-temporal areas. This also indicates that employing only fronto-temporal areas might be sufficient to implement a reliable CAD system for PTSD diagnosis, thereby facilitating the development of a more clinically practical CAD system.

In this study, we used EEG data measured in resting state during which default mode network (DMN) or salience network is more active than the task period (Choi et al., 2021), and thus DMN might be usefully used for PTSD diagnosis. We investigated the ROIs selected when the maximum classification accuracy was obtained using delta PSD features, and found that 16 of 18 DMN ROIs were selected together with 2 other ROIs, i.e., entorhinal and para hippocampal areas. Both entorhinal and para hippocampal areas are known to be related to clinical symptoms in PTSD patients, such as altered cognitive function and disrupted memory (Kerr et al., 2007). This result means that it is necessary to use the features extracted from the ROIs related to neuropsychological traits of patients together with those related to current brain state (resting state in this study) to obtain a relatively high diagnosis accuracy.

Machine Learning Approach With Interpretable Electroencephalogram Biomarkers

In recent years, machine learning approaches have received increasing attention in the development of an EEG-based CAD system to assist the accurate diagnosis of psychiatric disorders (Bzdok and Meyer-Lindenberg, 2018; Fazel and O'Reilly, 2020; Rutherford, 2020; Koutsouleris et al., 2021). In particular, it is important to use neurophysiologically interpretable EEG biomarkers in the development of an EEG-based CAD system to improve its reliability, as in deep learning (Sturm et al., 2016). In this study, we introduced low frequency EEG features that were closely associated with the neurophysiological characteristics of PTSD patients (Franke et al., 2016; Vergara et al., 2017; Newson and Thiagarajan, 2019), and thereby not only the proposed low frequency EEG biomarkers could significantly improve the performance of the CAD system, but also they were neurophysiologically interpretable. To evaluate the potential of low frequency EEG features for the performance improvement of the CAD system, we used a traditional machine learning approach using PSDs as features and SVM as a classifier, and the improved classification accuracy of 86.61% would not be still enough to be used in clinics. Even though we also used 3 different CNN-based deep learning algorithms for the diagnosis of PTSD patients as mentioned in the method section, we could not obtain a comparable classification accuracy to that obtained using the SVM approach (73.13% for Shallow ConvNet, 76.15% for EEGNet, and 77.03% for 13-Layers CNN). This result would be derived due to a relatively small number of samples (data amount) used in deep learning algorithms; traditional machine-learning models show better performance than that of deep learning models with relatively small samples (Kumar and Manash, 2019). Therefore, we will keep trying to improve the diagnosis accuracy by introducing more advanced algorithms, such as data augmentation to compensate a small number of samples in our future studies (Wang et al., 2018), thereby developing a clinically usable CAD system for PTSD patients.

Limitations

First, since all recruited PTSD patients were on medication, we could not control the compounding effects from medications. It has been reported that EEG characteristics can be changed by

medications. For example, antipsychotics increased slow waves, such as delta and theta (Amann et al., 2003), antidepressants modified alpha patterns (Bruder et al., 2008), and sedative-hypnotics enhanced low frequency power (< 15 Hz) (Ferri et al., 2017). All psychotic medications tend to increase PSD values in particular for relatively low frequency bands, but delta and theta PSDs for PTSD patients were still significantly lower than those of HC, as shown in **Figure 3**. Therefore, it could be reasonably thought that delta and theta PSDs of PTSD patients could be increased by medications, but which did not reach those of HC. Thereby, significant difference between two groups was still kept in terms of low frequency PSD values, and they were used as key features for accurate diagnosis. Our previous study also used same types of medications used in this study, i.e., antipsychotics, antidepressants, and sedative-hypnotics (Kim Y. W. et al., 2020). Because patients recruited in the previous study were independent from those recruited in the present study, it is impossible to directly compare the results of the two studies. However, assuming that similar medication impacts occurred on EEG characteristics due to using same types of medications for both groups of PTSD patients, it can be reasonably thought that the enhanced classification performance in this study would be caused by using optimal PSD features as compared to the previous study, not medication effects. Further studies should follow with drug-naïve PTSD patients to accurately investigate the medication impact on the development of an EEG-based CAD system, which can also allow for PTSD diagnosis at initial screening stages. Second, even though we controlled other psychiatric illness, we did not control comorbid depression. Third, we used a total of 40.96-s EEG data measured using 64 electrodes for data analysis (e.g., source estimation). It was reported that reliable source signal estimation can be possible using the EEG data acquired from more than 60 channels (Michel et al., 2004), and also reliable resting-state EEG data analysis with a data length of more than 40 s (Gudmundsson et al., 2007). However, since it is obvious that use of longer EEG data measured from a high-density EEG system with more channels (e.g., > 128) can allow for more reliable EEG data analysis, including source imaging (Haartsen et al., 2020), using such the high-density EEG data is necessary to improve the reliability and accuracy of EEG data analysis in future studies. Fourth, PTSD patients showed different EEG characteristics according to gender. For example, female patients showed enhanced alpha asymmetry than female HC (Metzger et al., 2004), while male patients showed decreased source activities in theta band and decreased alpha PSD as compared to male HC (Jokić-begić and Begić, 2003; Todder et al., 2012). Therefore, diagnostic performance could be further improved by considering gender-specific EEG features, which would be one of the interesting future topics.

CONCLUSION

We investigated optimal PSD frequency bands to improve the classification performances of a resting-state EEG-based CAD system for precise PTSD diagnosis. Low-frequency PSD features in delta and theta frequency bands showed significantly higher classification performances than relatively high-frequency PSD features (alpha, low-beta, high-beta, and gamma), and the best classification performance (86.61% and AUC of 0.93) was obtained when using delta PSD features. In addition, most meaningful features were extracted from fronto-tempora areas, which coincided with the neurophysiological findings of previous EEG-based PTSD studies. Although the present study showed relatively high classification performances between PTSD patients and HC, there is still room to improve the classification performance by introducing novel feature extraction and classification methods based on deep learning algorithms, which could be interesting future research topics.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Inje University Ilsan Paik Hospital (2015-09-018). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MS, S-HL, and H-JH: conception and design of the work and interpretation of data. MS and S-HL: data acquisition and analysis. MS, C-HI, S-HL, and H-JH: writing (original draft preparation and editing). All authors read and contributed to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fninf.2022.811756/full#supplementary-material

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REVIEW
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Integrative Brain Dynamics in Childhood Bullying Victimization: Cognitive and Emotional Convergence Associated With Stress Psychopathology

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Bullying victimization is a form of psychological stress that is associated with poor outcomes in the areas of mental health and learning. Although the emotional maladjustment and memory impairment following interpersonal stress are well documented, the mechanisms of complex cerebral dysfunctions have neither been outlined nor studied in depth in the context of childhood bullying victimization. As a contribution to the cross-disciplinary field of developmental psychology and neuroscience, we review the neuropathophysiology of early life stress, as well as general psychological stress to synthesize the data and clarify the versatile dynamics within neuronal networks linked to bullying victimization. The stress-induced neuropsychological cascade and associated cerebral networks with a focus on cognitive and emotional convergence are described. The main findings are that stress-evoked neuroendocrine reactivity relates to neuromodulation and limbic dysregulation that hinder emotion processing and executive functioning such as semantic cognition, cognitive flexibility, and learning. Developmental aspects and interacting neural mechanisms linked to distressed cognitive and emotional processing are pinpointed and potential theory-of-mind nuances in targets of bullying are presented. The results show that childhood stress psychopathology is associated with a complex interplay where the major role belongs to, but is not limited to, the amygdala, fusiform gyrus, insula, striatum, and prefrontal cortex. This interplay contributes to the sensitivity toward facial expressions, poor cognitive reasoning, and distress that affect behavioral modulation and emotion regulation. We integrate the data on major brain dynamics in stress neuroactivity that can be associated with childhood psychopathology to help inform future studies that are focused on the treatment and prevention of psychiatric disorders and learning problems in bullied children and adolescents.

Keywords: bullying victimization, cerebral functional activity, executive functions, memory, psychopathology, stress, theory of mind

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Abbreviations: ACC, anterior cingulate cortex; BA, Brodmann area; BV, bullying victimization; HPA, hypothalamic–pituitary–adrenal; PFC, prefrontal cortexprefrontal cortex; vl, ventrolateral; vm, ventromedial.

INTRODUCTION

Bullying victimization (BV) is a form of chronic psychological stress caused by interpersonal aggression that is repeatedly directed at a person who wields less power than their abuser (Olweus, 1994). Approximately one in 10 children worldwide are bullied regularly by their peers and another 30% of children are bullied on occasion (e.g., Nansel et al., 2001; National Academies of Sciences, Engineering, and Medicine, 2016; Biswas et al., 2020). Strong and growing evidence shows that BV is an intense psychological stress–targets of BV suffer chronic emotional distress which compromises their mental health and leads to persistent physical and social dysfunction, as well as poor academic achievement (e.g., Vaillancourt and Palamarchuk, 2021). The adverse correlates of BV similarly affect children worldwide (e.g., McDougall and Vaillancourt, 2015; Jantzer et al., 2021). Moreover, BV can lead to psychological adjustment problems and persistent mental health dysfunctions that extend well into adulthood (e.g., Arseneault et al., 2010; McDougall and Vaillancourt, 2015; van Geel et al., 2016; Moore et al., 2017; Vaillancourt and Palamarchuk, 2021).

Neurocognition plays a central role in the development of psychological stress. First, psychological stress is a physiological response to a salient stimulus that is perceived as a stressor such as a challenging event or behavior that disturbs the individual emotionally and thus triggers neurocognitive reactivity to adapt. Second, it is the cognitive appraisal that assigns the severity level to a stressor (i.e., interpretation of the stressor but not the nature of a stressor *per se*). If the stress is extreme and the stressor is uncontrollable, as is often the case with BV, neurocognitive stress reactivity can deviate toward maladaptation and psychopathology. In other words, the main effect of psychological stress is due to the perceived severity and controllability of the stressor, as well as the timing of the stressor (i.e., novelty/acuity/chronicity, Palamarchuk and Vaillancourt, 2021). That is why we hypothesize that the impact of BV on mental health will not be that different from the impact of other forms of psychological stress, such as childhood maltreatment, although we acknowledge that unique nuances may exist. We also recognize that the presence of physical/sexual abuse can aggravate psychological harm (e.g., Lereya et al., 2015); however, this interaction is beyond the scope of this review. Our reasoning for limiting our review to BV is because most neurobiological reviews have focused on child maltreatment and not on this other notable childhood stressor that affects far more children and youth worldwide (UNICEF, 2020).

Children are particularly vulnerable to the negative effects of psychological stress because the emotional and cognitive adjustment problems occur during a time when neuronal development is taking place (e.g., Sowell et al., 2004; Zhu et al., 2011; Cowell et al., 2015; Caballero et al., 2016). Emerging evidence supports that childhood BV has a profound effect on children's brain development. For instance, Du Plessis et al. (2019) found longitudinal links between childhood BV and structural changes in the right ventrolateral (vl) prefrontal cortex (PFC) moderated by cortisol levels in adolescent boys but not adolescent girls. High BV with low daily cortisol output and/or a steeper diurnal slope was associated with reductions in the right vlPFC surface area, and high BV with high daily cortisol output and/or a low flatter diurnal slope was associated with a larger right vlPFC surface area. These findings highlight the differential effects of BV on brain development. In particular, decreased cortisol levels in bullied adolescents might indicate stress adaptation because reduced surface area and increased cortical thinning of the PFC during adolescence are part of normal development. Quinlan et al. (2020) provided evidence that BV at age 14 was indirectly associated with generalized anxiety *via* reductions in volume of the left dorsal striatum (nucleus caudate and putamen) at age 19. Muetzel et al. (2019) found that 10-year-old targets of BV had increased cortical thickness in the left fusiform gyrus, yet the findings might relate to either the pre-existing differences or to the consequences of BV. Although it is important to compare the influence of BV across the lifespan, such as childhood vs. adulthood, we emphasize the integrative and inter-disciplinary research on childhood BV because the adult literature is limited. We predict that adulthood BV would differ from childhood BV mainly due to the distinct factors that hinder stress appraisal and increase stress sensitivity. Namely, the etiology of childhood BV sensitivity relates to the neurocognitive immaturity that contributes to the amygdala—PFC networking specifics, which are reviewed. Adulthood BV sensitivity likely relates to the frontrostriatal loop engaged in motivation, habits, reward-learning, and decision-making (for the nuances in general psychological stress, see Palamarchuk and Vaillancourt, 2021).

The primary focus of this review is to highlight childhood BV and psychopathology through the lens of neurocognitive functioning. The novelty is that we present BV research on executive functioning by outlining multiple levels of cognition including learning, semantic cognition, cognitive flexibility, and processing of social and emotional information that involves behavioral regulation (i.e., social cognition, Shany-Ur and Rankin, 2014). We offer an innovative approach to the topic of BV. Specifically, an integrative brain dynamics model of cognitive and emotional convergence is presented based on the neurophysiological evidence of the effects of early life and general psychological stress with the aim of helping explain why BV is likely so robustly linked to psychopathology. We also discuss the application of the results and provide examples to help with the design of future studies that are focused on the treatment and prevention of psychiatric disorders and learning problems in bullied children and adolescents. It is our cross-disciplinary contribution to cognitive neuroscience, social neuroscience, developmental neuropsychology, psychological pedagogy, and psychiatry interface, which distinguishes the innovation of this review.

STRESS-INDUCED NEUROPSYCHOLOGICAL CASCADE

Neuroendocrine Reactivity Related to Psychological Neuromodulation

Psychological stressors are salient stimuli, such as events and behavior, that can trigger aversive emotions and feelings. When a negative value is assigned to the salient stimuli (cognitive appraisal), psychological stress occurs. Psychological stress is a physiological response governed by the neurocognition to stay focused on the challenge and adapt. This stress response does not principally relate to the stimulus *per se*, but to the perceived stress severity and controllability that depends on the stressor's acuity (i.e., novel/unpredicted vs. homotypic/chronic). That is, the major difference between the stressor's influence on neuromodulation is not due to the nature of the stressor (e.g., childhood maltreatment vs. BV) but rather due to the interpretation of the stressor (see detailed review by Palamarchuk and Vaillancourt, 2021).

BV is a psychologically stressful experience for children (Vaillancourt and McDougall, 2013) and the stressor experienced is far too often extreme and/or prolonged. In general, psychological stress initially activates the hypothalamic–pituitary–adrenal (HPA) axis that alters glucocorticoid levels (i.e., circulated cortisol concentration), an effect seen in bullied children (e.g., Ouellet-Morin et al., 2011a,b, 2013, 2021; Vaillancourt et al., 2011; Kliewer et al., 2019). Human and animal studies show that increased cortisol levels in response to stress can over-activate the glucocorticoid receptors that prevail in the hippocampus and PFC, areas of the brain primarily associated with memory and learning. This activation can trigger neurocognitive responses linked to memory, mood, behavior, and executive functions, which should theoretically help the individual cope with the stressor (e.g., De Kloet et al., 1998, 2018; De Kloet and Derijk, 2004; Barsegyan et al., 2010; Vogel and Schwabe, 2016; Palamarchuk and Vaillancourt, 2021). Indeed, childhood adversity does not exclusively harm cognitive functions; it can also promote developmental adaptation to resilience *via* the improvement of some cognitive functions (Ellis et al., 2017). Nevertheless, stress-associated outcomes differ according to the intensity/severity and controllability of the stressor, which relates to cognitive appraisal (aforementioned stressor's interpretation), as well personal and environmental factors (e.g., Schneiderman et al., 2005; Palamarchuk and Vaillancourt, 2021). When a stressor is beyond the coping ability of the individual, impairment in cognition and metacognition (i.e., awareness of one's own thinking/learning) can occur (e.g., Lupien et al., 2005; De Kloet et al., 2016, 2018; Palamarchuk and Vaillancourt, 2021), which is often the case with BV (e.g., Ouellet-Morin et al., 2011b; Sinclair et al., 2012; Vaillancourt et al., 2013; Liu et al., 2016; Carroll et al., 2019; Gini et al., 2019). For example, there is mounting evidence documenting that bullied children and adolescents struggle academically in comparison to their non-bullied peers (Nakamoto and Schwartz, 2010; Espelage et al., 2013). Yet despite this well-replicated literature, the prevailing view regarding the association between BV and academic achievement does not account for a neurobiological mechanism (see exception, Vaillancourt et al., 2013; Vaillancourt and Palamarchuk, 2021). Rather, this association is typically attributed to poor school attendance or poor mental health mediating the link between BV and academic achievement.

The impaired neurocognitive functioning found in bullied children might also emerge from altered cortisol concentrations (e.g., Vaillancourt et al., 2008, 2011; Ouellet-Morin et al., 2011b, 2013, 2021; Östberg et al., 2018b; Palamarchuk and Vaillancourt, 2021), that in turn, affect neurochemical mechanisms (i.e., neurotransmission) of memory, mood, and behavior. Specifically, stress can impact dopamine signaling in the PFC-striatum circuits (e.g., Gamo et al., 2015; Reneaux and Gupta, 2018), which plays an important part in neuromodulation (i.e., the capacity of a single neuron to regulate wide-ranging populations of other neurons), reward-motivated behavior, reinforcement learning (e.g., Ikemoto, 2007), and representational mentalizing (i.e., theory of mind) development (Lackner et al., 2010).

In fact, the alteration of dopamine signaling is linked to childhood adversity (e.g., De Bellis et al., 1994; Oswald et al., 2014; Egerton et al., 2016; Bloomfield et al., 2019). Dopamine signaling can be moderated by environmental and genetic factors in adolescence, a developmental period that is particularly vulnerable to the effects of stress on the PFC's functions due to increased dopaminergic projections (Arnsten and Shansky, 2004; Oswald et al., 2014; Egerton et al., 2016). Polymorphism of the dopamine D4 receptor (DRD4) gene (i.e., shorter vs. longer alleles) was found to correlate with better representational mentalizing (Lackner et al., 2012), executive functioning, and social/emotional development in preschoolers (Pappa et al., 2015). Kretschmer et al. (2013) examined whether peer effects (negative vs. positive) on adolescent development were genetically moderated *via* the DRD4 polymorphism [the 7-repeat (7R) allele vs. the 4R allele]. Results indicated that the 4R allele was linked to higher susceptibility for the effects of BV and social well-being on later delinquency. Janssens et al. (2015) showed that peer rejection, a strong correlate of BV (Knack et al., 2012), was associated with rule-breaking behavior moderated by the dopamine transporter genotype (DAT1) in adolescents; in particular, the 10R-allele carriers showed more rule-breaking behavior in the context of high peer rejection, but less rule-breaking behavior in the context of low peer rejection. Cao et al. (2017) showed that functional polymorphism (TaqIA) in the dopamine receptor D2 (DRD2) gene (A2A2 genotype, i.e., no A1 allele) at age ∼12 years predicted higher levels of depression at age 14 years in bullied adolescent boys, but not bullied girls. In contrast, among adolescent offenders, carriers of the A1 allele were more likely to be severely bullied compared to non-carriers of the A1 allele (Vaske et al., 2011). Thus, altered dopamine signaling may be a predisposition to BV and its pervasive psychopathological impacts, especially in adolescents who

have poor executive functions, depression, and genetic risk factors.

Limbic Dysregulation and Emotion Processing

Besides the HPA axis, the principal stress response involves the locus coeruleus-norepinephrine system which relates to arousal, as well as fear supported by hyperactivation of the amygdala. This response has cognitive and behavioral effects, including arousal, attention, and cognitive flexibility (e.g., Skosnik et al., 2000; Morilak et al., 2005; Alexander et al., 2007; Valentino and Van Bockstaele, 2008). Although these effects can support careful assessment and strategic planning in unpredictable situations, they can also increase anxiety *via* the behavioral inhibition system associated with the lateral amygdala and the ventromedial (vm) PFC (e.g., Markett et al., 2018; Palamarchuk and Vaillancourt, 2021). In other words, behavioral inhibition ensues which involves the withdrawal from the danger, a reaction that is adaptive in this specific context. However, the continuation of inhibitory behavior is likely why we see the development of mental health problems including anxiety, depression, psychosis, psychosomatic, and eating disorders among bullied children (e.g., McDougall and Vaillancourt, 2015). For example, Weems et al. (2003) found that hyperarousal symptoms in adolescents with PTSD predicted the development of emotional numbing, which presumably relates to cognitive alterations in PTSD (see also Hayes et al., 2012; Palamarchuk and Vaillancourt, 2021).

BV is related to disturbed emotional processing and activation of the cerebral networks of social pain and monitoring (Rudolph et al., 2016; Will et al., 2016; Telzer et al., 2018, 2020; McIver et al., 2019). Within the cerebral networks of emotional processing, the essential role belongs to the amygdala, which if dysregulated, can contribute to psychiatric disorders (e.g., Monk et al., 2008; Brotman et al., 2014; Wegbreit et al., 2014). In bullied children, the amygdalar dysregulation can be seen in hyper responses to social exclusion (McIver et al., 2019), fearful faces (Swartz et al., 2019), and unexpected positive peer evaluation, in which high wariness in early childhood correlates with the severity of adolescent social anxiety (Jarcho et al., 2019). Moreover, amygdalar hyperactivity relates to the severity of BV and internalizing/externalizing symptoms, and to poor social self-esteem in bullied adolescent girls (Telzer et al., 2020). The neuronal mechanisms of anxiety are that repeated exposure to BV can facilitate the maintenance of extremely vivid aversive memories and evoke strong negative emotional responses such as acoustic perceptions or body sensations (Sansen et al., 2015). The traumatic memories can be intrusive as their activation can be triggered by even benign cues that remind the individual of the aversive event *via* an associative information network; this memory network can contribute to the development and maintenance of psychiatric dysfunctions such as social anxiety disorder (Iffland et al., 2014). Moreover, general anxiety levels have been shown to mediate a poor stress response in bullied children such as the negative relation between BV and cortisol levels during school lunchtime, a period of anticipated exposure to BV (Carney et al., 2010); whereas blunted cortisol responses to stress were found to be linked to increased social/behavioral problems in bullied adolescents (Ouellet-Morin et al., 2011b, 2021).

Stress-associated amygdalar dysfunction relates (but not exclusively) to the midbrain raphe nuclei and serotonergic (5-HT) pathways implicated in the downregulation of fear and anxiety (e.g., Bocchio et al., 2016; Palamarchuk and Vaillancourt, 2021) and in the enhanced active coping with inescapable stress in rodents in a time-locked manner (Nishitani et al., 2019). Uncontrollable stress is associated with the dysregulated dorsal raphe nuclei that affects 5-HT signaling (e.g., Amat et al., 2005), and alterations are linked to fear memory formation and retrieval (Sengupta and Holmes, 2019), as well as a higher risk for depression (e.g., Amat et al., 2005; Bocchio et al., 2016), greater susceptibility to future stress, and stress-induced anhedonia (Prakash et al., 2020). These serotonergic pathways can further compromise the physiology of various neuropsychological domains (e.g., Berger et al., 2009), including but not limited to those associated with BV— namely aggression (Bettencourt et al., 2013; Krygsman and Vaillancour, 2019), perception (Cole et al., 2014; Lavell et al., 2018; Östberg et al., 2018a), reward (Casement et al., 2014; Rappaport et al., 2019), attention/memory (Vaillancourt et al., 2011), appetite (Lee and Vaillancourt, 2018), and sleep (van Geel et al., 2016), with sleep problems mediating the link between delinquency and drug use in bullied girls (Sosnowski et al., 2016). In contrast, when a stressor is controllable, the vmPFC can inhibit the dorsal raphe nucleus' stress-response, which in turn prevents learned helplessness and behavioral depression (e.g., Amat et al., 2005; Maier et al., 2006). In other words, the vmPFC plays a major role in coping with stress (i.e., strengthening of the escape response) and its deficient inhibitory control over the limbic circuits triggered by aversive events determines the uncontrollability of the stressor that affects behavior. BV can be an uncontrollable stress per its association with behavioral /psychological maladjustments in childhood (Arseneault et al., 2010; McDougall and Vaillancourt, 2015; van Geel et al., 2016; Moore et al., 2017; Vaillancourt and Palamarchuk, 2021) and interpersonal needs-hopelessness sequela that can indirectly moderate suicidal ideation in adolescents (Shin et al., 2016; also see Hong et al., 2015; Silberg et al., 2016). Hopelessness results from anticipating more negative than positive events, unachievable goals, and surrender (Marchetti, 2019; see also Palamarchuk and Vaillancourt, 2021); its high levels correlate with increased inflammatory reactivity to social stress in bullied adolescents (Giletta et al., 2018). This is a psychological defeat with an alarming global effect. For instance, BV is a high-risk factor of adolescent suicide attempts (Koyanagi et al., 2019), which is the leading cause of death among youth in high-income countries, accounting for 17.6% of all deaths (UNICEF, 2020).

Neurocognitive Effects

BV exposure has been linked to significant memory impairment in children (Vaillancourt et al., 2011). Memory impairments are driven by glucocorticoid activation (Sauro et al., 2003) and cortisol-induced genomic mechanism that develops with the

stress-activated translocation of glucocorticoid receptors to the neuronal nucleus (Kino et al., 2009; Argentieri et al., 2017). Epigenetic mechanisms associated with stress primarily belong to the level of brain-specific DNA methylation, which leads to stable gene repression (e.g., Brenet et al., 2011) and has a distinguished impact in early life (e.g., Lubin et al., 2008; Szyf and Bick, 2013; Alexander et al., 2018).

Exposure to severe and/or chronic stressors can alter glucocorticoid signaling, resulting in dendritic atrophy and reduced spine density that affects synaptic plasticity (McEwen, 1999, 2000, 2001; Sousa et al., 2000; Moench and Wellman, 2015; Madalena and Lerch, 2017; Urban et al., 2019). Spine density is necessary for neuronal network formation, an essential component of cognition, including memory and learning that is promoted in the PFC and hippocampus (Amat et al., 2005; Gilabert-Juan et al., 2013). Stress can further induce cerebral morphological changes; for instance, early life adversity is associated with volume reductions in the amygdala, medial PFC, and hippocampus (in exposure to threat) and frontoparietal regions (in children exposure to deprivation; Kim et al., 2019; McLaughlin et al., 2019), as well altered white matter tracts (Kim et al., 2019), that increase the risk of developing PTSD (Yehuda, 2009) and neurodegeneration (Vyas et al., 2016). The alteration of cerebral morphology is moderated by interactions between childhood adversity and genes, such as the S allele of *5-HTTLPR* (for the hippocampus), Met allele of *BDNF* (for the amygdala, hippocampus, PFC, and rostral ACC), and *FKBP5* gene rs160780 (for white matter), which have links to depression (Kim et al., 2019). Consistent with this idea, several researchers have found links between BV and symptoms of depression and PTSD in children who are targeted by their peers (Vaillancourt et al., 2011; Idsoe et al., 2012, 2021; Nielsen et al., 2015; Lee and Vaillancourt, 2018). And, as is the case with the association between poor academic achievement and BV mentioned previously, the association between PTSD and BV likely has a strong neurobiological underpinning. We hypothesize that the PTSD and depression symptoms found in some bullied children are in fact due to neurophysiological changes that are like the ones found in maltreated children, resulting from suppressed neurogenesis, stress-associated delayed myelination, as well as distorted apoptosis (Kavanaugh et al., 2017).

Neurogenesis refers to neuronal growth and development, where key events occur in dendrites and axons when neurons differentiate and mature. In dendrites, it is seen in the morphological changes such as increases in size and branching that are necessary for signal flow between neurons. In axons, the changes involve growth cones that interact with cell adhesion molecules to anchor the tissue substrate, which are required components for primary outgoing pathways. Neurogenesis contributes to synaptogenesis and cortical thickness. Its suppression affects the amount and type of signals the neuron receives (Belsky and de Haan, 2011), whereas BV relates to the cortical thickness changes in adolescents (Muetzel et al., 2019). In a multicohort analysis of the community-based data (Brazil, Canada, and Europe), Parker et al. (2020) showed the variance in cortical thickness during maturation was linked to dendrite, spine, and myelin genes, which are also enriched in genes associated with psychopathology.

Myelination is the coating process of the axons that increases the speed of nerve transmission up to 100 times. Its delay has a significant impact on neuronal circuits' functioning (Belsky and de Haan, 2011). Delayed myelination can be detected by reduced fractional anisotropy in diffusion tensor imaging. In fact, maltreated children with PTSD present with reduced fractional anisotropy in the corpus callosum, the primary white matter tract with interhemispheric projections necessary for the processing of emotional stimuli and memory functions (Jackowski et al., 2008). Apoptosis is physiologically programmed cellular death, which is elevated during psychological stress due to neurochemical changes. In particular, animal studies suggest that severe stress causes neuronal apoptosis in the hippocampus related to the neurotransmitter imbalance (between glutamate and GABA), which may explain the findings pertaining to PTSD (i.e., inhibition of the HPA axis activation, anxiety, and poor learning; Gao et al., 2014). Although not directly assessed in bullied children, we hypothesize similar neurobiological findings will be obtained, because, like child maltreatment, BV is a form of interpersonal trauma (Idsoe et al., 2021).

From a therapeutic standpoint, we foresee that some neurocognitive dysfunctions may be reversible to a certain degree if the stressor is terminated (Conrad et al., 1999) and the neural harm is not related to a permanent loss of cells but rather is related to atrophy (McEwen, 1999, 2000; Sousa et al., 2000). The prediction is supported by the fact that while myelin plasticity substantially responds to chronic psychosocial stress (Laine et al., 2018), glucocorticoid treatment can initiate and enhance post-traumatic myelin formation, which is a cellular marker of cerebral regeneration (Chan et al., 1998). At the same time, glucocorticoid-related biomarkers (e.g., bedtime salivary cortisol and plasma dehydroepiandrosterone/cortisol ratio) are associated with the treatment of PTSD symptoms, including anxiety and depression in combat veterans (Yehuda et al., 2014).

Hippocampus-Dependent Learning

The neural factors of BV likely involve a complex interplay between hyperarousal, fear conditioning, and memory processing (i.e., learning and executive functions). Memory processing can be mapped onto the anterior cerebral network between the PFC, amygdala, hippocampus, and its adjacent cortex, the parahippocampal gyrus with peri- and ento-rhinal cortical areas (e.g., Raslau et al., 2014, 2015). In this anatomical network, the stress-response of the ventral hippocampus is distressed by the amygdala's projections that are linked to fear conditioning and affective processes (e.g., Anagnostaras et al., 2002; Cenquizca and Swanson, 2007). The amygdala plays a critical role in stress-induced memory impairment *via* fear conditioning and explicit/associative memory alteration (e.g., McGaugh, 2004; Rabinak and Maren, 2008; Robbins et al., 2008) that can alter hippocampus-dependent episodic memory when coupled with a stimulus that is perceived as emotionally evocative (e.g., Tulving and Markowitsch, 1998; Whitlock et al., 2006). Generally, psychological stress *via* fear activates the amygdala, which impacts proper hippocampal function that is

needed for encoding and working memory; severe stress can also lead to hippocampal atrophy and memory deficits (e.g., Bremner and Narayan, 1998). This stress-induced effect on hippocampus-dependent learning potentially contributes to the memory deficits found in bullied children (Vaillancourt et al., 2011; Sansen et al., 2015). Lee et al. (2018) showed that being verbally abused by peers in adolescence is associated with volume reductions in the left hippocampal subfields, a finding also documented with childhood maltreatment, another form of interpersonal trauma (Teicher et al., 2012; Riem et al., 2015). Emotional childhood neglect is linked to left hippocampal white matter reductions in adult patients with major depression (Frodl et al., 2010). As well, PTSD predicts right hippocampal volumes reductions (Carrion et al., 2007). PTSD symptoms inversely correlate with hippocampal and amygdalar (left) volumes in maltreated youth with PTSD compared to those without PTSD (Morey et al., 2016), whereas reduced hippocampal and amygdalar volumes partially mediate the relation between early life stress and behavioral problems (Hanson et al., 2015). Additionally, stress-induced hyperactivation of hippocampal glucocorticoid receptors suppresses neurotrophic factor and leads to atrophic changes in the dentate gyrus (a cortical region of the hippocampal formation; Amaral et al., 2007) that impairs long-term memory and promotes depressive symptoms in chronically stressed individuals (Malberg et al., 2000; Bekinschtein et al., 2008; Surget et al., 2011; Taliaz et al., 2011). Although these effects are well documented, more work is needed to clarify specifics of the brain structure and functioning linked to poor memory in bullied children and youth.

Striatum-Dependent Learning

Under stress, the amygdala also promotes functional connectivity with the dorsal striatum that reduces its functional connectivity with the hippocampus, impacting the quantity and quality of memory; in particular, the established coupling shifts memory encoding and retrieval from hippocampus-dependent ("cognitive") to striatum-dependent ("habit") mode (Packard, 2009; Vogel and Schwabe, 2016; Vogel et al., 2017; Zerbes et al., 2020). The dorsal striatum relates to the automatic/habit responses and attention regulation *via* the PFC and parietal cortical interactions (e.g., Lago et al., 2017) and the implication of this shift is that the memory process reduces flexibility and thus becomes more reflexive. Consequently, stress-induced poor attentional set-shifting (i.e., cognitive inflexibility), which is related to the medial PFC's dysfunction (e.g., Floresco et al., 2008; Butts et al., 2013; George et al., 2015), results in behavioral inflexibility that has been observed in bullied children. Specifically, the individual relies on old ways of responding, which might not be adaptive or appropriate because they are linked to threats from the past and not the present.

Although studies are needed to clarify the links between BV and striatum-related cognitive deficit, Telzer et al. (2020) showed that severe BV was related to hyperactivation in the amygdala, ventral striatum, fusiform gyrus, and temporoparietal junction, which was associated with increased internalizing and externalizing symptoms in adolescent girls. Of note, the ventral striatum is preferentially engaged in emotional processes and its dysregulation can lead to anxiety (Lago et al., 2017). The link between BV and anxiety is well noted in the literature and supported by meta-analytic findings (Reijntjes et al., 2010; Wu et al., 2015; Moore et al., 2017) and longitudinal studies (Takizawa et al., 2014; Sentse et al., 2017; Drazdowski et al., 2021). Silk et al. (2014) also has shown that reactivity of the ventral striatum (nucleus accumbens) and insula is moderated by depression in adolescence. Lee et al.'s (2020) findings are that BV has an indirect effect on adolescent depression *via* volume increases in the nucleus accumbens.

Developmental sensitization to stress differentiates children at risk for anxiety symptoms and internalizing disorders with profiles of sustained cortisol elevation (i.e., morning cortisol and during acute stress exposure; Laurent et al., 2015). Specifically, high levels of cortisol's response to stress are distinguished by anxiety and linked to the dorsal striatum (putamen) volume reduction during a sensitive period in neuroanatomical development in childhood. Of relevance, recall that Quinlan et al. (2020) showed that chronic BV was indirectly linked to generalized anxiety *via* reductions in the left dorsal striatum in adolescence. Egerton et al. (2016) found that childhood adversity was linked to increased striatal dopamine activity in adulthood. Oswald et al. (2014) showed positive associations between childhood trauma and current levels of perceived stress, which related to higher ventral striatal dopamine responses to amphetamine. Accordingly, difficulties in learning and adjustments found in BV targets (Nishina and Parra, 2019; Vaillancourt et al., 2011, 2013) could be related to the striatal-dependent shift in memory processing associated with cognitive inflexibility and anxiety, especially in adolescence.

Executive Functioning: Cognitive Flexibility

Executive functioning is a cognitive control based on the analysis of environmental information and processed sensory information to assist with information encoding and cognitive flexibility (e.g., rapid attention/task-shifting and proper behavioral adjustments), which is crucial for decision making and problem-solving in adaption to the challenges presented (e.g., De Kloet et al., 1998; Dajani and Uddin, 2015; Palamarchuk and Vaillancourt, 2021). This type of cognitive control relates the dorsal and vlPFC functioning (e.g., Wager and Smith, 2003; Barbey et al., 2013), that is linked to developmental increases in the frontoparietal and parietal-dorsal ACC functional connectivity in the right hemisphere, to a stronger degree in girls compared to boys (Langeslag et al., 2013). Stress can alter executive functions, which is seen in the aforementioned cognitive inflexibility such as poor task/set-switching ability linked to the medial PFC dysfunction (Floresco et al., 2008; Butts et al., 2013; George et al., 2015) that correlates to cortisol response (e.g., Plessow et al., 2012; Goldfarb et al., 2017). Cognitive inflexibility is related to binary decision-making, which is a defensive cognitive strategy to ergonomically conquer the social challenge by integrating insecurity predictability, reasoning, learning fortification, and monitoring behavioral strategies (Collins and Koechlin, 2012). Nevertheless, poor cognitive flexibility is associated with poor mental health, for instance, depression (Gotlib and Joormann, 2010; Murphy

et al., 2012; Hou et al., 2016) and BV. For example, Medeiros et al. (2016) showed that 10- and 11-year-old targets of BV had lower cognitive flexibility that was associated with poor "cold" executive functions (related to the logical-rational part of the cognition) compared to perpetrators of bullying. In another study, Jenkins and Canivez (2021) found a negative association between BV and executive functions, including cognitive flexibility, self-monitoring, emotion regulation, inhibition, and initiation in 6th Grade to 8th Grade. McQuade (2017) demonstrated that executive functions moderated the relation between BV and increases in aggression over a year in adolescents.

The neurobiological mechanisms are that psychological distress might modulate an individual's response to stress, whereas poor stressor controllability (capacity of the PFC, discussed above) relates to exacerbated fear and anxiety that affects cerebral networks of the executive functions seen in behavioral problems (e.g., Maier et al., 2006; Maier and Watkins, 2010). For example, this mechanism can be seen in the hippocampal-related approach—avoidance conflict in young healthy adults (O'Neil et al., 2015), amygdala-related greater risk-taking behavior in bullied adolescent girls (Telzer et al., 2018), and learned helplessness and behavioral depression related to the medial PFC in rodent models (Maier et al., 2006). In childhood, there are four common behavioral responses to stress: impulsivity (i.e., acting-out or overreactive), passive-aggressiveness (i.e., overly compliant or uncooperative), dependency (i.e., passive or demanding), and repression (i.e., withdrawn or anxious; Chandler and Shermis, 1986). These responses are commonly expressed in bullied children (Reijntjes et al., 2010, 2011), which we predict are likely shaped by altered executive functioning seen in a stress-induced narrowed attention that enhances binary discrimination where the focus is placed on the aversive details (i.e., attentional bias; Cohen et al., 2009; Byrom and Murphy, 2016; Otgaar et al., 2017; Palamarchuk and Vaillancourt, 2021). Relatedly, BV is associated with goal-oriented selective attention (Carroll et al., 2019) and greater negative appraisals of peers (Troop-Gordon and Ladd, 2005).

A potential contributor to the behavioral maladjustment of bullied children is stress-induced right amygdalar hyperactivity that alters ascending projections toward the PFC. This signaling can modify executive functions related to decision making in unpredictable conditions (Maier et al., 2006; Maier and Watkins, 2010; Gupta et al., 2011; O'Neil et al., 2015), which are governed by the dorsal/lateral PFC (e.g., Wager and Smith, 2003; Barbey et al., 2013). Adolescence is a particularly vulnerable period for the development of executive dysfunctions because of the peculiarities in brain development seen in the functional organization of cognitive networks (especially in the right hemisphere; Sowell et al., 2004; Zhu et al., 2011) that parallel with attentional shifting, working memory, response inhibition, and goal-directed behavior (Luna and Sweeney, 2004; Paus, 2005; Bunge and Wright, 2007; Yurgelun-Todd, 2007; Schmithorst and Yuan, 2010). For instance, white matter microstructure was found to be correlated with the impulsive behavior in adolescents, with sex-specific differences (Silveri et al., 2006). The consequence is that learning strategies and decision-making are suboptimal at a time when an individual needs full functioning of their faculties to deal with the toxic social stressor. Relatedly, Herringa et al. (2016) showed that childhood adversity predicted hyper responses to aversive images in adolescence seen in the right amygdala hyperactivity and increased the right amygdala → bilateral PFC (BA 9, 10), bilateral hippocampi → bilateral PFC (BA 8, 9), and right hippocampus → left ACC (BA 32) functional connectivity. Moreover, right hippocampus reactivity was negatively associated with internalizing problems, while childhood adversity predicted increased right amygdala-dorsal PF connectivity specifically for negative emotional stimuli for adolescents only with lower levels of internalizing problems. This could be related to the higher ability of the PFC to regulate amygdalar responses (i.e., the PFC → amygdala inhibitory control); yet more research is needed to clarify these findings.

Aversive stimulation/stress can activate (i.e., higher 5-HT release) the dorsal raphe nucleus (and consequently the amygdala and striatum) and reduce its inhibition *via* diminished input from the vmPFC (Maier et al., 2006; Christianson and Greenwood, 2014); whereas inhibitory control of the vmPFC over brainstem and limbic structures determines stressor controllability and resilience (e.g., Amat et al., 2005; Maier and Watkins, 2010). In analogy to mental resilience and coping with general psychological stress (Palamarchuk and Vaillancourt, 2021), we predict that the mental health issues associated with BV in childhood are related to an escalated perception of helplessness and loss of control (hypofunctioning of the dorsomedial PFC linked to hyperactivated midbrain raphe nuclei and altered serotonergic signaling) associated with being abused (hyperactivated amygdala and norepinephrine surge) and weaker executive functioning that results in poor behavioral control and depression. In other words, the PFC, which is responsible for emotional control of impulses and behavioral responses to stimuli (i.e., inhibitory control) is hijacked by the area of the brain responsible for more primal functions like survival (i.e., the limbic system, specifically the amygdala and insula) following fear conditioning (e.g., Etkin and Wager, 2007; **Figures 1–3**). *Vice versa*, poor executive functioning is also a risk factor for BV in young children. Preliminary results by Vargas et al. (2019) showed that in youth, increased exposure to BV was independently associated with lower volumes of the right medial orbitofrontal area (BA 11, which is a part of the vmPFC). As well, clinical high-risk youth, who displayed altered gray and white matter, were more exposed to BV compared to healthy youth. It may relate to the altered social skills and inhibition problems (i.e., impulsive behavior). In particular, Verlinden et al. (2014) demonstrated that the risk for being bullied in first and second grade was associated with inhibition problems at age 4, but did not relate to working memory, shifting (i.e., cognitive flexibility), planning/organization, or emotional control. Inhibition difficulties, for instance, can be seen in the amygdalar hyperactivity and greater positive functional connectivity between the amygdala and rostral ACC (i.e., social pain network) in children with attention-deficit/hyperactivity disorder (ADHD; Hulvershorn et al., 2014).

This functional pattern of the social pain network is similar to the one found in bullied girls by Rudolph et al. (2016). This might explain why children with ADHD—who also have more academic, educational, and social problems—are at higher risk for BV than non-ADHD peers (Loe and Feldman, 2007; Holmberg and Hjern, 2008; Wiener and Mak, 2009; Murray-Close et al., 2010; Taylor et al., 2010; Efron et al., 2021).

Executive Functioning: Semantic Cognition

We hypothesize that the stress children experience because of being bullied by their peers will also present as poor inhibitory control that negatively impacts semantic performance, i.e., the coherence or correspondence in comprehension and abstract thinking, which consequently impairs memory and decision-making ability. This hypothesis is based on PTSD studies demonstrating that stress-associated cognitive deficits are linked to altered memory formation due to retroactive interference (Yehuda et al., 1995). Retroactive interference relates to higher cross-categorization, which refers to altered patterning discrimination by individual cues that are present in correct and incorrect responses. The effect of retroactive interference—when a new memory interferes with the retrieval of an old memory—is more pronounced in children than in adults because children attend to too many details and cannot discern complex patterns if they are missing details or extraneous cues (e.g., Darby and Sloutsky, 2015). Consistent with this idea, Vaillancourt et al. (2011) found that altered cortisol levels in bullied adolescents predicted neurocognitive dysfunction in visual and verbal learning, as well as in executive tasks that were related to learning strategies and decision-making.

During maturation, semantic cognition capacity increases with the development of the anterior (rostral) PFC (orbitofrontal cortex, BA 10; Dumontheil, 2014), which have been found to be positively associated with levels of social anxiety in childhood and adolescence (Rosso et al., 2004). At the same time, intense stress can induce suggestive pressure or spontaneous semantic priming (e.g., interpretation of innocuous events as threats), and thus facilitate retrieval of false memories (Payne et al., 2007; Otgaar et al., 2017). Although the tendency to falsely remember events increases during normal development in human cognition due to a paradoxical complementarity effect—an increase in both accurate and false recall/recognition with age (Metzger et al., 2008; Howe et al., 2009; Brainerd et al., 2018), there are opposite developmental trends in false memory due to deficient cognitive ability to form semantic relations (i.e., semantic cognition; Ralph et al., 2017) in young children (Brainerd and Reyna, 2007). That is, false memory formation decreases with age for semantically unrelated information but increases with age for semantically related information. Besides the increase in the strength of these associative links between pieces of information (i.e., backward associative strength), its automaticity also prevents inhibition (forgetting) of false memories with age (McDermott and Roediger, 1998; Gallo and Roediger, 2002; Howe, 2005). In children, false recognition can be promoted by increased rates of similarity that leads to a gist extraction from semantically related information (i.e., fuzzy face theory; Brainerd and Reyna, 2005), as well by false identity judgment about distractors or decreased rates of non-identity judgment (Brainerd and Reyna, 1998).

These developmental nuances of false memory formation and maintenance hypothetically interplay with the stress-induced executive dysfunctions in bullied children. This complex cognitive interplay can explain the discrepancies between retrospective accounts of BV in relation to actual self-reports of BV. For example, Nishina and Parra (2019) found that students who overreported their abuse by peers had poor current (12th grade) psychosocial adjustment (i.e., higher depressive symptoms and social anxiety, and lower self-worth). Although the authors did not identify the underpinning cognitive mechanisms, they referred to an analogy with a mood-congruent memory bias (i.e., induced by emotional valence of the content/stimuli; Miranda and Kihlstrom, 2005) for over-reporters. Such biases relate to a triggered negative mood by aversive content that can distort memory; yet over-reporters presented with "naturally" negative mood (e.g., depressive symptoms) that can endorse false recall (Bookbinder and Brainerd, 2016).

We relate these types of discrepancies to false memories due to compromised semantic ability associated with psychological maladjustment following severe stress perception in over-reporters. Cognitive appraisal has a strong moderating effect on emotional dysregulation and is thus linked to psychiatric outcomes such as mood and anxiety disorders (Picó-Pérez et al., 2017; Zilverstand et al., 2017). For instance, Graham et al. (2003) showed that BV identified by self-reports but not peer-reports predicted similar psychological maladjustments as did BV identified by both self- and peer-reports for 6th grade students. Furthermore, studies that isolate functional activity uniquely to false memories (e.g., hit baseline) have shown that false memory retrieval occurs within the network of cognitive control/appraisal and emotion regulation, that includes the vmPFC (BA 24) and dorsal anterior cingulate cortex (ACC, BA 34/24, 32), as well extending to the frontoparietooccipital regions (BA 6/44, 40, 18/19) and the brainstem; whereas activity in verbal processing share same regions with semantic false memories, which includes dorsomedial (BA 32/8, 6/8) and vlPFC (BA 45/47), dorsal ACC (BA 32), and frontoparietal regions (BA 6/44, 40/7; see a voxel-wise quantitative meta-analysis by Kurkela and Dennis, 2016). Thus, verbal memory and false memory processing share a network with cognitive appraisal, which can be compromised by high rates of self-perceived stress that is associated with psychological maladjustments. Accordingly, Slattery et al. (2013) demonstrated the impact of low cognitive appraisal on poor cortisol response to a social stress test in adolescents at age 18; whereby internalizing disorders increased the links between poor verbal memory performance and poor cortisol reactivity. Woody et al. (2018) found that social stress elicited cortisol and cardiovascular responses to the speech stressor; while there were no associations between cognitive load/intelligence and stress cortisol responses (Sladek et al., 2016; Woody et al., 2018). Lastly, executive dysfunction may also explain why youth who underreported being bullied had poorer 6th grade adjustment (Nishina and Parra, 2019),

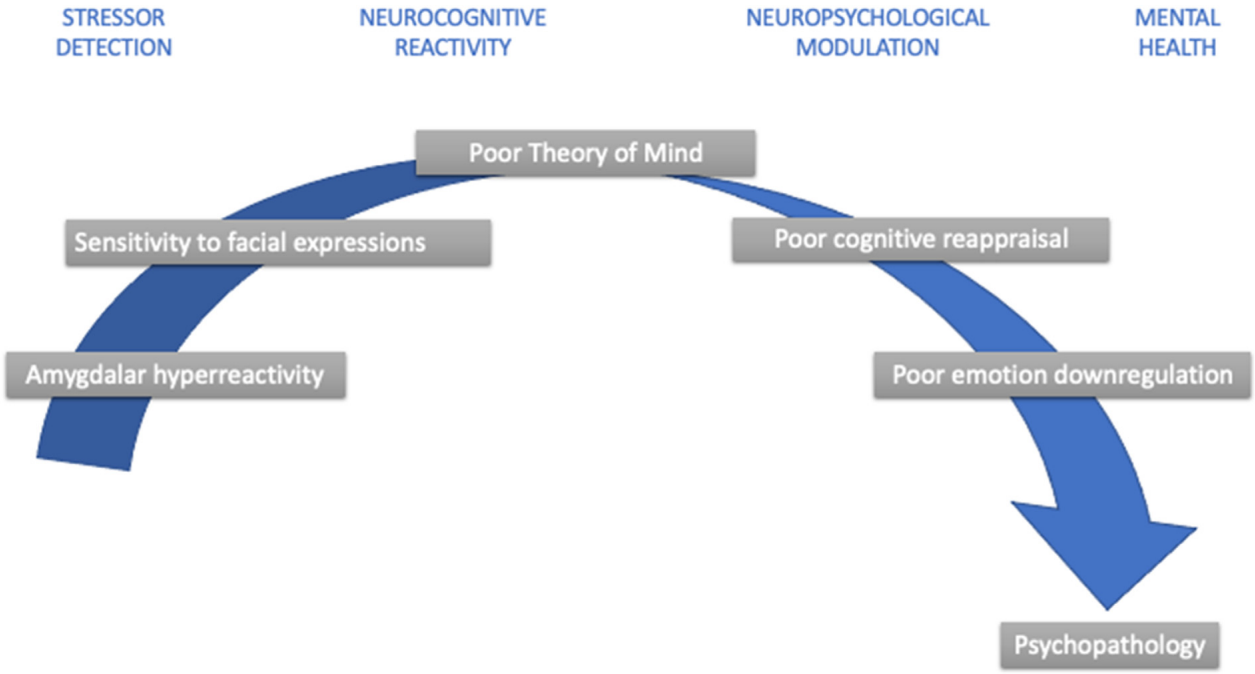

FIGURE 1 | Developmental aspects in childhood stress psychopathology. Note: This simplified diagram summarizes developmental nuances that contribute to childhood stress sensitivity and increase psychopathology risk.

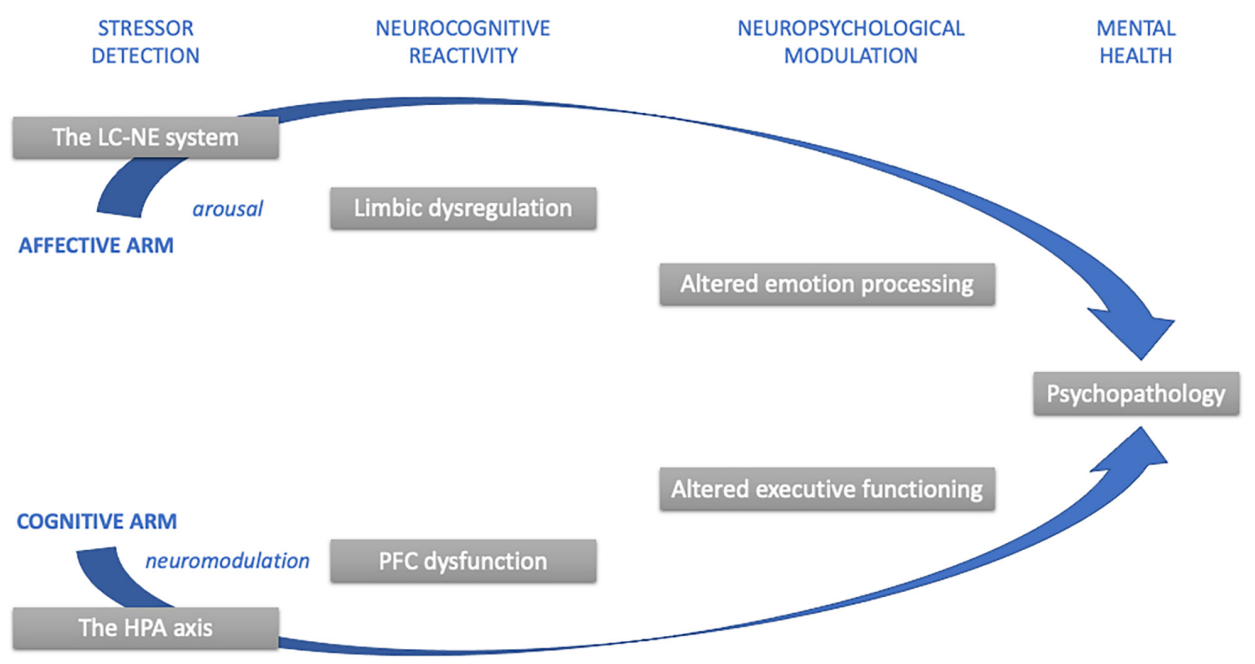

FIGURE 2 | Cognitive and emotional convergence associated with stress psychopathology. Note: This simplified diagram summarizes the pathways of cognitive and emotional convergence in stress that can lead to psychopathology. *Abbreviations*: HPA, hypothalamic–pituitary–adrenal; LC-NE, locus coeruleus.

which cannot be supported by a mood-congruent memory bias. Rather, the under-reporting could be due to poor post-stress recall; especially since true memories undergo retrieval-induced forgetting, which is less applicable for false memories in children (Price and Phenix, 2015). Baugerud et al. (2016) demonstrated that maltreated children had a poor true recall for both neutral

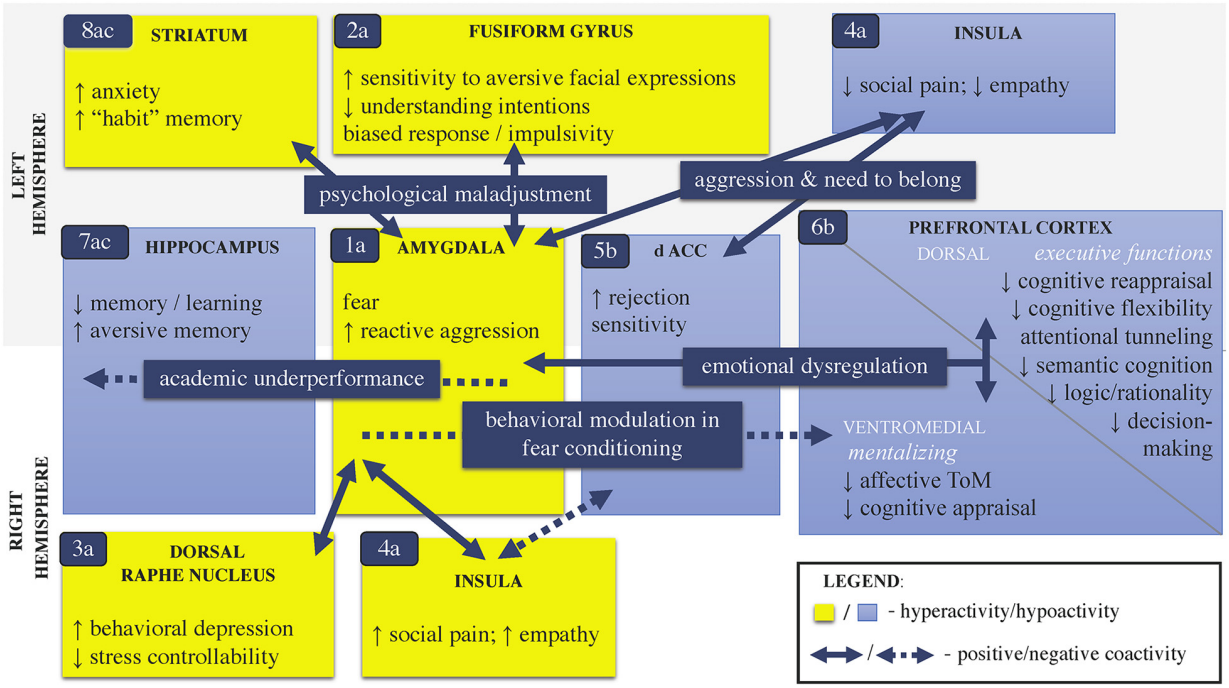

FIGURE 3 | Major brain circuits associated with childhood stress psychopathology. Note: This schematic model integrates the major cerebral circuits (1–8) of cognitive and emotional convergence in stress neuroactivity that can be associated with childhood psychopathology. The model is a simplified syntax of the evidence in: [a] general psychological stress; [b] childhood BV; and [c] early life adversity. [a] The brain dynamics stem from (1) a hyperactivated amygdala following fear conditioning, which is a common neural response to an acute psychological stressor (i.e., "alarm-to-threat" stage, Palamarchuk and Vaillancourt, 2021). The magnitude of the amygdalar stress-reactivity can be mediated by the activity of the (2) fusiform gyrus, (3) dorsal raphe nucleus, and (4) anterior insula. [b] Hypothetically, the amygdalar overactivity can suppress the (5) dorsal/rostral ACC, which further can lead to (6) hypofunctioning of the vmPFC and thus impair mentalizing in young targets of BV. [a,c] Generally, in intense/chronic psychological stress, the amygdalar hyperactivity can (7) alter hippocampal memory encoding and (8) promote striatum-dependent memorization, which together, exacerbate compromised PFC related to executive functions. If altered PFC has poor inhibitory control over the amygdalar reactivity, a vicious cycle can occur (1 ⇄ 8). Of note, besides environmentally mediated pathways, childhood BV can also be a risk factor if the maladaptive brain dynamics pre-date the stress. For instance, genetic factors and developmental nuances of greater amygdalar sensitivity to stress and/or immature functions of the PFC (e.g., poor ToM) can increase susceptibility to stress in early life. The cerebral networks potentially display hemispheric lateralization in functions that distinguish pure targets (the right dominance) from bully-victims (the left dominance). *Legend*: dACC, dorsal anterior cingulate cortex; BV, bullying victimization; ToM, theory of mind; ↑, increase; ↓, decrease.

and emotional information, but higher rates of false recall for aversive information compared to their non-maltreated peers. Altered post-stress recall could be a maladaptive strategy to deal with the stressor, the interpretation of that stressor, and the memory of the stressor. This hypothesis resonates with the findings that in bullied children, depression is an independent contributing factor to memory deficit including verbal domain, which is negatively correlated to the PFC's executive functioning in addition to elevated cortisol levels (Vaillancourt et al., 2011).

DEVELOPMENTAL ASPECTS AND INTERACTING NEURAL MECHANISMS IN CHILDHOOD STRESS PSYCHOPATHOLOGY

Sensitivity to Facial Expressions

Fear-conditioned memories of faces can impair the fusiform gyrus (Mueller and Pizzagalli, 2015), which functional significance is (but not limited to) in facial expression perception and face recognition (Kanwisher and Yovel, 2006; Iidaka, 2014). A recent study showed that high BV at age 8 years was associated with a "thicker cortex" of the fusiform gyrus in high-resolution structural magnetic resonance imaging (MRI) at age 10 years (Muetzel et al., 2019). Although true neural basis is unclear, in pediatric neuroimages, what appears to be larger cortical thickness, could in fact be due to delayed myelination. Myelination, the axonal insulation that facilitates signaling (action potentials) within synapses, is an essential neuronal networks component which undergoes critical developmental changes across ages 5–10 years. The increase in myelin deposition alters the contrast between gray (cortex) and white matter (which mainly contains long-range myelinated axons); thus, cerebral maturation can appear as cortical thinning after reaching peak by age ∼10 years (Shaw et al., 2008; Ducharme et al., 2016; Natu et al., 2019). The reported greater thickness of the left fusiform gyrus (Muetzel et al., 2019) may in fact be related to delayed myelination in bullied children. However, more research is needed to assess the impact of potential mediators and moderators. For example, socioeconomic status was found to

moderate trajectories of the cortical thickness decline, which had a curvilinear pattern (relatively steep decline in early childhood and subsequent leveling off during adolescence) for the low levels and linear pattern for the high levels, with the major effect in left superior temporal gyrus (Piccolo et al., 2016). As well, ADHD is associated with delayed maturation of the PFC (Shaw et al., 2007), whereas ADHD outcome mediates cortical thickness—the worse outcome is related to a "fixed" thinning of the left medial PFC at baseline (mean age 8.9 years) and the better outcome is related to the normalization of cortical thickness in the right parietal cortex at follow-up (mean age 5.7 years; Shaw et al., 2006).

Cortical thickness is an age-related parameter in structural MRI studies; however, the fact is that cortical thinning can relate to various factors of neurogenesis (e.g., synaptic reorganization and pruning; Shaw et al., 2008). As well, we see the interpretation during "thickening → thinning" transitioning period at age 10 years is especially challenging; conversely, longitudinal studies that define trajectories of cortical development and cortical thickness reached peaks would provide more insights into the underpinning cellular mechanisms. It cannot be explained solely by structural MRI and *in vivo*; an integration of other parameters is needed such as cortical pattern matching to account for gray matter variation and gyral patterning (Shaw et al., 2008; Tamnes et al., 2010). Maturation is also associated with functional connectivity transformations when cortical control within cerebral networks enhances and other cerebral regions, including the fusiform gyrus, lose centrality during the transition to adolescence period (Sato et al., 2015). Thus, we relate the reported greater cortical thickness (Muetzel et al., 2019) to delayed maturation that can be clarified with the cortical development trajectories in bullied children. The greater cortical thickness can also be a risk factor for cerebral functional connectivity due to local white matter alterations, which has to be further tested with functional MRI and fractional anisotropy.

Although Muetzel et al. (2019) do not account for the aforementioned neuroimaging specifics, that is, the delayed myelination rather than cortical hypertrophy, they did discuss that these cortical morphology changes in the fusiform gyrus could be related to a sensitivity to aggressive facial expressions developed in BV targets. Indeed, nonverbal facial expressions recognition involves emotional contagion (part of affective empathy) *via* brain-to-brain coupling and mimicry between individuals' emotion systems (Prochazkova and Kret, 2017), whereas the amygdala can increase the sensitivity of the fusiform gyrus to visual response to static fearful faces accordingly to the connectivity models of fear sensitivity by Furl et al. (2013). However, we predict that this stress mechanism likely extends beyond this type of amplified emotional response to fearful/aggressive faces and involves functional connectivity (white matter architecture) that alters the fusiform gyrus input into theory-of-mind (ToM) *via* developmental sensitization in BV.

Theory of Mind

Theory of Mind (ToM), also known as mentalization, refers to the ability to infer the mental states of others like their thoughts/beliefs (i.e., cognitive perspective-taking or cognitive ToM) and emotions/feelings (i.e., affective perspective-taking or affective ToM) and the analysis of related behavior that is essential in social interactions and problem-solving (Dvash and Shamay-Tsoory, 2014; Healey and Grossman, 2018). Both cognitive and affective ToM engage the temporoparietal junction, precuneus, and temporal poles. Yet, cognitive ToM may engage the dorsomedial and dorsolateral PFC; whereas affective ToM may uniquely engage the vmPFC, the amygdala, and basal ganglia (that includes striatum; Healey and Grossman, 2018). The fusiform gyrus facilitates understanding intentions during ToM processing *via* the visual perception of others' emotions (i.e., "reading" eye expressions or body language; Schurz et al., 2013, 2014; Thye et al., 2018; Tallarita et al., 2020). During social interactions, face processing relates to the activation of the fusiform gyrus, which is significantly influenced by emotional input from the amygdala; the latter produces fast perceptions of social–emotional feelings that are moderated by previous social experiences (i.e., subconscious threat-monitoring; Schultz et al., 2003; Duncan and Barrett, 2007; Herrington et al., 2011; Balderston et al., 2014; Frank et al., 2019). The amygdala's hyperactivity may therefore disturb networks with the fusiform gyrus and compromise ToM processing (insufficient input from the vmPFC), which can be seen in rigid habit-like behavior during stressful social interactions due to the limbic dysregulation. In fact, Frick et al. (2013) showed that increased sensitivity of the fusiform gyrus to fearful faces was accompanied by the changes in connectivity between the fusiform gyrus and amygdala (increased) and also between the fusiform gyrus and vmPFC (decreased) in social anxiety disorder. Rudolph et al. (2016) also showed that the fusiform gyrus over responded to exclusion (relative to inclusion) together with the amygdala and dorsal ACC, the brain structures which hyper responses consequently predicted internalizing symptoms in bullied girls. In contrast, conscious awareness (the dorsomedial PFC activation in emotional downregulation) of the fearful faces can reduce the amygdala-PFC functional connectivity; whereas the connectivity is increased when information is processed non-consciously and the amygdalar responses relate to a negative bias in the subsequent evaluation of neutral faces (Lapate et al., 2016). That is, cognitive behavioral therapy that facilitates proper evaluation of the fearful faces (i.e., cognitive reappraisal) may help with overcoming explicit biases and subconsciously/"automatically" triggered social anxiety.

The need to support cognitive re-appraisal may also relate to poor executive functions in BV. Du Plessis et al. (2019) showed that childhood BV was related to a structure of the right vlPFC (i.e., thickness and surface area, moderated by cortisol levels), yet only in adolescent boys. *Vice versa*, stress-associated impairment of ToM likely can promote attentional tunneling that reduces cognitive flexibility (i.e., poor functioning of the medial/ventral PFC affects the dorsal/lateral PFC activity), and in turn, contribute to academic underperformance of bullied children. Clemmensen et al. (2020) showed that ToM and involvement in BV were both independently associated with academic performance in children aged 11–12 years, even after accounting for IQ and shared variance. Moreover, in this study,

bullied girls had lower academic performance than non-bullied girls and boys and bullied boys.

Emotion Regulation: Prefrontal Cortical Descending Projections

The neurobiological background of ToM is that the medial PFC is critical for social cognition like judgment. Although the vmPFC is linked to understanding affective but not cognitive ToM (Shamay-Tsoory et al., 2006; Shamay-Tsoory and Aharon-Peretz, 2007), it is an anatomical key element for social, affective, and cognitive networks; and its disruption is linked to psychopathology and psychiatry (Hiser and Koenigs, 2018). Our hypothesis is that the complex interplay between altered vmPFC's circuits linked to ToM affects the amygdalar responses to aversive emotions and fear, which relates to stress sensitization and psychiatric outcomes in bullied children.

First, the vmPFC is a reward-sensitive region that promotes social connection to others, monitors safety, and inhibits emotional-related reactions (Delgado et al., 2006). The vmPFC and left amygdala coactivity (i.e., concurrent responses during emotion processing) occurs during active regulation of negative emotions (Diekhof et al., 2011; Yang et al., 2020). The evidence for adults is that negative coactivation between the vmPFC (i.e., hyperactivity) and right amygdala (i.e., hypoactivity) influences downregulation of negative emotions, which is a cognitive "minimizing" reappraisal (Banks et al., 2007; Morawetz et al., 2017; Yang et al., 2020). Specifically, the vmPFC encodes emotional value (e.g., perceived aversiveness and fear) and further modulates it during cognitive reappraisal (e.g., distancing from feelings or reward anticipation). The successful emotion downregulation concordantly hypoactivates the left amygdala (i.e., negative coactivation that suppresses emotional value) and extends to the nucleus accumbens (the ventral striatum) to suppress reward encoding (Phan et al., 2005; Staudinger et al., 2009; Mulej Bratec et al., 2017). Thereby, we may say that successful emotional regulation relates to the pattern of top-down (i.e., cortical → subcortical) negative coactivity: the vmPFC hyperactivity → the right amygdalar hypoactivity (then mirrored by the left amygdala) → the ventral striatum hypoactivity. Of note, the cerebral activity pattern differs during the cognitive "positive" reappraisal (i.e., finding the positive meaning in negative experiences) as displays positive coactivity in this functional architecture (Doré et al., 2017), which likely contributed to Dougherty et al.'s (2015) findings of the "divergent" effect in children reappraisal (see also"self-appraisal" vs. "stressor-appraisal"; Palamarchuk and Vaillancourt, 2021).

However, BV is associated with emotional dysregulation related to the PFC dysfunction. In bullied young adults, the medial PFC-amygdalar connectivity across inclusion and exclusion was found to be positive (i.e., the medial PFC hyperactivity → the right amygdalar hyperactivity; see **Figure 3**) and indicated insufficient inhibitory control over the amygdala; this positive connectivity also moderated the relation between BV and depressive symptoms (McIver et al., 2019). For bullied adolescent girls, Casement et al. (2014) showed that the medial PFC and striatal hyper responses to reward anticipation mediated the link between social stressors and concurrent symptoms of depression; while Telzer et al. (2020) found that severity of BV correlated to hyperactivity in the amygdala and ventral striatum (but not limited to these structures). In bullied adolescents, it was shown that emotion regulation moderated poor cortisol reactivity to stress (Kliewer, 2016); as well, emotion regulation was associated with the emotion awareness and later on with anger regulation and lower rates of BV during the 2-years follow-up (Riley et al., 2019). In young children, Park et al. (2021) showed that chronic stress exposure can affect resting-state functional connectivity: (1) decrease it between the medial PFC and ventral tegmental area, which is essential in reward cognition such as associative learning and motivational salience; yet (2) increase it between the anterior hippocampus and left inferior frontal gyrus (BA 44), which is a part of the vlPFC implicated in language production and phonological/speech processing. These brain dynamics support the aforementioned findings that chronic BV can impair academic performance and increase the vulnerability of young targets. Similarly, mood and anxiety disorders were found to relate to less successful emotional downregulation in cognitive reappraisal (i.e., distancing strategies related to the vmPFC; Picó-Pérez et al., 2017), while the amygdalar hyper responses were linked to depression (Zilverstand et al., 2017). That is, emotional dysregulation in bullied children is likely linked to poor ToM that compromises cognitive appraisal and exhibits the pattern of top-down positive coactivity the vmPFC hyperactivity → the amygdalar hyperactivity (**Figure 3**).

Second, a successful "minimizing" reappraisal circuit also involves the positive coactivation of the social pain network, the vmPFC, the ACC, and the insula (Diekhof et al., 2011; Winecoff et al., 2013; Doré et al., 2017). For instance, Phan et al. (2005) showed that increases in negative emotional experiences in healthy adults were related to hyperactivity in the amygdala and hypoactivity in the dorsal ACC, which was moderated (attenuated and augmented, respectively) during cognitive reappraisal that involved activation of the dorsomedial PFC. The ACC is a behavior-monitoring and optimal decision-making region that integrates contextual socio-emotional information such as perceived fairness/unfairness (e.g., Kennerley et al., 2006; Lavin et al., 2013; Rolls, 2019). Activity in the dorsal/rostral ACC for fearful faces is negatively associated with BV (Swartz et al., 2019). Of note, a rostral part of dorsal ACC (often termed as the rostral ACC) belongs to the vmPFC (BA 32). Changes in functional coactivity within the social pain network (**Figure 3**) may explain heightened rejection/interpersonal sensitivity in targets compared to perpetrators; that has been shown to mediate the association between BV and mental health symptoms (Williams et al., 2017; McDonnell et al., 2018). The hypothesis resonates with Kross et al.'s (2007) findings that self-reported ratings of rejection-associated distress negatively correlated with hyperactivity in the left dorsal ACC (BA 6, which activity correlated positively with the right dorsomedial PFC, BA 8) and right insula in young healthy adults. As well, compared to high rejection sensitivity, low rejection sensitivity was associated with hyperactivity in the lateral PFC (left BA 45, 9, and right BA 6). Baird et al. (2010) demonstrated that increases in the dorsal ACC (anterior, BA 24/32 and posterior, BA 31) and

bilateral dorsolateral PFC coactivity was associated with reduced sensitivity to relational aggression during an affect recognition task in bullied adolescent girls. This denotes a moderating role of the executive functions in emotion regulation. McIver et al. (2019) showed that social exclusion (compared to inclusion) increased the ACC-right insula functional connectivity in bullied and non-bullied young adults. However, positive connectivity between the left amygdala-ACC and left amygdala-right insula were attenuated in bullied adults, compared to non-bullied adults. That is, emotional dysregulation in bullied children is likely moderated by heightened rejection sensitivity with the pattern of top-down coactivity lateralized to the right: the vmPFC hyperactivity (i.e., poor affective ToM) → the dorsal ACC hypoactivity (higher rejection sensitivity) → the amygdalar hyperactivity → the right insula hyperactivity (**Figure 3**).

The dorsal ACC over responses to social exclusion in both BV and chronic peer rejection (Rudolph et al., 2016; Will et al., 2016), and its coactivation with anterior insula during social exclusion predicts increased internalizing symptoms and avoidance motivation in bullied girls (Rudolph et al., 2016). However, the reactivity of the left insula, as well as of the left nucleus accumbens (ventral striatum) appears to be additionally moderated by both adolescent depression (Silk et al., 2014) and bullying behavior (Perino et al., 2019). Importantly, executive functioning capacity also modulates the association between the activity in the left insula and dorsal ACC linked to aggression: low executive functioning predicts positive association and high executive functioning predicts negative association (Chester et al., 2014); whereas the dorsal ACC-insula functional connectivity correlates to target-changing behavior, which is linked to extraversion and thus, hypothetically, could facilitate increased interpersonal aggression (Takami and Haruno, 2020).

Perino et al. (2019) demonstrated that adolescents' self-reported BV was related to hyperactivation in the medial PFC, insula, amygdala, and ventral striatum; yet Beekman et al. (2016) revealed that the need to belong can moderate the stress-responses to social exclusion. Chester et al. (2016) showed that rejection, as compared to acceptance, was not directly associated with a greater need to belong, but it was indirectly *via* social pain. Specifically, higher activity in the dorsal ACC and the insula predicted affiliative behavior. Thus, specifics of the dorsal ACC-insula functional coactivity, which are moderated by both executive dysfunctioning and rejection sensitivity, may differ between pure targets and bully-victims (i.e., children who are bullied and bully others; **Figure 3**); and because bullied children can have an anxious need to belong, which may be why they become perpetrators (Barker et al., 2008; Haltigan and Vaillancourt, 2014; Underwood and Ehrenreich, 2014). The poor outcomes for BV targets might thus be due to a socioemotional asymmetry that is characterized by a higher sensitivity to rejection vs. the higher need to belong seen in perpetrators. Perpetrators of bullying have been shown to have status goals related to dominance and prestige (Sijtsema et al., 2009) and often enjoy greater power, dominance, and popularity than BV targets (Vaillancourt et al., 2003; Pouwels et al., 2018; Faris et al., 2020).

As well, bullying behavior may relate to greater impulsivity (related to cognitive inflexibility associated with the dorsal/lateral PFC, reviewed above), which was found to be associated with the cortical volume reductions (in the medial PFC and insula) but subcortical volume increases (in the ventral striatum, hypothalamus, and anterior thalamus), whereas cortical to subcortical volume ratio partially mediated the association between early adversity and antisocial behavior (Mackey et al., 2017). That is, in bully-victims, the dorsal ACC—left insula coactivity likely has stronger functional connectivity and relates to an increased need to belong, aggression, and impulsivity but lesser rejection sensitivity compared to pure targets (**Figure 3**).

The dorsal ACC-insula effects on the amygdalar responses related to impulsivity and rejection sensitivity that distinguishes pure targets from bully-victims may belong to their empathic ability; and their neural network is also regulated by these structures (Völlm et al., 2006; Decety et al., 2013a,b). Empathic ability is an emotional aspect of inferring and sharing the emotional experiences of others (Dvash and Shamay-Tsoory, 2014), which entitles affective empathy (i.e., affective ToM, the vmPFC) and emotional contagion (Healey and Grossman, 2018). During social interactions, empathy levels mediate spontaneous engagement of cognitive ToM (i.e., cognitive empathy) related to the dorsomedial PFC (Pluta et al., 2011; Wagner et al., 2011). Its deterioration in the right hemisphere is associated with not understanding others' emotions (Ratka, 2018). In mentalizing, the activation of the dorsomedial PFC is less pronounced in relation to negative social stimuli like social exclusion compared to more positive ones like social inclusion (Powers et al., 2013). This is likely an adaptive tool, a type of self-defensive mechanism that involves mental withdrawal to numb emotional pain. In fact, social exclusion is related to a higher tolerance for physical pain and "emotional numbness" such as reduced joy and empathy, which are intercorrelated (DeWall and Baumeister, 2006; Powers et al., 2013). Accordingly, we hypothesize that the aforementioned poorer ToM ability in children who are bully-victims compared to pure targets and pure perpetrators (Shakoor et al., 2012) is related to the vmPFC functioning being moderated by the dorsomedial and dorsolateral PFC activity (i.e., cognitive empathy and executive functioning; see **Figure 3**). This may also explain the findings that depression, anxiety, and psychosomatic symptoms were most frequently observed in bully-victims, but equally observed in pure targets and pure perpetrators (Kaltiala-Heino et al., 2000).

Behavioral Modulation: Amygdalar Ascending Projections

Meta-analytic findings of the emotional processing of fear in individuals with PTSD, social anxiety disorder, and specific phobia reveal hyperactivity in the amygdala and insula compared to healthy participants undergoing fear conditioning. Of note, the patterns of coactivation between the cortical regions of interest were observed only in individuals with PTSD (Etkin and Wager, 2007). Specifically, the patterns included: (1) negative coactivation between the right amygdala (its hyperactivity was then mirrored by left amygdala) and right dorsal/rostral ACC (its hypoactivity was then mirrored by the left cortex); and

(2) positive coactivation between the latter cortex and the right (and then left) vmPFC. Conversely, extinction of conditioned fear relates to hypoactivity in the amygdala and hyperactivity in the dorsal/rostral ACC and the vmPFC (Phelps et al., 2004). Considering the amygdala-PFC connectivity, the main pathway for behavioral modulation in bullied children hypothetically can be: fear conditioning → the right amygdala hyperactivity (then mirrored by left amygdala) → hypoactivated dorsal/rostral ACC → hypoactivated vmPFC → poor affective ToM, higher rejection sensitivity, and higher distress (**Figure 3**).

Phobia and distress can have a larger effect on the PFC in childhood than in adulthood. ToM ability is facilitated by the inferior temporal lobe extending into the fusiform gyrus, which emerges around the ages of 3–5 years and is solidified in most children by around age 7 (Sabbagh et al., 2009; Lackner et al., 2010). Yet, ToM reasoning activity in the medial PFC shifts from ventral to dorsal only in late childhood (Moriguchi et al., 2007) and the affective component of empathy develops earlier than the cognitive component (Decety and Svetlova, 2012). The neuroanatomical background is that ToM development relates to the white matter maturation in: (1) "local structure" of (near) the medial PFC, medial parietal (precuneus), and temporoparietal regions, and (2) connectivity between the inferior frontal and temporoparietal regions (Grosse Wiesmann et al., 2017); whereas white matter tracts of the frontotemporal regions have a pattern of late maturation (Tamnes et al., 2010). The cortical functional connectivity development is also asymmetrical—earlier in the right circuits and more expressed in the anterior (dorsal frontoparietal) regions (Sowell et al., 2004; Zhu et al., 2011).

Thus, the medial PFC (i.e., anterior region) is largely influenced by the right amygdala (i.e., subcortical region). However, the medial PFC is neither capable to execute sufficient inhibitory control over the amygdala during aversive stimuli nor is supported enough by the dorsal PFC related to cognitive reasoning due to its late maturation. Not surprisingly, the pre adolescent period is a high-risk time for stress to alter the maturation and functioning of the PFC (Caballero et al., 2016). This explains why the vmPFC (especially in the right hemisphere) is susceptible to behavioral modulation imposed by the amygdala's hyperactivation in fear conditioning/emotional face perception, which is greater in children than in adults (Monk et al., 2003; Guyer et al., 2008), and linked to psychopathology, such as major depression and bipolar disorder (Monk et al., 2008; Brotman et al., 2014; Wegbreit et al., 2014).

Barker et al. (2008) and Haltigan and Vaillancourt (2014) examined the joint trajectories of BV and perpetration across childhood and adolescence respectively and found that the evolution of bullying involvement emerged from victimization to perpetration and not the reverse. A meta-analysis by Walters (2021) further revealed that BV and perpetration are intercorrelated and have bidirectional cross-lagged longitudinal relations, i.e., perpetration ⇄ victimization. High BV seems to exacerbate poor affective reasoning and emotional control. Indeed, higher levels of BV in children are related to lower conscientiousness and higher neuroticism, which manifests as feeling angrier, blaming the perpetrator, and being less forgiving during conflicts (Bollmer et al., 2006), which could indicate ToM nuances linked to asymmetry in the PFC's control over the amygdalar responses to anger and fear that distinguish pure targets from bully-victims. In particular, poor ToM at age 5 years was found to be related to higher levels of reactive aggression (i.e., limbic/automatic response to a real/perceived threat) at age 6 years in BV targets; whereas better ToM at age 5 years was found to be related to higher levels of proactive aggression (i.e., a non-provoked/planned behavior for personal gain) at age 6 years in perpetrators (Renouf et al., 2010). As well, the amygdala's higher response to anger and lower response to fear predicts bullying behavior (Swartz et al., 2019). Regardless of the motivational direction (i.e., approach vs. withdrawal), higher levels of anger have been related to larger left lateralization in the PFC activity (Wacker et al., 2003; Stewart et al., 2008), while the right amygdalar hyperactivity is a frontline response to fear conditioning linked to hypoactivity of the right medial PFC (Etkin and Wager, 2007), and the latter correlates to impaired detection of deception (Stuss et al., 2001; Beekman et al., 2016). Moreover, emotional valence asymmetry in cerebral functioning is well-documented (Davidson, 1992); and the hypoactivity in the right cortex relates to poor recognizing of negative emotion such as envy, whereas hypoactivity in the left cortex relates to poor recognition of positive emotion (Shamay-Tsoory et al., 2007). We hypothesize that poor ToM is linked to the amygdala-medial PFC coactivity, which is moderated mainly by fear, and in the right hemisphere in pure targets compared to bully-victims, whose functional coactivity is moderated mainly by anger and in the left hemisphere (**Figure 3**).

DISCUSSION: STRESS VULNERABILITY NUANCES AND INTEGRATIVE BRAIN DYNAMICS

BV is associated with a significant psychological burden that triggers a neuroendocrine cascade of stress-response activated by elevated cortisol levels and the outcomes are seen in the long-lasting deterioration of mental health, including depression, PTSD, and suicidal ideations in children and adolescents (Ttofi et al., 2011; Moore et al., 2017; Vaillancourt, 2018; Vaillancourt and Palamarchuk, 2021). BV can develop into an interpersonal trauma (Idsoe et al., 2021) where psychopathology is a by-product of a multilevel neural phenomenon with the brain dynamics bidirectionally stemming from the cortical-subcortical cerebral circuits. Given the scarcity of research that can clarify the cognitive and emotional convergence underlying psychopathology associated with childhood BV, we review and integrate the evidence on brain dynamics in: (A) general psychological stress; (B) childhood BV; and (C) early life adversity (see schematic outlining of the major cerebral circuits in **Figure 3**), and in doing so, emphasize the following aspects:

(A). The integral role in cognitive and emotional convergence belongs to the amygdala functioning and its circuits triggered by fear in acute stress. Fear conditioning

can lead to psychiatric problems because the amygdala is the emotional hub of memory and influences the cognitive "defence" mechanism on a subconscious level, which is represented in the vm fronto-temporoparietal network (e.g., Palamarchuk and Vaillancourt, 2021).

(B). Perspective-taking abilities (i.e., ToM) play an essential role in social interactions. Both cognitive and affective ToM share the vm fronto-temporoparietal network with cognitive "defence" mechanism. Immature and/or stress-compromised ToM relates to the misperception of others' intentions and can contribute to explicit bias and anxiety (reviewed above). This can narrow attention with a feeling of losing control over the situation. At the same time, cognitive inflexibility and insufficiency in emotion regulation aggravates stress perception and worsens psychological disturbance (e.g., Palamarchuk and Vaillancourt, 2021). Not surprisingly then, BV is linked to poor ToM.

(C). From the early life stress viewpoint, there are several major nuances that can explain why BV is reliably associated with a risk of developing psychiatric disorders in childhood. First, the higher risk of psychiatric disorders in bullied children is likely supported by developmental aspects in the anatomical ToM signature besides emotion reactivity. In particular, the higher amygdalar response to fear (seen in a norepinephrine surge) and its stronger influence on the vmPFC (seen in the negative coactivity of these brain structures) in children compared to adults could increase the risk of disorders. Second, the disturbed emotional processing due to poor inhibitory control of the vmPFC can aggravate the amygdalar hyperactivity in stress. Third, stress-induced morphological and neurochemical changes within the cerebral networks are especially harmful in early life because of undergoing neurogenesis and myelination, which is likely compromised in young targets. Lastly, the susceptibility to psychiatric dysfunctioning among bullied children and adolescents can relate to the neuroepigenetics that moderate stress reactivity (e.g., blunted cortisol response).

Although we refer to the neural mechanisms of early life and general psychological stressors, the neuropathology in childhood BV may differ and longitudinal studies are required to test the brain dynamics models—integrative vs. specific—to better understand how BV gets under the skin to confer risk in young targets. Nevertheless, psychopathology in mental stress significantly relates to the cognitive appraisal of a stressor but not to the stressor *per se*; while the influencing factors are stressor's acuity, timing, and novelty (e.g., unpredicted/novel vs. homotypic, e.g., for details see review by Palamarchuk and Vaillancourt, 2021). We thus hypothesize that the brain dynamics associated with childhood stress psychopathology depend on a stress perception/comprehension to a larger extent, compared with a type of victimization (e.g., child maltreatment vs. peer victimization). The integrative brain dynamics for BV and early life adversity potentially would not be significantly distinct since many of the associations are extrapolated from research on childhood stress in the broader sense; and oftentimes, BV does not exist in a "vacuum". Although poly victimization is out of this review scope, we would suggest that children and youth who experience multiple types of victimization across different domains (child maltreatment, community violence, physical/sex abuse, etc.) are likely to be among those with the worst neurocognitive and psychiatric outcomes due to a complex stress response.

The goal of this review is to help inform future studies that are focused on the treatment and prevention of psychiatric disorders and learning problems in bullied children and adolescents. To move the research forward in this area, we offer the following recommendations. First, research on BV, as well as on any psychological stress, must identify the exact stressor(s), perceived stress severity, and perceived controllability of the stressor. Second, psychological/clinical symptoms must be well differentiated in BV target's anamnesis (e.g., occurred prior to a stressor/event, induced by a specific stressor, random/transient/chronic, and aggravating/relieving factors). Third, studies on cognitive re-appraisal aimed to define neural correlates in emotion regulation must consider different effects of *minimizing appraisal* vs. *positive appraisal* and *self-appraisal* vs. *stressor-appraisal* (Palamarchuk and Vaillancourt, 2021).

Our hope is that the proposed neurobiological mechanisms can be used in the prediction of risk for neurocognitive vulnerability and psychopathology following BV in childhood. For instance, consideration of dopamine-related behavioral maladjustments may facilitate proper sociomedical interventions focused on preventing children from becoming the target of BV or bully-victim. As well, a better understanding of the association between BV and academic achievement can help young BV targets with expedited sociopsychological help, including cognitive behavioral therapy focused on associative learning, cognitive flexibility, and reappraisal to develop a more appropriate reactivity to stressors and successfully cope with the stress.

CONCLUSION

BV is a psychological stress which can lead to pervasive interpersonal trauma that devastates far too many children worldwide. Being the target of BV places children at risk for a host of problems, most notably in the areas of mental health and learning. In our review, we delineate how exposure to this type of social pain is associated with a series of neurobiological responses that could result in psychopathology, as well as enduring structural and functional changes in the brain. We describe the complex cognitive and emotional convergence and synthesize the data in a form of integrative brain dynamics, as well as provide testable hypotheses to explain why children are more vulnerable to the adverse outcomes of BV than adults. Given how high the stakes are, the reduction of bullying must be prioritized.

AUTHOR CONTRIBUTIONS

TV encouraged and supported ISP to investigate the impact of psychological stress on cognition in bullied children. ISP

planned and carried out the project, the main conceptual ideas, developed hypotheses, and integrative model of the brain dynamics associated with bullying victimization. ISP designed the figures and wrote the manuscript with notable input from TV. Both TV and ISP provided critical feedback, helped shape the manuscript, and contributed to its final version. All authors discussed the results and agreed to be accountable for the content of the work. All authors contributed to the article and approved the submitted version.

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Dynamic Interrogation of Stochastic Transcriptome Trajectories Using Disease Associated Genes Reveals Distinct Origins of Neurological and Psychiatric Disorders

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The advent of open access to genomic data offers new opportunities to revisit old clinical debates while approaching them from a different angle. We examine anew the question of whether psychiatric and neurological disorders are different from each other by assessing the pool of genes associated with disorders that are understood as psychiatric or as neurological. We do so in the context of transcriptome data tracked as human embryonic stem cells differentiate and become neurons. Building upon probabilistic layers of increasing complexity, we describe the dynamics and stochastic trajectories of the full transcriptome and the embedded genes associated with psychiatric and/or neurological disorders. From marginal distributions of a gene's expression across hundreds of cells, to joint interactions taken globally to determine degree of pairwise dependency, to networks derived from probabilistic graphs along maximal spanning trees, we have discovered two fundamentally different classes of genes underlying these disorders and differentiating them. One class of genes boasts higher variability in expression and lower dependencies (High Expression Variability-HEV genes); the other has lower variability and higher dependencies (Low Expression Variability-LEV genes). They give rise to different network architectures and different transitional states. HEV genes have large hubs and a fragile topology, whereas LEV genes show more distributed code during the maturation toward neuronal state. LEV genes boost differentiation between psychiatric and neurological disorders also at the level of tissue across the brain, spinal cord, and glands. These genes, with their low variability and asynchronous ON/OFF states that have been treated as gross data and excluded from traditional analyses, are helping us settle this old argument at more than one level of inquiry.

Keywords: embryonic stem cells, transcriptome, neurological, psychiatric, tissues, autism, Parkinson, schizophrenia

INTRODUCTION

The question of whether a distinction should exist between psychiatric and neurological disorders predates the time when psychiatry was not even a formal discipline as we know it today. Back then, motor movements were used as criteria to identify mental disorders, by observing and describing patients in a motor-informed way (e.g., catatonic, hyperactive, etc.) (Rogers, 1992). Under the spell of Freud's psychoanalyses and following Descartes's dualism, this type of physical-motor criterion lost influence in favor of elaborate descriptions of mental and emotional states inferred by other, non-motor-based criteria. There was more judgment added on to the perception of the patient; for example, terms such as deviant, opposing, defiant, socially inappropriate, behaviors, etc., entered the descriptions of children with atypical neurodevelopment. This judgment took place solely based on external observation, without any additional description of internal states of their nervous systems.

The distinction broadened between mental illness and disorders that affected the person's function beyond dysfunction of the central nervous system, thus prolonging the ongoing physiological and medical debates (Mehta, 2011); it also impacted the perception of other members of society with regards to one or the other (Torres, 2018). A recent revival of this debate underscores the side of the argument that psychiatric disorders are not just "mental" but are physical, too, identifying neurobiological substrates of mental illness (Goodkind et al., 2015). These substrates are in line with the current US National Institute of Mental Health Research Domain Criteria (RDoC), a framework that cuts across research domains (Bernard and Mittal, 2015) but still has room for improvement (Huys et al., 2016; Torres et al., 2016; Friston et al., 2017; Torres, 2020, 2021).

An example of the brain's affected tissues that are amenable to separating psychiatric from neurological disorders is provided in **Figure 1A** and results from this recent resurgence of the debate (Crossley et al., 2015). Yet, others have contested such neuroimaging distinctions between the disorders, on the grounds that medications can alter brain structures (David and Nicholson, 2015). Specifically, the argument is centered on the ambiguity that medication brings to the studies that are based on brain structure by, for example, increasing basal ganglia volume or increasing volume loss in general, in the case of traditional antipsychotics (David and Nicholson, 2015).

The interactions between diagnosis and medication are also mentioned in the Diagnostic Statistical Manual DSM-5 to justify the exclusion of motor criteria from diagnosis. Nevertheless, several of the mental disorders on a spectrum, like autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), and schizophrenia do have functional neuromotor issues with neurobiological bases, i.e., of the neurological type, even when medication was never used (Torres and Denisova, 2016) or was sparsely used (Nguyen et al., 2016). Thus, the confounds between medication and psychiatry- or neurology-based diagnoses are palpable at the clinical level and confusing at the level of basic brain research.

One avenue that we could explore to try and distinguish psychiatric and neurological disorders is by re-examining brain (and bodily tissues) from the standpoint of dynamically changing gene expression in early embryonic stages of pluripotency, as cells transition into neuronal classes. In this context, we could use different levels of inquiry. For example, we could interrogate the genes with an eye on their roles in fundamental processes at the molecular or channel level, or perhaps at the systems level or at the level of tissues, etc., not as a role of the gene in isolation but rather as its role with respect to interactions with other genes. Regardless of the framework of choice, addressing possible differentiation between psychiatric and neurological disorders through genes' dynamic interactions and their expressions on brain and bodily tissues critical for the person's functioning may have new utility to aid in developing targeted treatments. Such treatments may be precisely aimed at mitigating such adverse effects on the brain and on the control of the movements that make up the behaviors examined by these observational diagnoses in the first place.

In this paper we leverage recent advances in the modeling of neurodevelopmental stages involving human embryonic stem cells (hESC), which have made transcriptome data from early development available to the scientific community. Such sharing of data from cultures validated by primary developing tissue offers new opportunities to advance analytical and visualization tools that can potentially facilitate the study of the dynamics of cell differentiation across multiple developmental stages. It can help us shed light on the question of differentiating pools of genes associated with disorders of the nervous systems that may or may not rise to the level of mental illness.

An example of such open access work is by Yao et al. (2017), which modeled the early stages of human brain development, including early regional patterning and lineage specification. These authors described cell characterizations amenable to providing benchmark datasets to advance our understanding of the origins of disease of the human brain. Here we use their data to design new visualization tools inclusive of all genes' fates in the transcriptome and genes' states across differentiation of self-emerging patterns recorded several times over 54 days. The results available from single-cell RNA sequencing (scRNA-seq) or single cell transcriptomics offer gene-expression data from tens of thousands of genes across hundreds of cells evolving and differentiating toward neuronal stages. These data, combined with identification of disease-associated genes and their expression on human brain and bodily tissues, may help us track the origins of differentiation between psychiatric and neurological disorders.

Analyses of such data often entails dimensionality reduction and visualization of the reduced set [e.g., after Principal Component Analyses, PCA initialization and t-distributed stochastic neighbor embedding, *t-SNE* (van der Maaten and Hinton, 2008)]. Often, during several of the steps leading to the embedding and visualization in much lower dimensional spaces (of two or three dimensions), thousands of genes may be discarded owing to low variability and/or asynchronous expression across various reading days. These data that are discarded may, however, be key to cases where atypical development takes place. Consequently, genes that may be critical to early development toward neuronal stages could be potentially disregarded by current popular methods like t-SNE. This approach would miss an opportunity to examine

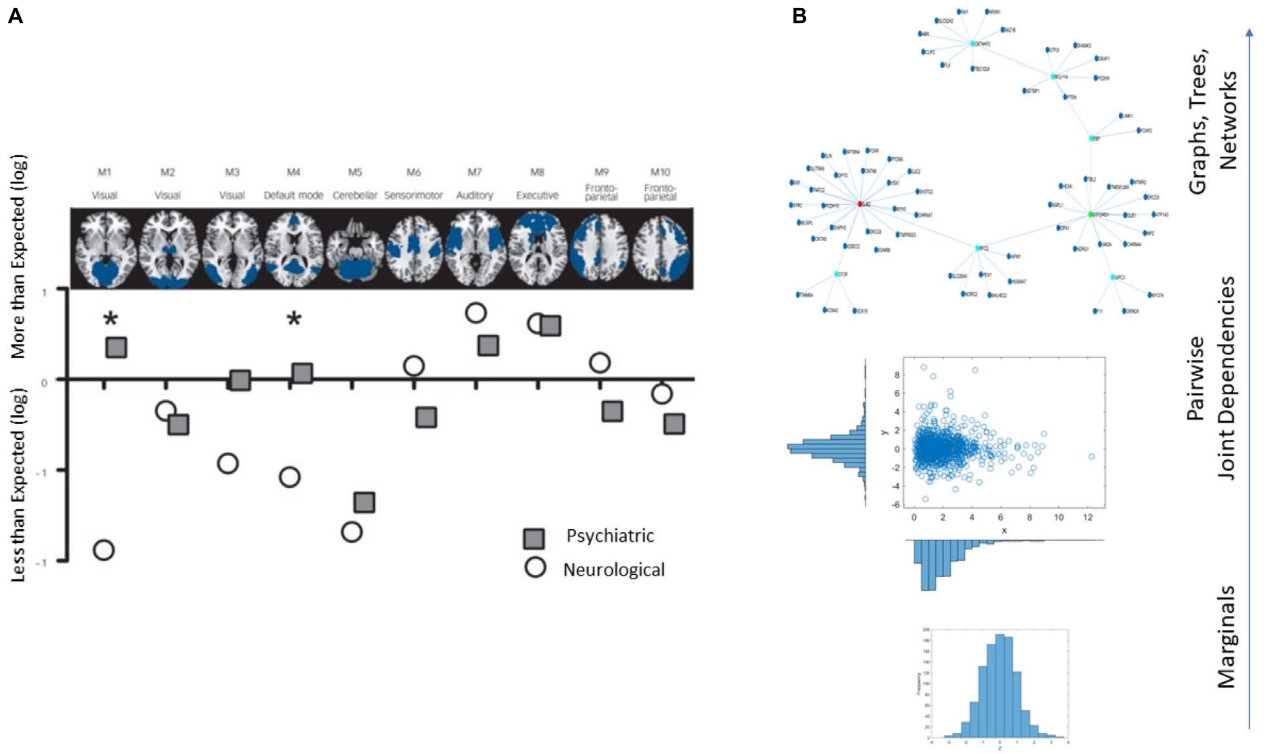

FIGURE 1 | Different lines of inquiry to study the differentiation between psychiatric and neurological disorders. **(A)** Brain structural differentiation reported by Crossley et al. (2015) (Figure from Crossley et al., 2015 with permission). **(B)** Approach used in this work, from simpler to more complex levels of interrogation and the consideration of important dynamically changing statistical co-dependencies across genes' expressions. Marginal distributions of different genes' expression across cells followed by studies of pairwise genes' relations and evaluation of degree of genes' co-dependencies in joint distributions, followed by analyses of complex, dynamically changing, interconnected networks of genes, across days of embryonic stages of cell differentiation toward neuronal states.

the transcriptome data from the vantage point of inter-related nodes in a network, using a stochastic approach that goes beyond locally selected neighboring interactions of genes with systematic variability to leverage (and understand) the dynamic, asynchronous nature of many otherwise discarded genes during pluripotent neuronal differentiation.

We propose new methodologies (**Figure 1B**) that treat a set of genes as a network entity whose parts interact with each other over the course of cell development. To that end, we use a layered approach. First, we identified genes associated with a plurality of psychiatric and neurological disorders, and which also overlapped, thus being associated with phenotypes that are considered comorbid with, for example, autism spectrum disorders (ASD), attention deficit hyperactivity disorder (ADHD), cerebral palsy (CP), etc. (Torres, 2020, 2021). Then, we consider the cumulative expression of the genes across four readings through 54 days, as the cells transition to neuronal classes. For each gene, we derived marginal distributions of expression across cells and tracked pairwise dependencies to interrogate the full transcriptome dynamically on each reading day. We did so within another layer of inquiry, as the genes formed part of a probabilistic interconnected graph.

This network of interacting interconnected genes associated with a plurality of psychiatric and neurological disorders cannot be *separated* into a disjointed collection of data points, since the topological properties of the graph determine expression and differentiation. We therefore found relationships between local and global properties of this network by defining metrics that quantified the "fate" of each gene during cell differentiation and the degree of interdependency between all cells. Furthermore, our approach was dynamic, i.e., we looked at gene expressions on multiple days for a culture of cells. This approach allowed us to shed some light on how changes in the "state" of the expressions of different genes during the embryonic stages determined the clinical phenotype and characterized different pathologies of the CNS.

This characterization based on the fate and state of the genes' expressions led to the proposition of a general mechanism and new paradigm that traces the origins of differentiation and commonalities between neurological and psychiatric disorders back to the early stages of embryonic development. This is the point when cells differentiate and become fully matured neurons that will make up the different systems of the nervous system. In this sense, we transformed the current psychiatric vs. neurological disorders debate into an opportunity to explore when, during these early embryonic stages, the genes expressed as one disorder or another as a function of their degree of interdependencies. We discuss the implications of our results while considering the notion that gross data such as low-variability and asynchronous genes expression, which

are often discarded as superfluous, may in fact hold the key to many unknown aspects of neurological and/or psychiatric disorders. Developing new methods to harvest and utilize their dynamically changing stochastic activities may be critical to understanding the mechanisms guiding us in the design of treatments to cure diseases.

DATASETS AND CODES

Single-cell RNA-seq data during neural differentiation of hESCs were provided by the study of Yao et al. (2017). They revealed a multitude of neural cell classes with a range of early brain regional identities. They analyzed 2,684 cells with >20,000 transcripts, as assessed by unique molecular identifiers (UMIs). Cell types were named by point of origin as progenitor (P), transitional (T), neuronal (N), or other tissue (O). We focussed on the evolution of 24,046 genes' expression across the neuronal type examined at days 12, 19, 40, and 54.

The code that implemented the Kernel Statistical Test of Independence and was used for the present analysis is available in Gretton and Gyorfi (2010) as free source material on their website (https://www.gatsby.ucl.ac.uk/~gretton/).

MATERIALS AND METHODS

Disorders

From the genetic database of the Simons Foundation Research Initiative (SFARI) we gathered the set of genes that, according to the literature, are linked to the behavioral diagnosis of Autism. We also compiled several sources found in the DisGeNet portal and identified the lists of genes linked to a variety of neurological [Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS), Dystonia, Cerebral Palsy (CP), Ataxia Syndrome, Tourette's, and Late and Early Onset Parkinson's disease (White et al., 2012)] and psychiatric disorders [Schizophrenia, Depression, Obsessive Compulsive disorder (OCD), bipolar disorder, and Post-Traumatic Stress Disorder (PTSD)]. The psychiatric disorders under consideration are defined in the DSM, whereas the neurological ones are comorbid with autism spectrum disorders (ASD) across the human life span. Some disorders like CP and Tourette's are also included in the DSM.

Transcriptome Data

Data matrices expressed as M cells × N genes containing at each entry transcription in log Counts/million. The matrix is transposed to express each of the 24,046 genes as it expresses across cells. This is represented as $X^D_{N\times M}$, where N is the number of genes and M the number of cells for day D. **Figure 2A** shows the counts across neurons at day 40 (top) and at day 54 (bottom) for one gene. Notice that cells may not necessarily be the same on each day, but we work on the genes' expression space. Next, we took the expression of each gene at each cell as peaks in a series (shown in red) and normalized them using Eq. 1. Notice that the cell order is preserved from the original matrix of cells by genes. It is not a temporal order (it is not a time series), and as such, order does not matter in our calculations:

$$Normalized\ Count = \frac{count_i}{count_i + \frac{Avrg_{Global}Count}{Max_{Global}Count}} \quad (1)$$

here $count_i$ is the count value of the $gene_i$ and $Avrg_{Global}Count$ is the overall average of the matrix of values taken along the columns and the rows. $Max_{Global}Count$ is the maximum count value, also taken globally across the matrix values. We take each such value and apply Eq. 1 to scale all expressions of that gene across all cells, each day. The output of the normalization is shown in **Figure 2B** for cells with expression close to 1. These are cells where the ratio of $Avrg_{Global}$ Count to Max_{Global} Count is very small, so the denominator is only a small margin greater than the numerator. The inset stem plot in **Figure 2C** (inside the top and bottom histograms) represents all values of the gene across all the cells, scaled as spikes ranging between 0 and 1. These include values for which the ratio of $Avrg_{Global}$ Count to Max_{Global} Count is large and the overall normalized value is small. There is not a temporal order, it is just a series of fluctuations whereby the frequency histogram of all those values (smaller and larger) is of interest.

We then obtained the similarity distance between the two frequency histograms corresponding to the gene (across all cells read out each day) using the Earth Mover's Distance (EMD) (Monge, 1781; Rubner et al., 1998).

Genes' Fate: Quantifying It Through the Cumulative Earth Mover's Distance

The EMD is a measure of the distance between two probability distributions and is the 1st Wasserstein distance from the family of Wasserstein distance metrics. Essentially, the EMD is the minimal cost of transforming one distribution into the other.

Consider the unknown probability spaces $\left([0,+\infty], F_i^{D_A}, P_i^{D_A}\right)$ and $\left([0,+\infty], F_i^{D_B}, P_i^{D_B}\right)$ that define the statistical behaviors and corresponding probability distributions for a gene on two different days, D_A, and D_B during cell differentiation. The probability distributions can be approximated through histogram fitting from the available normalized cell expression data. Then, $EMD_{i,A\to B}$ is the EMD between $P_i^{D_A}$ and $P_i^{D_B}$ and is indicative of the departure of the statistical behavior of the gene i as we move from day D_A to D_B. Consider the days 12, 19, 40, and 54 of hESC differentiation. Then we define the quantity:

$$CumEMD_i = EMD_{i,12\to 19} + EMD_{i,19\to 40} + EMD_{i,40\to 54} \quad (2)$$

as the cumulative EMD distance of the gene or the "fate" of the role of that gene throughout embryonic stem cell development and differentiation. Then, the average EMD or "fate" of a set of genes $_{i=1,\ldots,N}$ associated with a particular pathology is the quantity:

$$AverageCumEMD = \frac{\sum_{i=1}^{N} CumEMD_i}{N} \quad (3)$$

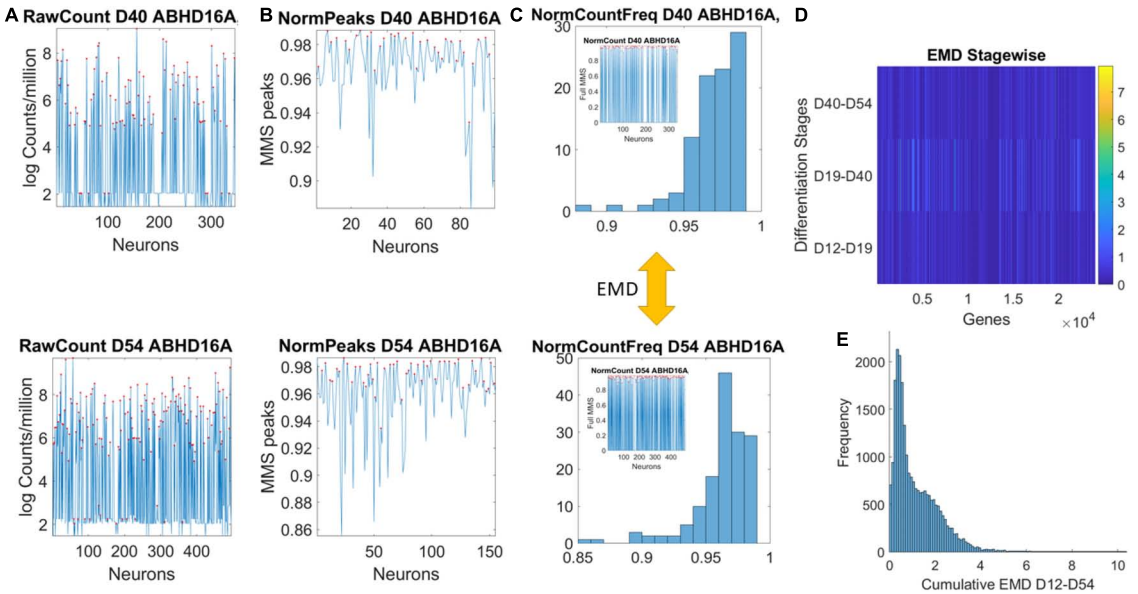

FIGURE 2 | Pipeline to estimate genes' fates according to expression across cells. **(A)** Sample gene's raw count across 496 cells on day 40 and day 54. **(B)** Normalized gene expression series for range of values obtained using Eq. 1. **(C)** Frequency histogram of scaled values across all neurons (represented as spikes between 0 and 1 in the inset). **(D)** The earth mover's distance (EMD) is taken between the frequency histogram of consecutive readings (e.g., between the histograms of Day 40 and Day 54) and obtained for each gene (across the columns of the matrix). EMD values range according to the color bar. **(E)** The cumulative EMD from day 12–54 is then obtained for each gene and a frequency histogram of all genes reveals the distribution of all values in fate space. Each bin in this histogram boasts a level of its genes' expression as they accumulated along the developmental trajectory of the cells. Which genes make up each bin can be easily obtained to further understand genes' interactions according to fate.

Figure 2D shows a color matrix with all genes across the columns (horizontal axis) and three rows representing, for each gene, the EMD obtained between two consecutive days.

We focused here on graphical modeling techniques and kernel-driven statistical independence hypothesis testing to determine the level at which these genes co-depend (Gretton and Gyorfi, 2010). This kernel technique was used to build a parameter space representing 16 disorders sorted out according to their levels of statistical co-dependency at the earliest and latest stages of the cell evolution. Our data of interest were the cumulative EMD traveled representing the gene's fate.

Kernel Statistical Test to Determine the Level of Interdependence Between Genes Associated With Each Disorder at Different Times During Cell Maturation

On a specific day during cell maturation, for any two genes A and B we had available their expression levels in N cells. We wanted to test whether the expression of gene A was statistically independent from the expression of gene B. Before we present the solution to this problem, let us first introduce the general framework for measuring independence, based on cross-covariance operators in *Reproducing Kernel Hilbert Spaces* (RKHSs).

Cross-Covariance Operator and Hilbert–Schmidt Independence Criterion

Different methods for measuring statistical independence between random vectors have been proposed over the past decades. Non-parametric approaches to this problem can be traced back to Hoeffding (1948), when he proposed a test statistic that depended on the rank order of the identically and independently distributed sample data. Techniques that constructed statistics based on empirical characteristic functions were later developed. Modern methods introduced the concept of Kernel Independence Measures, which have found applications in Independent Component Analysis (Bell and Sejnowski, 1996, 1997), fitting graphical models and feature selection. However, such measures do not necessarily ensure statistical significance. Hence, we decided to use a Kernel Statistical Test of Independence that was developed by Gretton and Gyorfi (2010), which allowed us to perform hypothesis testing on whether two datasets were independent, and which we applied on the gene expression dependence problem. We briefly present their methodology in the next few paragraphs.

Consider the Hilbert space F of functions from a measurable space $X \to R$. To each point $x \in X$ there corresponds an element $\varphi(x) \in F$ such that $< \varphi(x), \varphi(x^{'}) >_F = k(x, x^{'})$ where $k : X \times X \to R$ is a positive definite kernel. If we assume that F is separable, then F is a RKHS.

Note, that F is the completion of the set of all functions that are linear combinations of these feature functions. To evaluate the value of any function $f \in F$ at some point $x \in X$ one can simply take the inner product between the function f and the feature function $\varphi(x)$ mapping of the point x. This is known as the Reproducing Property, hence the term Reproducing Kernel Hilbert Space. Similarly, we define a RKHS space G of functions

from a measurable space $Y \to R$ with feature map $\psi(.)$ and kernel $l(.)$.

Now, assume the probability spaces $(X, \mathcal{F}_x, P_x)$ and $(Y, \mathcal{F}_Y, P_x)$ where $\mathcal{F}_x$, $\mathcal{F}_Y$ are the Borel σ-fields on X, Y, respectively, and P_x, P_y the corresponding probability measures. Then, for any two functions $f \in F$ and $g \in G$ the cross-covariance operator $C_{xy} : G \to F$ is defined as:

$$< f, C_{xy} g \; >= E\left(\left[f(x) - E\left(f(x)\right)\left[g(x) - E\left(g(x)\right)\right]\right]\right) \quad (4)$$

Or with respect to the feature mappings $\varphi(x)$, $\psi(x)$:

$$C_{xy} = E_{xy}\left[\left(\varphi(x) - E_x\varphi(x)\right) \otimes \left(\psi(y) - E_y\psi(y)\right)\right] \quad (5)$$

It can be shown that X and Y are independent if and only if the largest singular value of the operator C_{xy} is zero. As a measure of independence, the authors consider the Hilbert–Schmidt norm (i.e., the Hilbert–Schmidt Independent Criterion, HSIC) of C_{xy}, which is equal to the sum of squared singular values of C_{xy} and has a population expression (Gretton and Gyorfi, 2010):

$$HSIC\left(P_{xy}, F, G\right) = E_{xx'yy'}\left[k(x, x')\, l(y, y')\right] + E_{xx'}\left[k(x, x')\right]$$
$$E_{yy'}\left[l(y, y')\right] - 2E_{xy}\left[E_{x'}\left[k(x, x')\right] E_{y'}\left[l(y, y')\right]\right] \quad (6)$$

where x' denotes an independent copy of x and $k(.)$ and $l(.)$ are the kernels previously defined. The authors derive an empirical estimate of this independence criterion that follows the V-statistics and has expression:

$$HSIC_b(Z) = \frac{1}{m^2} trace(KHLH) \quad (7)$$

where Z is a sample of (x, y) pairs drawn independently from the distribution of $X \times Y$, with size m, K is the $m \times m$ matrix with entries k_{ij}, L is the $m \times m$ matrix with entries l_{ij} and $H = I - \frac{1}{m}\vec{1}\vec{1}^T$, where $\vec{1}$ is a row vector of ones.

Then, they proceed to construct a statistical hypothesis testing protocol to test whether X is independent of Y based on samples $(x, y)^m$ drawn from the probability space $\left(X \times Y, \mathcal{F}_x \times \mathcal{F}_y, P_{xy}\right)$. The null hypothesis is $H_0 : P_{xy} = P_x P_y$ and the alternative hypothesis $H_1 : P_{xy} \neq P_x P_y$

Finally, they approximate the independence criterion with a gamma distribution:

$$mHSIC(Z) \sim \frac{x^{k-1} e^{-\frac{x}{\theta}}}{k^{\theta}\Gamma(k)},$$
$$where\ k = \frac{(E(HSIC_b(Z)))^2}{var(HSIC_b(Z))},\ \theta = \frac{mvar(HSIC_b(Z))}{E(HSIC_b(Z))} \quad (8)$$

If $mHSIC(Z)$ is above the threshold determined by the level of significance that we choose for the test, the null hypothesis is rejected. **Supplementary Figure 0** depicts this pipeline.

The Construction of Gene Expression Statistical Dependency Networks for Days 12, 19, 40, and 54 of Neural Cell Maturation

For each disorder, we have a set of genes associated with it (extracted from DisGeNet). On a particular day D of cell maturation, we have the data $X^D_{N \times M}$, where N is the number of genes and M is the number of cells. Each row of our data refers to a specific gene and each column to a specific cell.

Define the unknown probability spaces $([0, +\infty)$, $\mathcal{F}_i$, $P_i)$ and $([0, +\infty)$, $\mathcal{F}_j$, $P_j)$ for the expressions of any two genes i and j, $(i, j = 1, \ldots, N)$ and the probability space $([0, +\infty)^2$, $\mathcal{F}_i \times \mathcal{F}_j$, $P_{ij})$ for the joint expression of the two genes. Here, $[0, +\infty)$ and $[0, +\infty)^2$ are the measurable spaces for the gene expressions and joint gene expressions, respectively, $\mathcal{F}_i, \mathcal{F}_j$ the Borel σ-fields generated by the measurable spaces of gene expressions of i and j, P_i, P_j the corresponding probability measures for the two genes and P_{ij} the joint *probability* measure. Consider the sample $Z = \left(X^D_{i,*}, X^D_{j,*}\right)$, i.e., the pairs of the two gene expressions in the cells. Choosing a level a of statistical significance, we can apply the Kernel method on the sample Z and determine whether the genes i and j are independent of each other on that specific day.

We perform the independency test on all undirected pairs (i, j), $i = 1 \ldots N-1$, $j = i+1, \ldots, N$ of genes and we construct the graph:

$G = (V, E)$, V is the set of nodes and E the set of edges, $|V| = N$, where $|.|$ denotes the cardinality of a set. An edge belongs to the graph, i.e., $e_{ij} \in E$ if and only if the genes i and j are statistically dependent according to the kernel independency test.

If the graph were fully connected it would have number of edges $\frac{N(N-1)}{2}$, and in this case all genes would be fully dependent upon one another. In search of a metric that shows how interconnected the genes related to the disorder of interest are, we define the *dependency index*:

$$DI = \frac{2|E|}{N(N-1)} \quad (9)$$

Therefore, for a specific disorder d, by constructing the dependency graphs G^d_{12}, G^d_{19}, G^d_{40}, G^d_{54} for each day of the disorder we can derive the dependency indexes DI^d_{12}, DI^d_{19}, DI^d_{40}, DI^d_{54}. Furthermore, we define:

$$initial\ state = DI^d_{12}$$
$$final\ state = DI^d_{54} \quad (10)$$
$$Dependency\ Increase = DI^d_{54} - DI^d_{12}$$

Then, we can map each disorder to a parameter space of dependencies throughout the course of the cell development to track how the interdependence between the genes associated with each disorder evolves through time, from the state of pluripotency to the state of full neural maturation. This allows us to stratify the spectrum of neurological and psychiatric disorders with regards to the complexity of their genotypical expressions.

Using the database DisGeNet, we identified the genes that are associated with each of the following disorders: Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS), Dystonia, Cerebral Palsy, Ataxia Syndrome, Tourette's, Late Onset Parkinson's disease (Late PD) and Early Onset Parkinson's disease (Early PD) and Schizophrenia, Depression, Obsessive Compulsive disorder (OCD), bipolar disorder, and Post-Traumatic Stress Disorder (PTSD).

For days 12, 19, 40, and 54 of the hESC differentiation to neural cells, we had the expressions of 24,046 genes from the human genome in log TCM (log transcripts count per million). For days 12, 19, 40, and 54, we had available the expression of all those genes in 263, 168, 346, and 495 different cells, respectively. Therefore, for the sets of genes associated with each of the disorders of interest, we had the datasets $X^D_{N\times 263}$, $X^D_{N\times 168}$, $X^D_{N\times 346}$, and $X^D_{N\times 495}$ for the 4 days, where D denotes the disorder of interest and N the number of genes associated with it. From these datasets we calculated the Dependency Indexes (see Section "Materials and Methods") for each day. **Figure 3A** shows the schematics of this procedure.

Network Analyses

Undirected Graphical Modeling for Evaluating the Dependency Between Gene Expressions

Graphical models have been extensively researched and used to describe statistical dependencies between random variables. These models can be either undirected graphs or directed graphs; in the latter case, we could derive cause-effect relationships between the variables. In the current project we were interested in undirected graphical models.

Formally, for any set of random variables $X = (X_1, X_2, \ldots, X_N)$, a graphical model attempts to associate the joint random vector X drawn from the probability space $(\Omega_1 \times \Omega_2 \times \ldots \times \Omega_N,\ F_1 \times F_2 \times \ldots \times F_N,\ P_X)$ with a graph $G = (V, E)$, where V stands for vertices and E for edges. Here, $\Omega_1, \Omega_2, \ldots, \Omega_N$ are the sample spaces for each random variable and $\Omega_1 \times \Omega_2 \times \ldots \Omega_N$ the joint space of the random vector X. $F_1, F_2, \ldots, F_N$ are the corresponding generated Borel σ-fields to denote the sets of all possible random events for each random variable and P_X is the probability measure on the random vector X. The set of nodes V represents the random variables $X_1, X_2, \ldots, X_N$ drawn from the probability spaces (Ω_1, F_1, P_{X_1}), (Ω_2, F_2, P_{X_2}), $\ldots$ (Ω_N, F_N, P_{X_N}), with their respective Borel σ-fields and probability measures. An edge $e_{ij} \in E$ if and only if the random variables X_i and X_j depend on each other.

If two nodes u_i and u_j are not connected with an edge it implies that the two variables X_i and X_j are conditionally independent, i.e., statistically independent *given* all other nodes:

$$X_i \perp X_j |\ X_{V/\{u_i,u_j\}}$$

This property of the graphical model is known as the *global Markov property*.

General Estimation of a Graphical Model Using Chow–Liu Trees

If we want to factorize the joint probability distribution in a dependency graph that has a tree structure, i.e., every two nodes are connected by no more than one path (in other words there are no loops in the graph), then the joint density of the random vector X factorizes with respect to the pair-wise joint and marginal densities as:

$$f(x) = \prod_{e_{ij}\in E} \frac{f_{ij}(X_i, X_j)}{f_i(X_i) f_j(X_j)} \prod_{u_i \in E} f_i(x_i) \tag{11}$$

It turns out that, in the case in which all variables are categorical and take values from a finite set, it is very easy to find the optimal tree that factorizes the joint distribution. Let N_x be the number of times a realization x of the random vector X appears in a collection of independent and identically distributed (*i.i.d.*) samples. The tree G that optimally factorizes X, given the sample data Z of size n, will be the one with the maximum log-likelihood (MLE):

$$G = argmax\ L(G) = \sum_{x\in Z} N_x \log\left(\hat{f}_G(x)\right) \tag{12}$$

which turns out to be:

$$L(G) = n \sum_{e_{ij}\in E} I(\hat{f}_{ij}(X_i, X_j)) + \mathrm{C} \tag{13}$$

where C is a constant and $I(\hat{f}_{ij}(X_i, X_j))$ the empirical mutual information between X_i and X_j. Therefore, by choosing the appropriate subgraph G that maximizes the sum of the empirical mutual information estimates, we obtained the optimal tree structured graphical model. Since the model is a tree, we simply needed to find the Maximum Spanning Tree from the mutual information network, for example, by using Kruskal's algorithm. The process we just described is known as the Chow–Liu algorithm and the extracted conditional dependency tree that factorizes X is the Chow–Liu tree.

Gene's Co-dependencies Graphical Models Spanning (Chow–Liu) Trees

For a specific set of diseases, in this case neurological and psychiatric, we had a set of genes associated with them. On a particular day D of cell maturation, we had the data $X^D_{N\times M}$, where N is the number of genes and M the number of cells. Each row of our data referred to a specific gene and each column to a specific cell. We treated each column (corresponding to a cell) as an *i.i.d.* sample drawn from the joint probability space of the expressions of that set of genes, and we wanted to generate the undirected graphical networks G_{12}, G_{19}, G_{40}, and G_{54} for days 12, 19, 40, and 54.

In the case of continuous variables, the methods used to estimate the Chow–Liu tree usually involves Kernel Density Estimation (KDE) of the joint and marginal probability densities (Gretton et al., 2007). However, in our case we wanted to factorize the joint probability density of a gene expression network with number of cells on the order of magnitude $\sim 10^2$. The application of KDE, given the dimensionality of the data (number of genes), would require in this case several samples far exceeding the available number of ESCs. Therefore, we resorted to extracting the Chow–Liu Trees by estimating the mutual information through binning and histograms on the available data (Drton and Maathuis, 2017). Once the Chow–Liu Tree corresponding to the

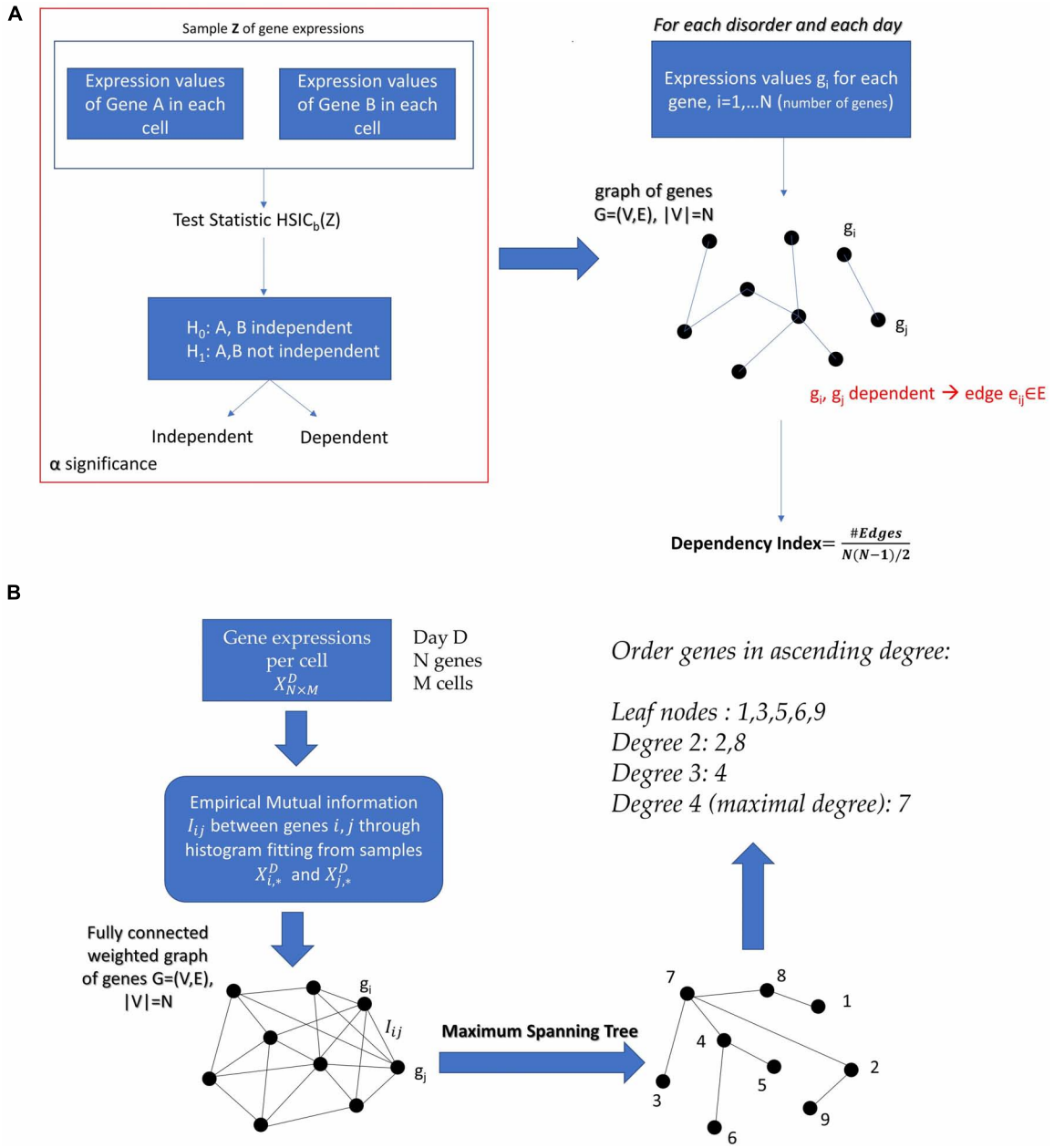

FIGURE 3 | (A) The construction of a graphical model after performing the Kernel Statistical Independency Test on every pair of gene expressions associated with a particular disorder on a specific day of cell differentiation. The Dependency Index characterizes the degree of interdependence of genes that define the nodes of the graph. **(B)** Proposed pipeline for the factorization of the joint probabilistic behavior of a network of genes and for determining the significance of each gene in the network.

factorization of the gene set of interest was obtained, we ordered the nodes in ascending degree. The proposed methodology can be appreciated in **Figure 3B** of the Section "Materials and Methods."

What do we achieve with this ordering? It is obvious that the higher the degree of a gene is, the more co-dependent its expression is with the expression of other genes in the network. This implies, in a statistical sense, that mutation or deletion of this gene is bound to immediately affect the expressions of many other genes. Note that this rationale simply states the co-dependency between the gene expressions, but the actual (causal) mechanisms through which this statistical relationship takes place are open to investigation.

The Gene's State Through a Binary Code

We obtained the average degree across the network's nodes each day. We then set it as the threshold value to determine ON or OFF state for the gene each day. Across 4 days, we had 16 possible binary states (2^4) that provided the state trajectory of the gene (in addition to its fate). This information served to classify cells according to the genes type. To that end, for each cell, we obtained

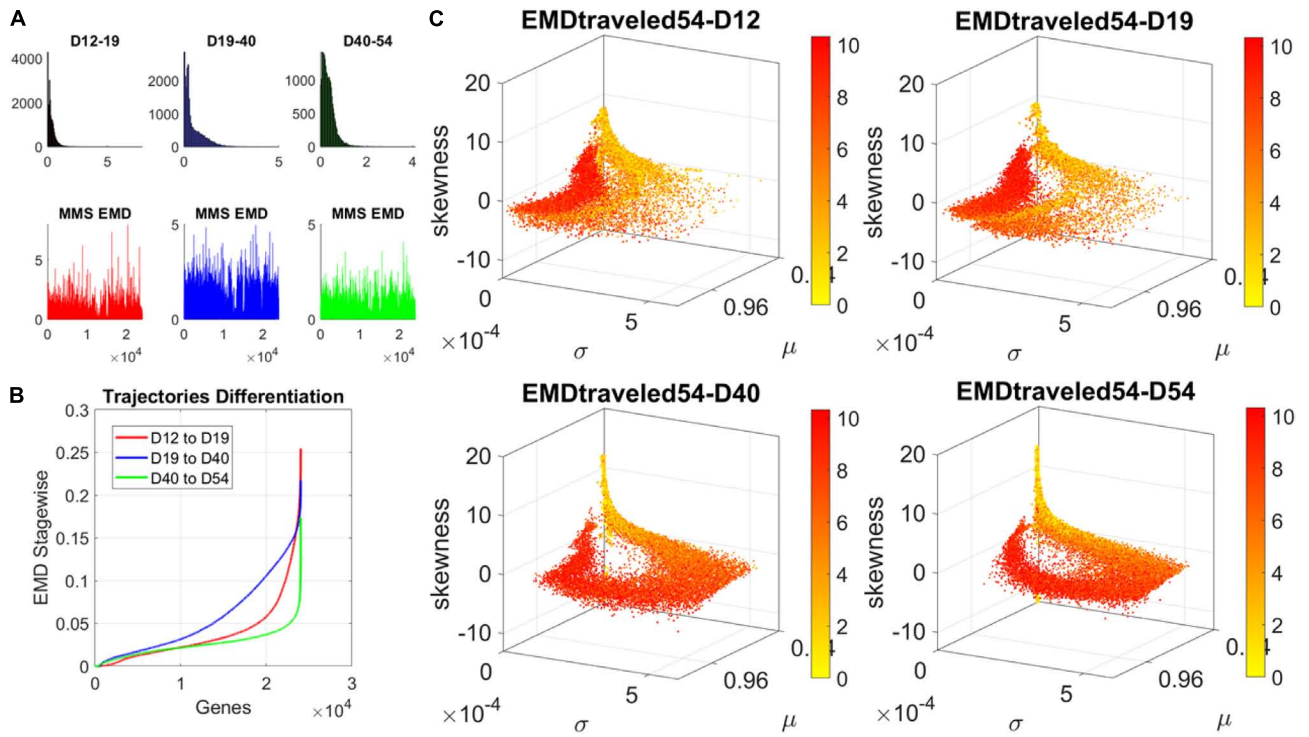

FIGURE 4 | Dynamic gene's motion trajectory in fate space. **(A)** The distributions of EMD obtained for each measurement between days (top frequency histograms) for all 24,046 genes and bottom values across genes each day. EMD based on normalized counts using Eq. 1. **(B)** Sorted values of change in EMD across genes showing the trajectory from measurement to measurement, for each gene (horizontal axis). Order is based on the cumulative EMD at day 54; then we plotted the values of EMD at each stage (D12–D19, D19–D40, D40–D54). **(C)** Full transcriptome representation on fate space is obtained by representing each point as the empirically estimated Gamma PDF moments for the best fitting shape and scale parameters (in an MLE sense). The mean μ is represented along the *x-axis*, the variance σ along the y-axis and the skewness along the *z-axis*. The kurtosis is proportional to the marker size (larger represents more kurtotic distribution). Color bar reflects the value of the cumulative EMD at day 54 when the cells are neurons. Then, we trace back where the gene was in fate space at the initial stages, D12, D19, and D40.

the frequency histogram of counts corresponding to each class of genes, [0000], [0001],. . ., [1111]. Then, we used MLE to obtain the probability distribution best characterizing the histogram and obtained a parameter space to represent the shape and scale parameters thus determined as points along a scatter. Since each gene has a binary configuration, for a given day, we could then retake the cells × genes matrix and ask which cells cluster in this space according to the 16 possible states.

RESULTS

Cumulative Earth Mover's Distance Captures Dynamic Evolution of the Genes' Expression in Fate Space

The contributions of each gene to the overall evolution of the hESCs as they reached neuronal stages were well captured by the stochastic characterization of their normalized count taken for each gene across cells each day. These marginal distributions can be seen evolving across all genes in **Figure 4A**, which shows the frequency histogram of all EMD values obtained for each comparison. **Figure 4B** shows the values sorted out across the genes, providing a sense of the overall trajectory of changes in gene expression across all cells. Also notice that, since each day the number of cells changes, we normalized the EMD quantity to range between 0 and 1 when superimposing the data across days (**Figure 4B**).

We then accumulated the EMD value by summing up to day 54 and building a colormap to visualize the change across all genes in the transcriptome. For each gene, we took the frequency histogram of the normalized gene expression across the cells and, using maximum likelihood estimation (MLE), we obtained the continuous family of probability distributions that best fit the histogram in an MLE sense. This was the Gamma family, which spans a broad range of shape (a) and scale (b) values (ranging from the memoryless exponential $a = 1$, to distributions with heavy tails, to symmetric, Gaussian-like distributions). We estimated the Gamma moments of the empirical distribution corresponding to each gene. Each day we plotted them on a parameter space, whereby the mean is represented along the *x-axis*, the variance is represented along the *y-axis*, the skewness is represented along the *z-axis,* and the kurtosis is proportional to the size of the marker (higher kurtosis represented by larger marker size). We then colored each gene with the cumulative EMD at day 54, when the cells had reached neuronal state. This

enabled us to visualize the "motion" dynamics of the 24,046 genes in the transcriptome and, retrospectively, see where the most active and least active genes were located. This information is shown in **Figure 4C**, as the cells evolved to neuronal stages.

Different Evolution of the Dependency Index Values Throughout Human Embryonic Stem Cells Maturation for Psychiatric vs. Neurological Disorders

We obtained the quantity Dependency Increase, as explained in the methods, by taking the shift in the Dependency Index at Day 12 versus the increase in the Dependency Index until full neuronal maturation at day 54. We then plotted that value for each disorder along the *y-axis* of a parameter space, as a function of the Dependency Index at the initial stage and at the final stage. This is represented as a vector field (rate of change) in **Figure 5A**. A pattern emerged across disorders whereby all disorders that were classified as neurological, except for Late Onset Parkinson's Disease, tended to be characterized by a lower dependency during the first stage of cell differentiation and a high increase in dependency at the neuronal stage. The genes associated with these neurological disorders tended to increase their co-dependencies as the cells matured into neurons, but early in the process they were less co-dependent (lower values of Dependency Index on the *x-axis*). These results can be appreciated in **Figure 5B**.

In contrast, disorders classified as psychiatric in the DSM-5, as well as Late Onset Parkinson's Disease (and except for Infantile Schizophrenia), tended to be characterized by a higher dependency during the first stage (higher values along the *x-axis*) but a lower increase in dependency as the cells matured into neurons. Tourette's, a psychiatric disorder according to the DSM-5, was also traditionally classified as neurological and often labeled as ASD. In this parameter space, ASD seemed to lie on the border between neurological and neuropsychiatric disorders, whereas infantile schizophrenia (which used to be defined as autism earlier in DSM history) lined up with ASD along the early value of the Dependency Index, but with a much higher dependency increase as cells matured into neurons. We highlight this duality with the black edge of the marker in CP and Tourette's on panel 5B using a parameter space that we explain next.

Negative Correlation Trend Characterizes Genes Associated With Neurological and Psychiatric Disorders, With Complementary Features in Autism Spectrum Disorder and Parkinson's Disease Associated Genes

For the genes associated with each disorder, we calculated the average cumulative EMD at Day 54 (*y-axis*) and plotted it as a function of the Dependency Index at Day 54 (*x-axis*) in **Figure 5B**. We noticed a negative trend whereby the variability of expression, as quantified by the cumulative EMD from measurement to measurement, tended to decrease for Depression, PTSD, OCD, CP, Tourette's (all DSM disorders). In this cluster, early and late PD, which are neurological disorders, appeared amid psychiatric DSM-classified disorders.

Neurological disorders such as FXTAS, FX, Dystonia, and Ataxia were high in cumulative EMD, thus implying higher cumulative changes in variability of genes' expression. However, these genes tended to have lower dependency indexes than DSM-classified disorders. ASD, currently classified as a DSM psychiatric disorder, appeared among the neurological cluster with a high cumulative EMD at day 54, but a lower dependency index during this final state.

Infantile schizophrenia and bipolar disorder, both DSM disorders, were the exception to this negative trend, as they were both high in EMD expression and dependency index. Further details are shown in **Supplementary Figure 1** comparing the two classes of genes at final fate and state.

Visualization in Fate Space of Genes Associated With Psychiatric and Neurological Disorders

Using the visualization in **Figure 4C**, we tracked the evolution of the genes associated with the neurological and psychiatric disorders in fate space. We found that they moved from a spread-out configuration in earlier days toward more localized regions along a path of high variability in EMD and an opposite region of low variability in EMD. As explained previously, the EMD measured the change from one frequency histogram (marginal distribution) on gene's expressions across the cells on one day, to the frequency histogram on the next day. As such, cumulatively they reflect the overall variability in gene expression toward the cells' fate. We call those with the higher cumulative EMD High Expression Variability (*HEV*) *genes* and those with the lower cumulative EMD, Low Expression Variability (*LEV*) *genes*.

By day 54, two distinct lobes are obvious in **Figure 6**, which we projected in **Figure 7** along the plane spanned by the first two empirically estimated Gamma moments, mean vs. variance. There we saw the high mixture between the neurological (green) and psychiatric (black) disorders. We then asked if there were distinctions across the genes that we could visualize using the Chow Liu maximal spanning trees, treating their network interactions according to probabilistic graphs.

The results from the visualization prompted us to further examine these intermixed genes and their evolution along the Gamma mean (μ) and Gamma variance (σ) dimensions of fate space. Projecting these values on a mean, variance parameter plane, clearly showed their evolution and convergence to two distinct lobes in **Figure 7** (Day 54), whereby the lobe of HEV genes with higher variability in EMD quantifying probability transitions, separated from the lobe of LEV genes with lower variability. The composition of these lobes was highly intermixed, with 2,613 genes total, 946 in the lower, line like shaped lobe, and 927 in the upper, curved shaped lobe. That count included all disorders, whereby a gene could be counted multiple times if it was associated with multiple disorders. We then extracted 512 *unique* HEV genes and 927 *unique* LEV genes. These genes might be associated with more than one psychiatric and/or neurological

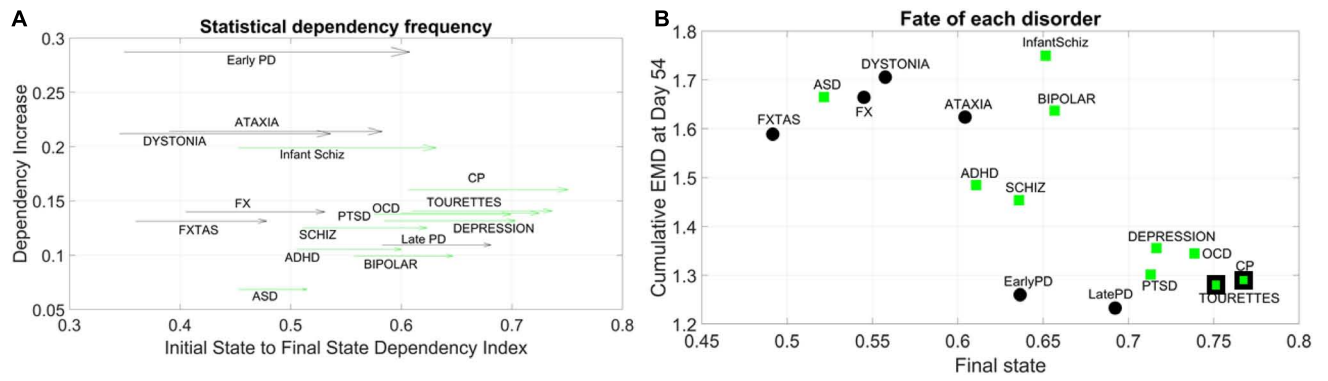

FIGURE 5 | Dynamic evolution of genes' co-dependencies in neurological vs. psychiatric disorders. **(A)** A negative trend is observed between Dependency Increase and Dependency Index value at the initial state. Psychiatric (green arrows) disorders tend to cluster away from neurological (black arrows) disorders and exhibit a high degree of dependency in the initial state. ASD lies on the border between most neurological and psychiatric disorders with regards to the dependency metric. Late PD lies among the psychiatric (DSM) disorders. Vector represents the change from Day 12 to Day 54. **(B)** Disorders diagnosed by the DSM (green squares) as psychiatric tend to cluster toward high interdependency and lower variability and level of expression. These include depression, OCD, CP, Tourette's, and PTSD, which group with late and early PD (neurological disorders). CP and Tourette's are also considered neurological by some physicians, so the edge of the marker is colored black to represent the mix. At the other end, neurological disorders (FX, FXTAS, Dystonia, and Ataxia) cluster with ASD (a DSM disorder with neurological underpinnings). ADHD is separable from ASD in this parameter space, despite their allowed comorbidity by the DSM-5. ADHD is closer to schizophrenia, whereas bipolar and infantile schizophrenia stand on their own in the middle of the graph and away from the general trend. These disorders have associated genes with mid-level of interdependencies and high EMD at fate, signifying higher variability in expression profile.

disorder, or just with one or the other (see **Supplementary Table 1** and expanded **Supplementary Table 2**). **Supplementary Figure 1** shows the dependency indexes and fate across disorders split according to genes in these different lobes. We further analyzed the composition of the two lobes, but first, we had a look at the network analyses.

Differentiation of Network Evolution of High Expression Variability vs. Low Expression Variability Genes in Psychiatric (DSM) vs. Neurological Disorders

The Chow Liu maximal spanning trees for each of the lobes identified in **Figures 6**, **7** were obtained and the degree associated with each gene was quantified to represent in **Figures 8**, **9** the network evolution of the HEV and LEV genes, respectively. These graphs show the genes blindly, without the disorders' labels, to give a sense of the differentiation between the two lobes of intermixed genes in both neurological and psychiatric disorders.

Several important conclusion emerge from these representations of the genes' fate evolution. First, these two lobes have fundamentally different evolution in genes' interactions. Second, the network has a handful of genes with high number of genes connected to it (the network's hubs). These hub genes are such that if a link is disrupted between two of these hubs, the network is disconnected with potential cascade effects (**Figure 8**). In this sense, this is a fragile architecture that remains so at each registered day. Third, days 12 and 54 have fewer hubs than intermediate days. The latter is true in both lobes but more so in the lobe of HEV genes. The lobe of LEV genes has a far more distributed network in intermediate days 19 and 40, suggesting a more robust architecture. This result reveals the importance of such genes in the overall evolution of the transcriptome toward neuronal states.

The network patterns prompted investigation of the hubs' evolution across neurological and psychiatric disorders. For each lobe, we built matrices of hub genes along the rows, sorted by graph degree, and disorders across the columns, sorted by neurological (early PD, late PD, Dystonia, Ataxia, FXTAS, and FX) and psychiatric (DSM diagnosed including infantile Schizophrenia, Schizophrenia, ADHD, ASD, Bipolar, PTSD, CP, Depression, OCD, and Tourette's). Each entry was color coded with the node's (gene's) degree (in log scale) to help visualize the colors better, since they ranged non-linearly from 1 to 470. In the case of degree 1, two genes co-depended on each other's expression patterns. In the case of degree 2 or more, the node had an edge with co-dependency with two or more genes. In **Figure 10** we focused on the HEV genes from the curved lobe (with genes colored in green representing those associated with psychiatric disorders and those colored black associated with neurological disorders).

Here the numerator sums over the number of genes with degree above 3 in each of the neurological disorders under consideration and the denominator sums over the number of genes with degree above 3 in each of the psychiatric disorders under consideration. If in a psychiatric disorder (in the denominator) 0 genes participate, the contribution of the denominator is $e^0 = 1$. Otherwise, the ratio will reflect the balance of genes in one vs. the other, obtained for HEV and LEV genes separately.

Figure 10 (HEV genes) and **Figures 11**, **12** (LEV genes) depict the matrices whose entry is the scalar value of the ratio (plotted as a color map in logarithmic scale). **Supplementary Figure 2** depicts the HEV genes values and **Supplementary Figures 3**, **4** do so for the LEV genes. **Figure 10B** shows the HEV genes lobe

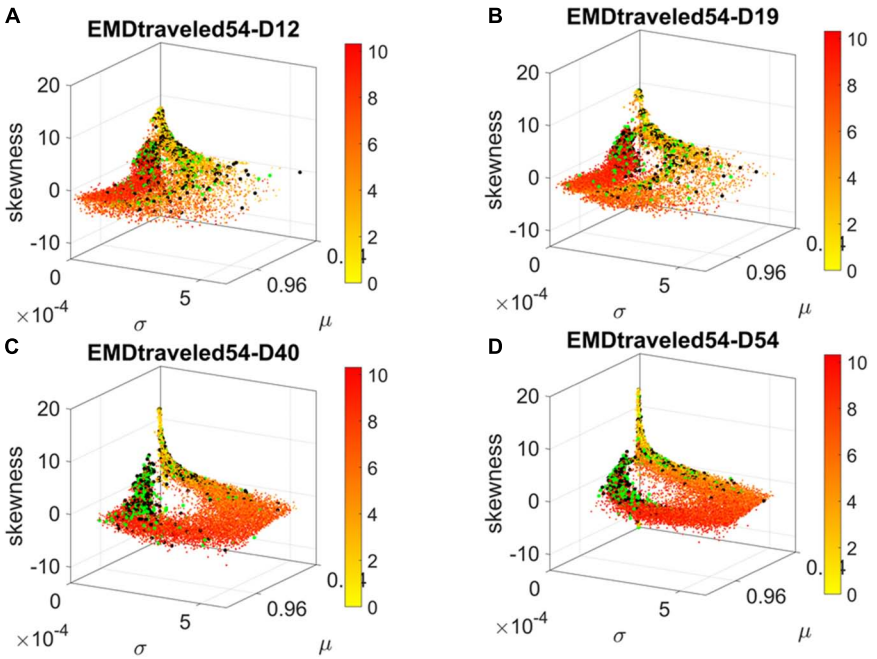

FIGURE 6 | Visualizing the trajectories of genes associated with psychiatric (black) and neurological (green) disorders as they evolve in fate space. Color gradient represents the cumulative EMD at day 54, with yellow representing low values owing to low variability and lower expression whereas red represents high variability and higher expression across cells. At day 54, two lobes emerge along the high values space (higher μ) and central tendency (toward 0 skewness) with higher concentration in lower variance regions (along the σ dimension). Notice the high change from Day 19 to Day 40, with clearly two lobes with intermixed genes from both classes of disorders in day 54. **(A)** Day 12. **(B)** Day 19. **(C)** Day 40. **(D)** Day 54.

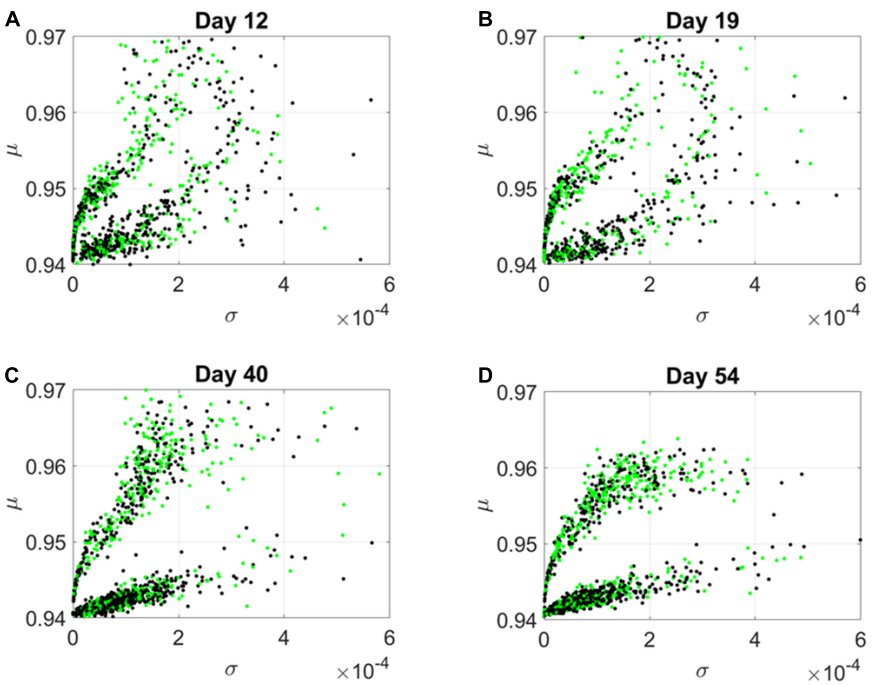

FIGURE 7 | Projection of transcriptome genes associated with neurological (green) and psychiatric (black) disorders on the Gamma moments plane spanned by the empirically estimated Gamma μ and Gamma σ reveals two classes of genes. At each measurement day the genes move to eventually form two distinct lobes corresponding to the HEV and LEV genes on the curved and line-like scatters, respectively. **(A)** Day 12. **(B)** Day 19. **(C)** Day 40. **(D)** Day 54.

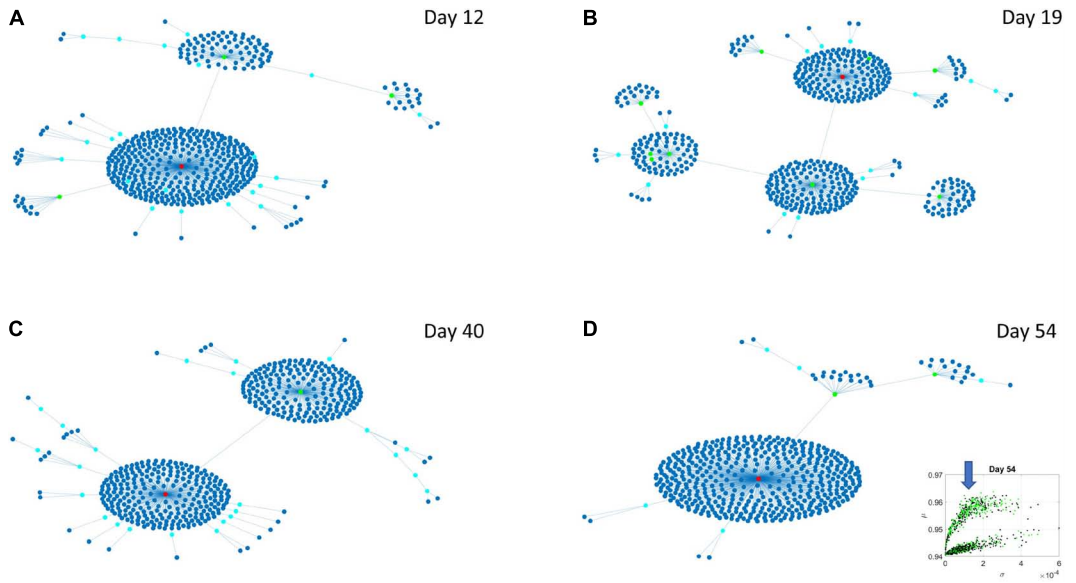

FIGURE 8 | Evolution of statistical dependencies of the HEV genes. Hubs with two or more nodes but fewer than 10 are cyan; those with more than 10 degrees but not the maximum are colored green. The maximum degree is colored red. In the inset in the lower right panel, the arrow points at the lobe of HEV genes comprising the dynamically evolving network. The Chow Liu Maximal Spanning Tree represented as an interconnected network evolving from Day 12 to Day 54, for the HEV genes (located on the line like lobe as marked by the lower right-hand inset). The inset in the lower right panel indicates the lobe of HEV genes comprising these dynamically evolving networks. **(A)** The network has two main hubs on Day 12. **(B)** More major hubs appear on Day 19. **(C)** As the network evolves most genes are concentrated once again around two main hubs. **(D)** On Day 54 we have one major hub.

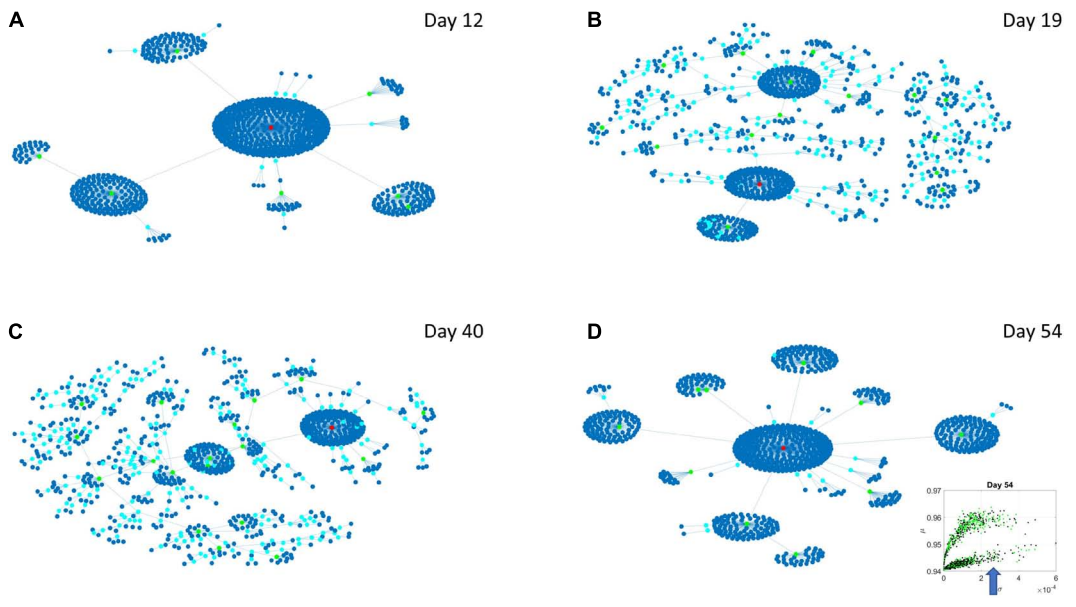

FIGURE 9 | Evolution of statistical dependencies of the LEV genes. Hubs with two or more nodes but fewer than 10 are cyan; those with more than 10 degrees but not the maximum are colored green. The maximum degree is colored red. In the inset in the lower right panel, the arrow points at the lobe of LEV genes comprising the dynamically evolving network. The Chow Liu Maximal Spanning Tree represented as an interconnected network evolving from Day 12 to Day 54, for the LEV genes (located on the line like lobe as marked by the lower right-hand inset). The inset in the lower right panel indicates the lobe of LEV genes comprising these dynamically evolving networks. **(A)** On Day 12 the network is centralized with genes tightly concentrated around few major hubs. **(B)** On Day 19 the network is more distributed with a high number of small hubs. **(C)** This behavior persists on Day 40. **(D)** On Day 54 the network is once again centralized but there are more major hubs than on Day 12.

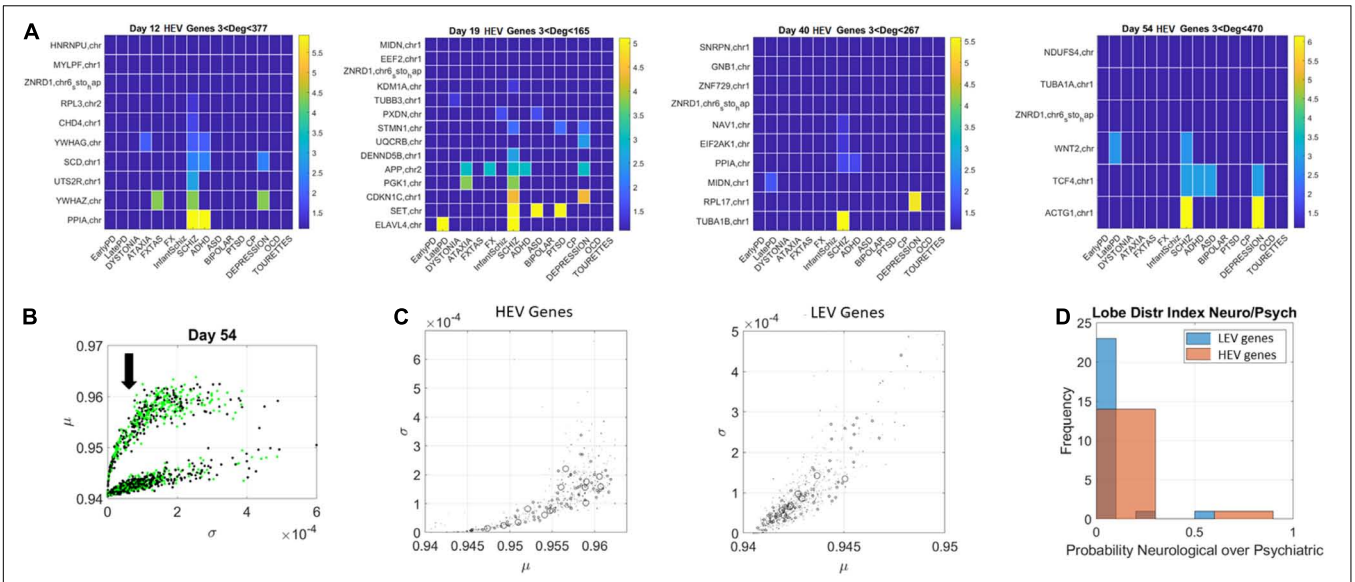

FIGURE 10 | Evolution of genes' degree distributions across neuropsychiatric and neurological disorders. **(A)** Days 12–54 evolution of gene hubs (≥3 degrees) on the curved lobe of HEV genes indicated in panel **(B)**. Rows are genes and columns are disorders (neurological followed by DSM psychiatric). **(B)** The arrow marks the lobe of LEV genes used to build the matrices. **(C)** Index *r* of Eq. 14 was obtained to ascertain the involvement of each disorder type per lobe. The marker size is proportional to this index, with larger markers representing more involvement in neurological disorders. Both lobes are represented, and, despite the larger number of psychiatric disorders, a higher proportion of neurological involvement is featured in HEV genes. This is represented in the probability distribution graph in panel **(D)**.

as depicted on **Figures 6**, **7** for day 54, whereas **Figure 10C** provides the same plot (rotated) for genes in DisGeNet found on the transcriptome at day 54. In this figure, the size of the marker is proportional to the scalar ratio, reflecting the balance between neurological and psychiatric genes for each class of genes. **Figure 10D** provides the probability graphs depicting the higher probability for HEV genes in neurological conditions. This higher proportion comes despite the far larger number of DisGeNet genes associated with psychiatric disorders like Schizophrenia [as captured by the colormap matrices in 10A (HEV genes) and 11–12 (LEV genes)].

Similarly, for the LEV genes, we plotted the color map matrices involving disorders along the columns and genes along the rows. To aid with visibility, these are split between **Figure 11** for Days 12 and 19, and **Figure 12** for Days 40 and 54. In days 19 and 40, more genes partake as hubs and sub-hubs, with a more distributed network topology than in the case of HEV genes (as shown by the networks in **Figures 8**, **9** for HEV and LEV genes, respectively).

We saw that schizophrenia (being the disorder associated with the highest number of genes across these disorders) was the one with the highest number of hubs. We also observed the involvement of a hub across multiple disorders of the neurological class and of the psychiatric class. Furthermore, we noted that psychiatric disorders in general spanned more hubs, and these hubs had a higher degree than neurological disorders. Hubs with degree of 3 and above that were common to both classes of disorders and / or in only one class abounded and are listed in **Supplementary Table 1** and in the expanded **Supplementary Table 2** listing also function and other information about the genes.

To further investigate the balance between HEV and LEV genes in neurological vs. psychiatric disorders, we quantified the disease ratio,

$$r = \frac{e^{\sum_{i=1}^{genes} NeuroDegree}}{e^{\sum_{i=1}^{genes} PsychDegree}} \tag{14}$$

Results From Binary Barcode (State Space) and Cells' Subtypes

Figure 13 shows representative results of tracking the gene's state according to a binary code that sets the state ON if the node's degree at a given day is above the average degree of the network that day, and OFF otherwise. With four readings we have 2^4, 16 possible states, and each gene is assigned one possible state according to its network dynamics. In this figure (for simplicity) we plot two examples of genes with state [1111] remaining each day above the average degree of the network, taken across all nodes. Likewise, the example of [0100] is of a gene that only raises above the average degree of the network in day 19. The other configurations (dashed trajectories) on each plot reflect the trajectories of other genes in other binary state vectors.

Using this information, we could build clusters of the cells that primarily had genes in a binary state. The use of both the fate and state dynamics of the gene enabled the tracking of the stochastic activity over time.

Further Similarities and Differences Between Neurological and Psychiatric Disorders

Besides tracking genes expression and their variability in fate space along with the binary ON/OFF states of each gene and

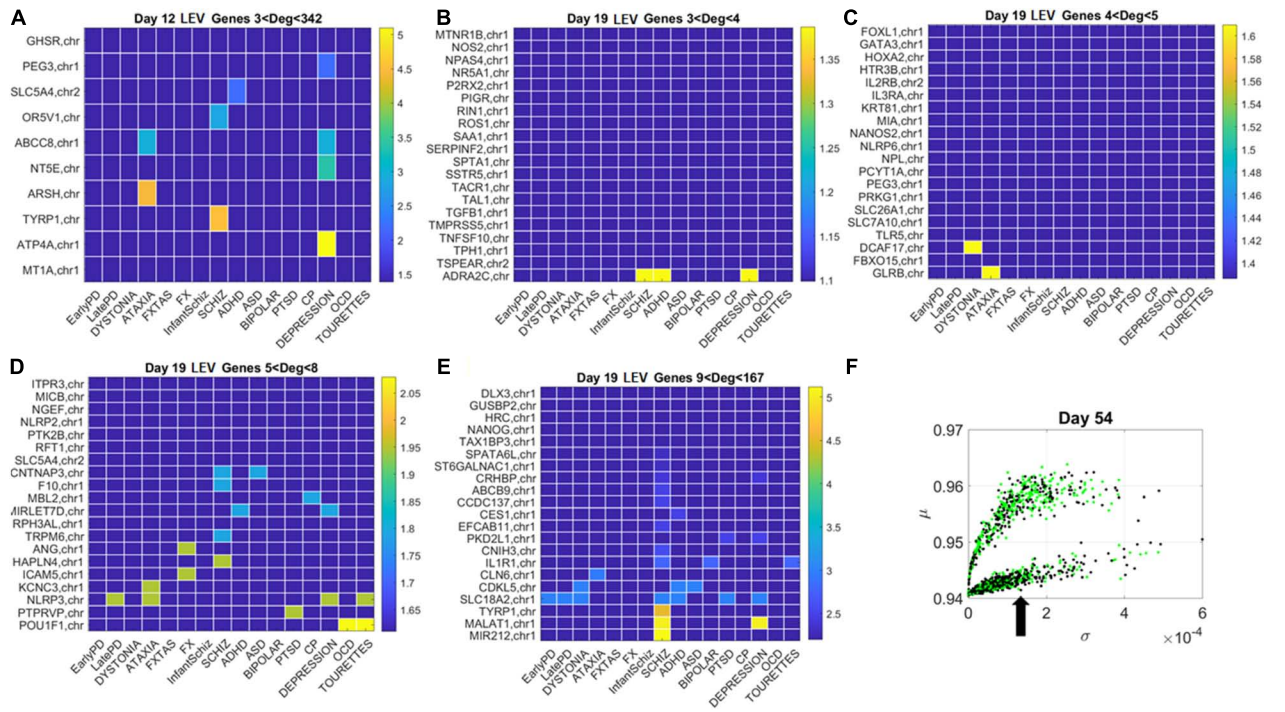

FIGURE 11 | Tracking the LEV genes in the lower (line-like) lobe in Days 12 and 19 according to disorder (as in **Figure 10**) reveals the participation of several hubs in both neurological and psychiatric disorders. **(A)** Day 12 LEV genes with hubs having less than 342 connections. **(B)** Day 10 LEV hub genes with up to 4 connections. **(C)** LEV hub genes with up to 5 connections. **(D)** Day 19 LEV hubs with up to 8 connections. **(E)** Day 19 LEV hub genes with up to 167 connections. **(F)** Lobes of HEV and LEV genes with arrow pointing to the LEV genes used to build these color map matrices and those in the next figure.

their involvement in neurological vs. psychiatric conditions, we selected the following clusters of tissues to evaluate genes' involvement:

- Cortical Regions: Cortex, Frontal Cortex.
- Neuromotor System: Caudate Basal Ganglia, Cerebellar Hemisphere, Cerebellum, Nucleus Accumbens, Putamen Basal Ganglia, Substantia Nigra.
- Limbic System: Amygdala, Anterior Cingulate Cortex, Hippocampus, Hypothalamus.
- Spinal Column: Spinal Cord Cervical C1, Nerve Tibial.
- Glands: Adrenal and Pituitary gland.

We found statistically significant differences at the alpha 0.05 level ($p < 0.04$, t-test). **Figure 14** shows the bar plots comparing the outcomes for HEV and LEV genes for psychiatric (red) and neurological (blue) disorders.

The psychiatric disorders included ADHD, ASD, Schizophrenia, Bipolar, Depression, Infantile Schizophrenia, PTSD, and OCD. The neurological disorders included FXTAS, Early PD, Late PD, Ataxia, FX, Dystonia and CP, and Tourette's, given the involvement of the neuromotor systems in the last two disorders. Maximal differences in percentile change and in the reshuffling of disorders (which we plotted sorted by percentile in **Figure 14**) accounted as well for statistically significant differences between the tissues for the HEV vs. LEV genes ($p < 0.04$, t-test).

DISCUSSION

This paper uses genomic information to reframe a recently revived debate on the possible differentiation between neurological and psychiatric disorders. We reframed the question by addressing whether, despite a shared genomic pool, psychiatric and neurological disorders could be differentiated at very early embryonic stages. To pursue our question, we took advantage of the validation strategies used by Yao et al. (2017) (i.e., the fact that they compared their two-dimensional *in vitro* model to primary tissues from atlas data and cortical cells from mid-gestation human fetal embryos). Their work generated a transcriptome-based lineage that allows for studies of human brain development and for the modeling of human neurodevelopmental disorders.

With these results in mind, we here developed and implemented a three-level approach that interrogated the early development human transcriptome trajectories of hundreds of hESCs as they reached neuronal state. Each level of inquiry offered new insights on the complex genetic origins of psychiatric and neurological disorders, highlighting fundamental differences between the two types of disorders. We uncovered and characterized two classes of genes with essentially different dynamics (distinct ON/OFF states) and fate (cumulative expression variability) featured throughout differentiation. Furthermore, using these classes of genes, we pointed at commonalities between the motor and limbic

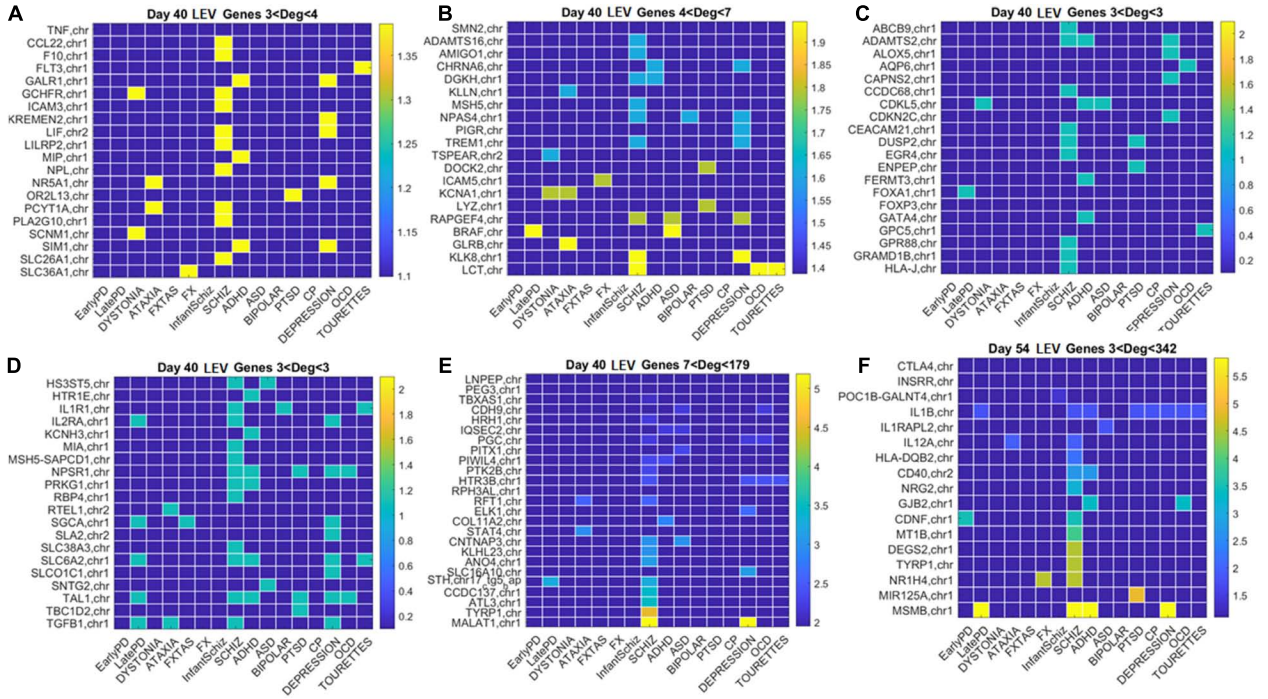

FIGURE 12 | Low expression variability genes Days 40 and 54 (same format as **Figure 11**). **(A)** Day 40 LEV hub genes with up to 4 connections (degree). **(B)** Day 40 LEV hubs with up to 7 connections. **(C)** Day 40 LEV hubs with 3 connections. **(D)** Day 40 LEV hubs with 3 connections. **(E)** Day 40 LEV hubs with more than 7 and up to 179 connections. **(F)** Day 54 LEV hubs with over 3 and up to 342 connections. Color bar is in log (degree).

systems for one class (but not the other) that could possibly explain the current confounds in observational criteria. These analyses provide evidence supporting the notion that psychiatric disorders have substantial neurological underpinnings and yet their associated genes' network interdependencies are significantly different at the early embryonic stages of cell differentiation. We next discuss each level separately.

Level 1: Marginal Distributions

Traditionally, transcriptome interrogation aims at uncovering different classes of cells with some genomic composition. This general approach tends to do away with genes that have low variability or asynchronous behavior, i.e., they may be turned OFF in the initial stages, or have such low expression that their contribution is presumed negligible. We thought differently here and, instead of first trying to uncover cells' classes, we transposed the cells × genes matrix and expressed each gene as a function of the cells' expressions. Then the question was, for each gene, how was the gene's expression across cells cumulatively contributing to the final neuronal fate. Furthermore, how was the gene's state evolving across these different readings? We reasoned that some registered cells might not be the same from day to day, yet the expression of the genes would change across the transcriptome in some way that would lead to self-emergent patterns, found without discarding any genes. For each gene we then obtained the marginal probability distribution of its expression to neuronal fate and measured across days the departure in expression variations, using the EMD metric appropriate to quantify differences or similarities between such frequency histograms.

Tracking this information without discarding any genes allowed us to visualize the genes associated with psychiatric and neurological disorders embedded in the full transcriptome. The new visualization tool revealed *two fundamental subtypes of genes* (as depicted in the two lobes of **Figure 7**). Both lobes had a mixed composition of genes associated with psychiatric (according to the DSM-5) and neurological disorders. The question that emerged then was, what contribution was each set of genes (neurological vs. psychiatric) making to each lobe? We will defer that question to the third level of inquiry.

One lobe is characterized by genes of highly varying expression and high cumulative EMD whereas the other contains genes of low expression variability and low cumulative EMD. Here it may be worthwhile mentioning that traditional methods such as *PCA* and *t-SNE* would have likely missed the LEV genes of the second lobe, with lower expression and lower variability. And as we will see at the third level of inquiry, that would have missed an opportunity to capture the real evolution of these genes from the vantage point of probabilistic nodes interacting across a network.

Level 2: Co-dependent Genes

Moving on to a higher level of complexity, we studied the pairwise interactions between genes by focusing on the joint probability distributions of all possible pairs for each distinct group of genes associated with the various psychiatric and neurological

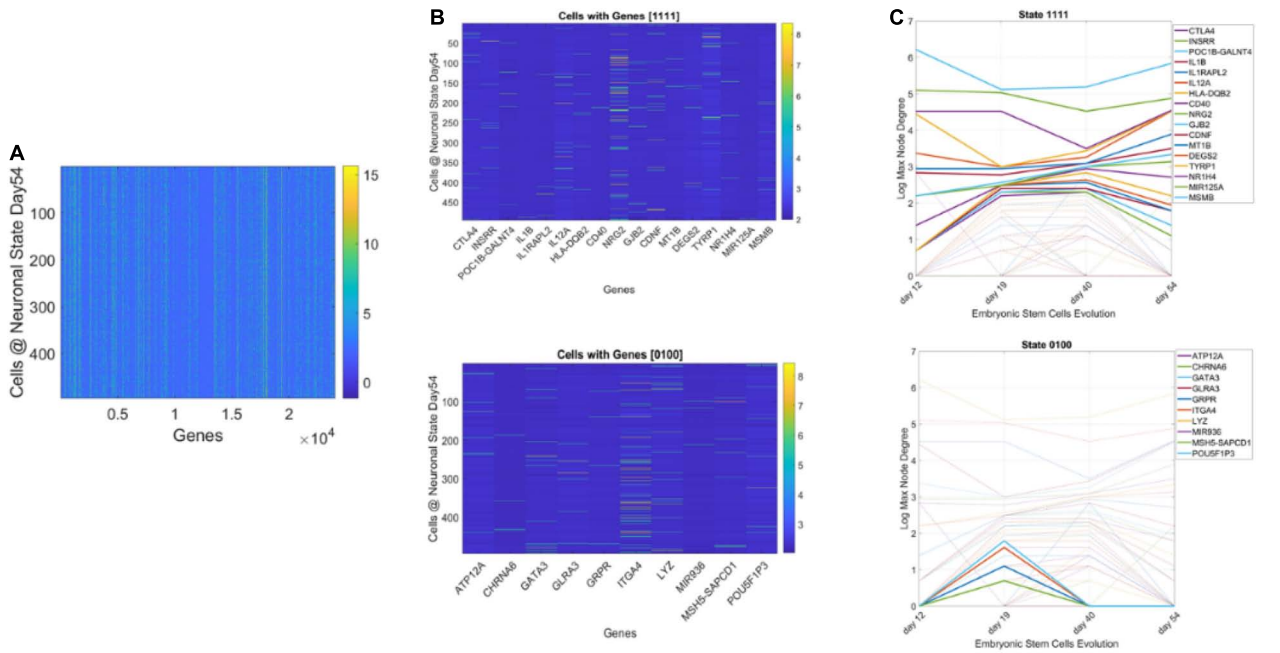

FIGURE 13 | Genes' states and cells' hierarchical trees. **(A)** Matrix of 495 cells × 24,046 genes at day 54 of neuronal stages of hESCs. Each entry of the matrix is the counts in transcripts per million capturing the genes' expressions at day 54. **(B)** Sample genes states [1111] and [0100] whereby the gene's degree in the network is above the average degree each day (always ON, above connectivity threshold) and the case whereby the gene is OFF on day 12, then ON day 19, and OFF the remaining time. **(C)** Dynamic trajectory reflecting the log of the maximal degree of the node (gene) on the *y-axis* and time (days 12–54) under consideration on the *x-axis*. Dashed trajectories reflect the profile of all genes across binary space. Continuous lines on the top graph reflect the genes that remained ON above threshold (average degree) across all days vs. top graph genes with [0100] OFF/ON profile.

disorders, according to sources found in the DisGeNet portal. By employing a robust statistical independency test, we were able to quantify the average degree of pairwise dependencies in each network of gene expressions. The initial degree of interdependence between genes associated with different pathologies (**Figure 5A**) allowed us to differentiate between neurological and psychiatric disorders.

Two main clusters appeared in this vector field, representing the increase in dependency from the initial to the final states. One cluster boasting a higher dependency increase was primarily neurological: early onset PD, Ataxia, Dystonia, FX and FXTAS. Yet, several DSM (psychiatric) disorders appeared at that level as well. These included disorders that are detectable in infancy, such as infantile schizophrenia, CP, OCD, and Tourette's. They share a highly compromised somatic-sensory-motor system and profound issues with the limbic system that without a doubt will impact the overall neurodevelopment of the individual. This pool of genes with high dependency gains across early embryonic stages of neuronal cell differentiation suggests rather early origins of such disorders and the highly interconnected evolution of their associated genes. They also showed a larger rate of change from the initial to the final state.

At a lower level of dependency increase, we saw mostly DSM psychiatric disorders, except for late onset PD. This is interesting considering the high incidence of dementia, hallucinations, and other altered mental states in late PD and other related tauopathies (Koga et al., 2022; Tu et al., 2022). These rates of change were more modest than those in the other group of disorders with a higher dependency increase. In particular, ASD, which lies approximately midway along this vector field, had the lowest dependency increase, signaling an altogether different signature of genes' co-dependency during early embryonic stages of neuronal differentiation.

Indeed, ASD seems to lie on the border between the class of neurological and psychiatric disorders, which confirms at the genetic level that the spectrum of autism comprises pathologies of the nervous system that underlie its phenotypic conceptualization as a behavioral/mental disorder. This is supported by extended research showing biorhythmic patterns in autism with a unique signature of noise-to-signal ratio derived from fluctuations in signal amplitudes and timing. This motor code is bound to impact the kinesthetic reafferent feedback from the periphery to centers of central control (Torres et al., 2013a, 2020; Torres and Denisova, 2016; Wu et al., 2018; Torres, 2021). Here we observed the origins of such departures at these early embryonic stages of neuronal cells' differentiation, whereby the genes associated to ASD manifested the smallest shift in dependency index value from the initial to the final state, accompanied by the smallest increase in genes' statistical dependency. The change in dependency index (i.e., the range of values where the arrow denoting rate of change lands) overlapped with those ranges in FX, FXTAS, Ataxia, Dystonia, and Early PD along the neurological disorders, and with infant Schizophrenia, along the psychiatric ones. This is interesting in view of recent

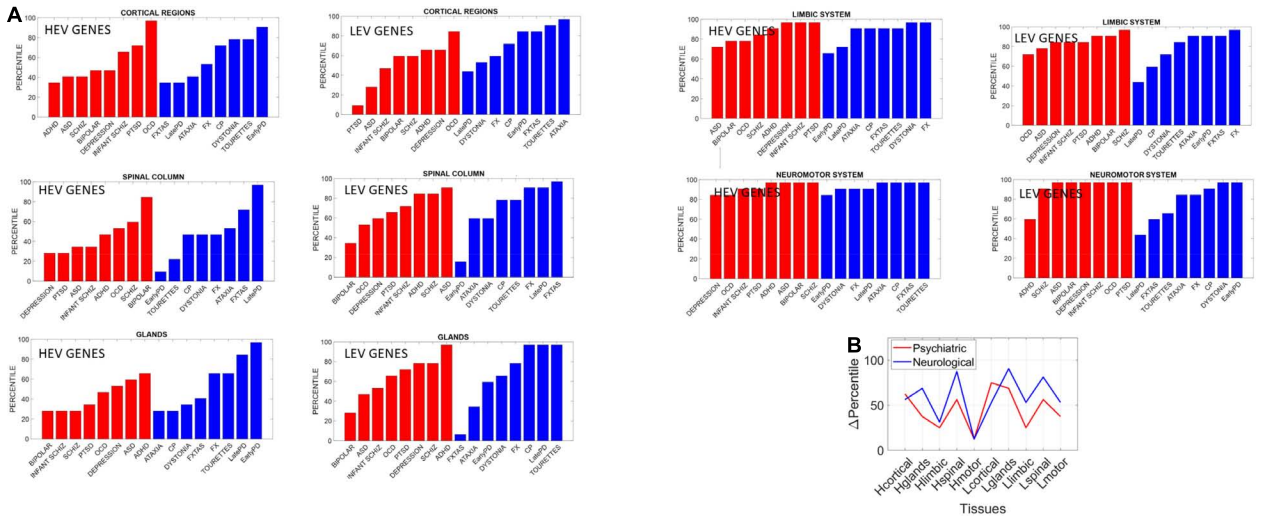

FIGURE 14 | Differentiating psychiatric from neurological disorders by genes' expression in tissues, for the HEV and LEV genes classes. For each disorder in each clinical category (psychiatric or neurological) we removed the genes that would maximally express on tissues from the brain, the spinal cord, and glands, as described above. Upon removal, we calculated (as in Torres, 2020) the resulting normalized changes in overall gene expression across these tissues. Then, we found in which percentile, taken over all tissues, each cluster of tissues belonged and identified the range of the change obtained within each cluster of genes (HEV vs. LEV). We computed the difference in percentile for each cluster of tissues to compare neurological vs. psychiatric disorders. **(A)** Percentile of change in genes expression on tissues for each disorder (red psychiatric vs. blue neurological) for tissues reported by the Consortium atlas of genetic regulatory effects across human tissues, GTEx v8 atlas of 54 tissues (GTEx Consortium, 2020) here grouped into regions, cortical, spinal cord, limbic and neuromotor systems and glands (see text). **(B)** Changes in percentile between psychiatric and neurological disorders are statistically significant at the 0.05 level and reflect the differences between HEV ("Htissue") and LEV genes ("Ltissue") for each set of tissues comprising these regions of interest. The changes in limbic and motor tissues were minimal in HEV genes, but appreciable in the LEV genes (often discarded).

work examining digital biomarkers of FX carriers who, despite their young age, manifested gait patterns present in Ataxia and FXTAS much like participants in the autism spectrum did (Torres et al., 2020; Bermperidis et al., 2021). In this recent work, according to causal stochastic analyses, the kinesthetic feedback loops estimated from their gait largely departed (in both ASD and young FX carriers) from the neurotypical age-matched controls. They resembled instead the gait of patients with PD (Bermperidis et al., 2021). Our results here confirmed that the presence or absence of a disorder was not due to the mutation of a specific gene but rather resulted from the degree of co-expression among multiple genes. Therefore, in the context of the evolving human transcriptome, what truly separates neurological from psychiatric disorders is that the latter show much higher complexity of co-expression and interconnectedness in the early stages of cell differentiation, as compared to the genes associated with the former.

This hypothesis is reinforced if we observe the relative positions on this plane of Infant Schizophrenia and Schizophrenia, Schizophrenia and Bipolar Disorder, Tourette's and OCD, PTSD and Depressions as well as ASD and ADHD, and ASD and Infant Schizophrenia. Once again, we have a transition in the initial degree of dependency from what characterizes Infant Schizophrenia as a neurodevelopmental disorder to what characterizes Schizophrenia as a disorder appearing in early adulthood. Also, OCD and Tourette's have a high comorbidity, and symptoms of the latter may appear in the symptomatology of OCD. The same is true for Depression and PTSD, whereas both Schizophrenia and Bipolar Disorder belong in the psychotic spectrum of psychiatric diagnoses, according to the DSM-5. Hence, the proven clinical proximity in all three pairs recapitulates here in their proximity in our parameter space. Interestingly, ASD was once labeled as "Infantile Schizophrenia," a clinical labeling that receives support in our parameter plane: ASD and Infantile Schizophrenia have nearly the same initial degree of dependency in our parameter space. Finally, Tourette's is clustered among the psychiatric disorders. Indeed, there is ample debate on whether Tourette's is a psychiatric or a neurological disorder, despite generally being classified as the latter (Sandor, 1993; Kerbeshian et al., 1995; Brovedani and Masi, 2000).

When examining the cumulative changes in expression variability according to the EMD, a negative trend emerges in both types of disorders. The higher the genes' variability accumulated toward Day 54, the lower the dependency index in this final state. However, ASD appears among the neurological disorders and Early and late PD appears among the psychiatric (DSM) ones (near to PTSD, Depression, OCD, CP, and Tourette's). Furthermore, ADHD and Schizophrenia lie midway of this scatter and Infantile Schizophrenia and Bipolar disorders depart from the psychiatric group along the axis of variability, i.e., their average cumulative EMD expressions are among the highest levels, along with those of the neurological disorders, at Day 54.

From these patterns, it may be possible to perceive that networks that reach a highly interconnected and complex state are characterized by genes that have more constancy in their

statistical behavior. We revisit this proposition shortly, at the third level of inquiry. We reasoned here that, intuitively, this would make sense, since highly interconnected networks could dictate a gene's behaviors in a distributed way, whereas more disjointed networks would allow genes to behave in a more independent manner.

Our second level of inquiry considers pairs of genes, isolated from the rest of the network, and then integrates over all pairs to derive the degree of interdependency. We next explore a full probabilistic graphical model and visualize the global behavior of these genes. We characterize the topology of this network and identify the importance and role of different genes in the evolution of the network and subsequently on the origins of different disorders. To that end, we consider the multivariate probabilistic behavior of the two different subtypes of genes that we discovered at the first level of analysis.

Level 3: Genes' Networks

By employing factorization techniques and graphical modeling, we were able to capture the evolutions of the networks of HEV genes (**Figure 8**) and LEV genes (**Figure 9**). In both graphical trees, we observed a hierarchical structure, with genes that were central (hubs) and associated with many other genes as well as genes that were leaf nodes. One key difference between HEV and LEV genes was that the latter have a less hierarchical organization on Days 19 and 40, with many small hubs and "clouds" of genes forming around the dominant hubs of the network. Note that the topology of the networks implies fragility, since removing a central hub or removing edges that connect large hubs would result in disconnected graphs, with the network of HEV genes being the most fragile of the two.

We then cataloged the degrees of genes that were significant in each of the eight networks (two networks $\times$ 4 days for each genes' class) and belonged to different neurological and psychiatric disorders (see **Figures 10–12**). Despite a vast majority of high degree genes being consistently associated with psychiatric disorders such as Schizophrenia (owing to the very high number of associated genes reported in DisGeNet), we found a far higher proportion of neurological involvement in HEV genes (**Figures 10A,D** showing the probability distributions along with **Supplementary Figures 2–4**). This result, along with those in **Figures 11**, **12** for the LEV genes, pointed to a degree of overlapping of genes associated with neurological disorders with those associated with psychiatric disorders. This is captured as well on **Supplementary Table 1**, which we expanded **Supplementary Table 2**, to catalog the genes' function, location, and phenotypes. The results also unambiguously separated genes involved in neurological disorders from those associated with psychiatric disorders in that the former were probabilistically more HEV (**Figure 10D**). In contrast, the latter tended to be (probabilistically) LEV, yet forming more robust and distributed networks. From the network analyses and the index ratio quantifying neurological over psychiatric predominance, we concluded that, probabilistically, underlying psychiatric disorders have more LEV genes and underlying neurological disorders have more HEV genes.

The Level of Average Genes' Expression on Tissues

The fundamental differences quantified between psychiatric and neurological disorders using the different lobes of HEV vs. LEV genes extended to the tissues, as we probed these genes' expressions on the 54 tissues from GTEx. Here we grouped tissues into brain regions (cortical, motor-subcortical and limbic), the spinal cord, and glands (see main text for details) and measured the percentile change in HEV vs. LEV genes upon removal of those genes in each of the disorders under consideration. We then grouped these disorders according to psychiatric and neurological clinical classification and compared the change in percentile between the two types of disorders.

Our analysis showed that, across different groups of tissues that typically serve different roles in the autonomic, central, and peripheral nervous system, fundamental differences emerged between the two types of disorders under consideration. The genes associated with psychiatric disorders expressed on these tissues differently than they did in neurological disorders. These differences were statistically significant between HEV and LEV genes in general. They were also statistically significant when considering the genes as part of psychiatric or neurological disorders. To that end we pooled the changes in percentile across all tissues and genes' type of each disorder class (psychiatric or neurological) and found that the HEV vs. LEV genes classification served to separate psychiatric from neurological disorders. Furthermore, motor, and limbic regions were minimally different in HEV genes (relative to LEV genes) when comparing psychiatric to neurological disorders (**Figure 14B**). This suggests that LEV genes, traditionally discarded, contribute to such statistically significant differences between psychiatric and neurological disorders. This result, paired with the probabilistically higher prevalence of neurologically associated genes across the HEV lobe and their presence in psychiatric disorders, supports the notion of motor involvement in mental pathologies. It is in this sense that one could argue that psychiatric disorders are also neurological disorders.

A main corollary of these results is that the LEV genes, which under traditional methods are likely discarded and excluded from the analyses, make an important contribution to the distinction between psychiatric and neurological disorders. This can be appreciated in **Figure 14B** whereby HEV genes (likely the ones entering the analyses under traditional techniques) do not separate motor and limbic systems between the two disorder types. It is the LEV genes that do so in the motor and limbic systems, and in other tissues as well. Further development of new analytical techniques that also include these genes with lower expression variability and asynchronous ON/OFF states may open new lines of inquiry across diseases in general.

Considering the Gene's State Through a Binary Barcode

Interrogating the fate of the genes gave us a sense of ways to automatically cluster groups of genes according to the evolution of their expression variability. But, what about the changes in gene's state? Once we reached the third level of inquiry and

determined the gene's degree of codependency at the network level, it was possible to examine the average degree of the gene to determine its hub activity level as above or below that average threshold (on a logarithmic scale to capture the non-uniform degree distribution). In 4 days of measurements (in this case), we found genes that were always ON [1 1 1 1] in their hub networking role. These stood in contrast to those that were always OFF [0 0 0 0] in this role. Then, we found genes in other categories, thus building a barcode of binary states that allowed further examination of the genes embedded in the full transcriptome. Here we studied these pools of genes associated with neurological and psychiatric disorders, but the same type of method could be employed to interrogate the network's state dynamics of genes associated with other disorders, with different frequency of recordings (here 2^4 in 4 days of recordings, yielding 16 types that resulted in different cell classes expressing those main types). However, other refinements of this method might offer new dynamics information with higher granularity of cell's state that includes the low- and odd-varying genes with asynchronous states (such as the class of LEV genes uncovered here). In this sense, further work is warranted to formalize a new embedding algorithm that considers the full transcriptome fate and state code, to reveal topological invariants of the genes' classes associated with known diseases.

Recapitulating Phenotypical Information Cataloged From Multiple Sources

We took our investigation one step further and compiled information from the OMIM online source for genes of degree >3 of both gene lobes (HEV and LEV). Accordingly, we examined three categories of genes that, according to their association with both neurological and psychiatric disorders (overlapping roles), were associated with neurological disorders only and those associated with psychiatric disorders only. The "Neurological only" category consisted of only two such genes with degree higher than 3. Information about these classes of genes can be found in the **Supplementary Table 1** and in the expanded version of this table, including the inheritance of the identified genes and the functional properties of the proteins they encode in the **Supplementary Material**.

Both sets of genes associated only with psychiatric disorders and those associated with both the neurological and psychiatric ones were found to play critical roles in the developing nervous systems overall, fetal brain development, neurogenesis, neuronal differentiation, neuromodulation, synaptic organizations, healthy function of the senses, cortical development, and neuronal migration in the context of development. The psychiatric only-type genes were mainly associated with serotonin (5-HT), glutamate (NMDA), and nicotinic acetylcholine receptors (nAchRs), whereas the neurological and psychiatric-type genes were associated with adrenergic (norepinephrine) and dopaminergic pathways. In both groups, specific genes were important for the GABAergic system.

In an interesting outcome (summarized in **Supplementary Figure 5**), genes with a high network degree (large hubs) turned out to be crucial to the immune system. These genes are associated with autoimmune disorders and neurodegeneration, synthesis of growth factors, cancer and metastasis, inflammatory responses, and allergies. Notable cases are the HEV gene ELAVL4, with a network degree of 165. This hub is associated with Late Onset Parkinson's disease and Schizophrenia and is related to paraneoplastic neurological disorders (PND) and autoimmune neuronal degeneration. Another gene, LIF, in the LEV genes lobe, with a network degree of 4, has been hypothesized to play a functional role at the interface between the immune system and the nervous system. Here we recapitulated its association with Schizophrenia and Depression.

According to the OMIM literature, many of the identified high-degree genes (the hubs) play key roles in basic molecular and cellular functions, such as signal transduction, ATP synthesis, mitosis, cell-to-cell adhesion, intracellular signaling pathways, differentiation, proliferation, and transcription regulation. These large hubs also participate in these processes by influencing other genes' functions (as predicted by the network's topology) implicated in the formation of key components of the cell, such as the cytoskeleton and the extracellular matrix. These hub genes are crucial to the survival of the eucaryotic cell.

These findings imply that the genes associated with neurological disorders are no more fundamental than the genes associated with psychiatric disorders or that are at the intersection of both disorders. According to the transcriptome data used here, validated using primary tissues from atlas data and cortical cells from mid-gestation human fetal embryos, and to the analyses that we performed, the origins of both types of disorders can be traced back to the embryonic stages of differentiation and development. These involve the emergence of fundamental neural pathways and general biochemical cascades that characterize eucaryotic cell life. Moreover, in both types of disorders, key genes support the role and interaction of the immune system with the developing nervous system. The interaction between the two sub-systems is being explored and investigated by various researchers (Ashwood and Van de Water, 2004; Ashwood et al., 2006; Michel et al., 2012; Meltzer and Van de Water, 2017), and their findings will shed light not only on the mechanisms of emergence of neurodevelopmental and neurological disorders but also on what the DSM-5 characterizes as "mental disorders."

Amidst such heated debate on differentiating between neurological and psychiatric pathologies, perhaps, as synthesized by the OMIM literature behind their associated genes in our networks, a fundamental distinction is delineated by the neurotransmitter receptors and pathways linked to these genes. For example, the degeneration of dopaminergic pathways in the striatum is responsible for Parkinson's disease-associated tremor, which is, at some point of the disorder's progression, observable to the naked eye, thus shifting the focus to the "neurological" nature of PD (while ignoring the progressive dementia associated with it). In contrast, low serotonin in Depression leads to a "psychiatric" phenotype that sidelines motor control issues in these patients. On the other hand, high dopamine levels in Schizophrenia seem characteristic of this disorder, which despite being labeled "psychiatric" has a definite motor component (Rogers, 1992; Walther and Strik, 2012;

Nguyen et al., 2016; Slowinski et al., 2017; Walther and Mittal, 2017; Walther et al., 2020, 2022).

Possible Utility of Reframing the Question of Psychiatric vs. Neurological Disorders From the Genomics Standpoint

Two important theoretical constructs could be proposed to further disentangle these disorders at the level of modeling system's behaviors. One theoretical construct would have to borrow mechanisms from the immune/autoimmune systems to frame models of possible mechanisms. Given the synthesis of OMIM information, this line of inquiry will be important in future computational work.

Another avenue of theoretical inquiry explaining mechanisms to differentiate neurological from psychiatric disorders at the behavioral level would be related to the general framework comprising internal models of action (IMA) in the field of neuromotor control (Kawato and Wolpert, 1998; Wolpert and Kawato, 1998). We refer specifically to the "*principle of reafference*" (Von Holst and Mittelstaedt, 1950) and related computational modeling. According to this basic principle, every time a movement is initiated by the nervous system, information is sent to the motor system and a copy of the signal is created, known as the *efference copy*. This enables the CNS to distinguish sensory signals stemming from (exogenous) external environmental factors from (endogenous) sensory signals coming from the body's own actions (Jekely et al., 2021). According to the IMA, the efference copy is provided as input to a forward model, which predicts the sensory consequence of a motor command and measures the error between desired and attained outcome (Kawato and Wolpert, 1998; Wolpert and Kawato, 1998). Although IMA focuses only on error-correction and targeted-directed actions, complex movements are richly layered (Brincker and Torres, 2018). As such, endo-afference can be further separated into at least two components based on different classes of movements (Torres, 2011). One type of endo-afference is classically associated with voluntary actions, i.e., those deliberately aimed at a goal and operating under an error correction code (Kawato and Wolpert, 1998; Wolpert and Kawato, 1998). Another type of endo-afference, however, is associated with spontaneous or incidental actions, encompassing signals that transmit information about contextual variations associated with exploratory learning (Torres, 2013; Vaskevich and Torres, 2022). Reafferent signals also include pain and temperature afference, a far more complex and elusive code that needs to be distinguished from the motor code in current biosignals' analytics, e.g., as revealed in Elsayed (2021) and Ryu et al. (2021).

The principle of reafference allows us to distinguish between different levels of *mental intent* (Torres, 2013; Torres et al., 2013b; Choi and Torres, 2014; Ryu and Torres, 2020) and *physical volition* (Torres and Lande, 2015; Nguyen et al., 2016; Torres, 2016, 2017). This distinction can be conceptually mapped onto psychiatric and neurological issues, respectively, to inform hypothesis testing and theoretical modeling, possibly expanding criteria beyond subjective observation and opinion. However, we feel that the theoretical mechanisms stemming from immune/autoimmune systems will non-trivially add to our understanding of genomic differentiation between these classes of disorders. As such, they should be incorporated in a new internal model framework amenable to address different genes' classes analogous to deliberate vs. spontaneous or error-corrective vs. exploratory modes of neuromotor control and learning, respectively (Torres, 2011; Vaskevich and Torres, 2022). Here understanding recurrent loops of genes modulating other genes will be critical to forecast and track the onset timing of these disorders.

In summary, we have demonstrated at the level of hESCs that there are fundamental differences between psychiatric and neurological disorders when considering the full transcriptome. The inclusion in our analyses of genes associated with these disorders that nevertheless present odd or low variability and asynchronous ON/OFF states proved essential to making this differentiation. Considering only HEV genes would have missed this dichotomy. Furthermore, these distinctions extended to human tissues commonly studied in genomics. It is our hope that the multilayered, more inclusive approach offered in this work paves the way to open new lines of inquiry and help advance basic research in mental and physical health in general.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://doi.org/10.5281/zenodo.6299834.

AUTHOR CONTRIBUTIONS

TB analyzed data, designed analyses, and wrote manuscript. SS curated data and edited manuscript. FHG supervised work and edited manuscript. TS supervised work and provided computational resources. ET supervised work, conceived work, analyzed data, and wrote manuscript. All authors contributed to the final state of the manuscript and agreed to its publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2022.884707/full#supplementary-material

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