AUTISM SPECTRUM DISORDER WITHIN NEURODEVELOPMENTAL DISORDERS: CATCHING HETEROGENEITY, SPECIFICITY AND COMORBIDITY IN CLINICAL PHENOTYPES AND NEUROBIOLOGICAL BASES

EDITED BY: Eugenia Conti, Mirko Uljarevic, Marco Turi and Francesco Craig

PUBLISHED IN: Frontiers in Neuroscience

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88976-952-0
DOI 10.3389/978-2-88976-952-0

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AUTISM SPECTRUM DISORDER WITHIN NEURODEVELOPMENTAL DISORDERS: CATCHING HETEROGENEITY, SPECIFICITY AND COMORBIDITY IN CLINICAL PHENOTYPES AND NEUROBIOLOGICAL BASES

Topic Editors:
Eugenia Conti, Stella Maris Foundation (IRCCS), Italy
Mirko Uljarevic, The University of Melbourne, Australia
Marco Turi, Fondazione Stella Maris Mediterraneo Onlus, Italy
Francesco Craig, University of Calabria, Italy

Citation: Conti, E., Uljarevic, M., Turi, M., Craig, F., eds. (2022). Autism Spectrum Disorder within Neurodevelopmental Disorders: Catching Heterogeneity, Specificity and Comorbidity in Clinical Phenotypes and Neurobiological Bases. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-952-0

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frontiers | Frontiers in Neuroscience

TYPE Editorial
PUBLISHED 03 August 2022
DOI 10.3389/fnins.2022.958650

OPEN ACCESS

EDITED AND REVIEWED BY
Yusuke Takatsuru,
Toyo University, Japan

*CORRESPONDENCE
Marco Turi
turi.marc@gmail.com

SPECIALTY SECTION
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

RECEIVED 31 May 2022
ACCEPTED 24 June 2022
PUBLISHED 03 August 2022

CITATION
Conti E, Turi M and Craig F (2022) Editorial: Autism spectrum disorder within neurodevelopmental disorders: Catching heterogeneity, specificity, and comorbidity in clinical phenotypes and neurobiological bases. *Front. Neurosci.* 16:958650. doi: 10.3389/fnins.2022.958650

Editorial: Autism spectrum disorder within neurodevelopmental disorders: Catching heterogeneity, specificity, and comorbidity in clinical phenotypes and neurobiological bases

Eugenia Conti[1], Marco Turi[2]* and Francesco Craig[3,4]

[1]Child Neuropsychiatry Unit, ASL Toscana Centro, Empoli, Italy, [2]Fondazione Stella Maris Mediterraneo Onlus, Matera, Italy, [3]Department of Cultures, Education and Society, University of Calabria, Cosenza, Italy, [4]Scientific Institute, IRCCS E. Medea—Unit for Severe Disabilities in Developmental Age and Young Adults, Brindisi, Italy

KEYWORDS

heterogeneity, autism spectrum disorder, phenotypes, comorbidity, neurobiological mechanism

Editorial on the Research Topic
Autism spectrum disorder within neurodevelopmental disorders: Catching heterogeneity, specificity, and comorbidity in clinical phenotypes and neurobiological bases

Autism spectrum disorder (ASD) refers to a group of complex neurodevelopment disorders (NDDs) characterized by impaired social interaction and communication, and the occurrence of restricted interests and repetitive behaviors (APA, 2013). ASD is characterized by heterogeneity in terms of behavioral expression, onset, treatment-response, and comorbidities, along with heterogeneous genetic and neurobiological underpinnings (Lombardo et al., 2019). Although hinted at in pioneering descriptions by Kanner (1943) and later argued by Wing and Gould (1979), heterogeneity remains poorly defined. Therefore, to understand specific etiologies and inform individualized treatments, it is crucial to move away from the single entity toward addressing "heterogeneity" in ASD. In this frame, it is of utmost importance to keep in mind the "specificity" concept to differentiate ASD from other NDDs and enlarge knowledge about "comorbidity," too.

Recent state-of-the-art neuroimaging and electrophysiological tools, combined with advanced person-centered analytical and computational approaches, allow our field to start addressing heterogeneity from genetic and neural, to clinical units of analysis. Recent developments have also suggested the importance of going beyond the Diagnostic

and Statistical Manual of Mental Disorders (DSM-5) defined categorical boundaries and attempting to identify homogenous clinical subgroups across individuals.

From these statements derives the inspiring idea of creating the present Research Topic, in order to give a collection of contributions under the frame of "heterogeneity," "specificity" and "comorbidity" at clinical and neurobiological levels toward a better understanding of etiology and treatment. Indeed, the scope of the Research Topic has been to shed light on ASD, either at the clinical or neurobiological level, keeping in mind the frame of NDDs. This multi-disciplinary effort aimed at bringing together contributions ranging from basic science to sophisticated latent variable modeling approaches to facilitate clarification of distinct aspects of clinical phenotype, thus paving the way to clinical translatability.

The Research Topic has been taken into consideration by several research groups and finally consists of 20 papers (2 review papers and 18 original studies). In this editorial, we will discuss different themes that we have identified across the contributions: (1) Genetic variations and biological implications; (2) Neuroimaging and electrophysiological application; and (3) Characterization of the clinical phenotype. We conclude with a discussion on future directions in the field.

Genetic variations and biological implications

As far as genetic variations and biological implications, it can be asserted that "synaptopathies", involve disruption to genes expressed at the synapse and account for between 0.5 and 2% of autism cases. The Phelan McDermid Syndrome (PMS, also known as 22q13 deletion syndrome) and NRXN1 deletions (NRXN1ds) are two synaptopathies associated with autism. PMS often incorporates disruption to the SHANK3 gene, implicated in excitatory postsynaptic scaffolding, whereas the NRXN1 gene encodes neurexin-1, both implicated in trans-synaptic signaling in the brain.

Cooke et al. describe the Synaptic Gene (SynaG) study from the AIMS2-TRIALS project, which adopts a gene-first approach and comprehensively assesses these two syndromic forms of autism.

In the second paper focused on PMS, Isenstein et al., reported a habituation electrophysiological study (EEG-ERP) on PMS subjects to understand hyporesponsiveness in this clinical population. This study suggests that while neural response and habituation are generally preserved in PMS, genotypic and phenotypic characteristics may drive some variability.

Autism is often associated with potential risk factors that may alter the expression of certain receptors; for example, Liu et al., in a study of *in vitro* human neural progenitor cells, demonstrated that maternal diabetes may be correlated with the onset of ASD. Indeed, they showed that hyperglycemia, due to maternal diabetes, induces suppression of oxytocin receptors that contributes to social deficits in offspring, through a process of oxidative stress and epigenetic methylation.

Along a similar line of research Mariggio et al. focused on the genetic expression of the dopaminergic system (DS), possibly involved in the pathophysiology of ASD and attention-deficit/hyperactivity disorder (ADHD). Specifically, single nucleotide polymorphisms (SNPs) DRD1 and DRD2 dopamine receptors might be considered as potential risk factors for ASD and ADHD, thus being only DRD2-12 (rs7131465) significantly associated with a higher risk for the ASD/ADHD overlap.

Among biological risk factors also potential involvement of the immune system in the etiopathogenesis of ASD has been investigated. Specifically, De Giacomo et al. investigate the levels of immunological markers in peripheral blood of children with ASD founding that regulatory B cells and T cells were decreased in ASD subjects having a possible role in ASD pathophysiology.

Neuroimaging and electrophysiological application

In this Research Topic neuroimaging and electrophysiological applications have been particularly used to study the effects of treatment and to understand how alterations in the brain correlate with clinical aspects of the autistic phenotype.

Both functional and structural MRI methodologies are represented in the studies included. Lan, Xu, Yu et al., based on resting-state data, reveal a possible relationship between atypical visual attention and poor learning ability in subjects with ASD, while the fMRI entropy connectivity method used by Yu et al. reveals that a combination of abnormal top-down and bottom-up information processing accelerates the deoptimization of brain networks and potentially affects cognitive activities in patients with ASD. Lan, Xu, Wu et al. reported spontaneous activity changes in terms of fMRI acquisition, in the visual and language-related brain regions in the ASD population, while the correlation with multiple clinical indexes did not appear significant.

Though in smaller sample sizes cohorts, preliminary interesting results are reported in structural MRI studies, especially in terms of structure-function connections. A correlation with clinical indexes (in terms of ADOS and ADI-R scores) has been reported in Lucibello et al., where cortical thickness and gyrification alterations in preschoolers with ASD are reported. In a longitudinal cohort of subjects at risk for autism, Godel et al. detected a regional structural MRI index of gray-white matter contrast (GWC), founding that early onset of ASD symptoms (i.e., prior to 18 months) was specifically associated with slower GWC rates of change during the second year of life, in areas related to the central executive network. In the paper from Chen et al., significant differences between the

ASD group and the TD group in surface area, cortical volume, and cortical thickness were found and correlated with language, considering two ASD subgroups according to their response to speech ABR results, were detected, too.

Interestingly, two papers report (Sun et al.; Yu et al.) the application of neuroimaging/electrophysiology respectively serving as treatment monitoring or rehab intervention. In particular, Yu et al. reported a correlation between the reduction of socio-communicative symptoms and Default Mode Network activity in 3–6 years children with ASD after a 12-week mini-basketball training program. Another article in our Research Topic, by Sun et al., proposed a trial through tDCS stimulation in the dorsolateral prefrontal cortex area of children with ASD (4–12 years) and analyzed EEG mismatch negativity response in pre and post-treatment; even though a trend was detected, they did not found significant differences between ASD and healthy subjects, while a meaningful correlation of mismatch negativity response and symptomatology was detected.

The functional infrared-spectroscopy (fNIRS) technique has been recently reported as an efficient tool to investigate brain activity in the autism field (Zhang and Roeyers, 2019); in this Research Topic, Conti et al. systematically reviewed 13 papers applying fNIRS focused on young subjects (preschoolers) with ASD or infants at high risk of developing ASD, either in resting-state or task-evoked conditions. Findings confirm that the fNIRS application can represent a promising tool for potentially detecting autism traits, even in this very young population.

Characterization of ASD clinical phenotype

Four articles in this Research Topic are focused on the characterization of ASD phenotype and related clinical implications. In particular, Operto et al., compared adaptive skills, emotional/behavioral problems, and parental stress among children with different severity levels of ASD symptoms, reporting a strong role of adaptive behavior in modulating the presence of internalizing problems, thus suggesting the importance of taking it into account in the rehabilitation program and family support.

Beyond emotional/behavioral problems, DSM5 (APA, 2013) introduced the possibility of double diagnosing ASD and ADHD conditions, and existing literature suggests shared neurobiology between the two conditions (Di Martino et al., 2013). Interestingly, Aiello et al., tried to disentangle the clinical phenotype and specificity of the two co-occurring conditions in relation to autism traits [C-AQ-(Auyeung et al., 2008)] and empathy [C-EQ (Auyeung et al., 2009)], by comparing children with ASD with and without comorbid ADHD with children presenting ADHD only and children with typical development. The reliability of the C-AQ and C-EQ as behavioral markers to differentiate ASD (regardless of comorbid ADHD) from an ADHD condition and TD was confirmed.

Considering the dimensional approach to the autism spectrum, it is of interest the detection of autistic traits in the general population, too. In this frame, Vaiouli and Panayiotou used regression models to establish cross-sectional associations between autistic traits, alexithymia, and social-emotional difficulties in 275 young adults (e.g., college students), thus providing evidence of the influence of different alexithymic facets on the relationship between autistic traits and social-emotional challenges in young adults.

Research on ASD parents is another issue of great interest in the field, thus often representing a direct window on the broader autism phenotype (Sucksmith et al., 2013) and related shared neurobiological bases, too (Billeci et al., 2016). In the paper from Uljarevic et al., the relationship between social motivation in children with ASD and their parents was investigated, through the administration of the Social Responsiveness Scale (SRS). The study established that low social motivation in children with ASD may be driven, in part, by lower social motivation in one or both parents.

First-degree relatives of individuals with ASD may show mild deficits in cognitive flexibility as reported by Cheng et al. Indeed, the authors investigated first-degree relatives of individuals with ASD, either at the clinical level in terms of BAPQ (Piven et al., 1997) and CFI (Dennis and Vander Wal, 2010) or at the neuroeletrophysiological level (ERP), reporting cognitive flexibility deficits at both levels in ASD parents. The cognitive flexibility difficulties were related to autistic traits, thus representing a neurocognitive endophenotype of ASD.

Conclusion and future directions

The published papers on this Research Topic highlight the complexity of performing research in the field of ASD because of neurobiological heterogeneity and phenotypic expression. To conclude, we would like to mention the review paper from Nordahl et al. that is entirely in line with the core intention of the Research Topic. As a matter of fact, the authors summarize the findings of the Autism Phenome Project (APP), a longitudinal multidisciplinary study since 2006, that now includes over 400 subjects from 2 to 19 years of age investigated from medical, behavioral, and neuroimaging perspectives, with the final aim to catch heterogeneity of autism. This approach represents an effective model to investigate autism, showing the importance of translational contribution in developing better-tailored treatments and preventive strategies.

As suggested by Nordahl et al., the identification of subgroups is not meant to divide the autism community, but rather to improve individual care plans and redesign the care and social services for autistic people and their families.

All the published manuscripts emphasize future perspectives and ongoing challenges in the field. Hence, we would like to thank the contributors for their interesting and significant contributions and wish that this Research Topic stimulates further research potentially impacting the autistic community.

Author contributions

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

Funding

This work was supported by the European Research Council (ERC) under the European Union's Horizon 2020 Research and Innovation Program (Grant No. 801715) (PUPILTRAITS) and (Grant No. 832813) (GenPercept) to MT and by AIMS-2-TRIALS network to EC.

Conflict of interest

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frontiers in Neuroscience

ORIGINAL RESEARCH
published: 09 February 2021
doi: 10.3389/fnins.2021.634781

Maternal Diabetes-Induced Suppression of Oxytocin Receptor Contributes to Social Deficits in Offspring

Jianbo Liu[1†], Yujie Liang[1†], Xing Jiang[2], Jianchang Xu[1], Yumeng Sun[1], Zichen Wang[2], Ling Lin[1], Yanbin Niu[2], Shiqi Song[1], Huawei Zhang[2], Zhenpeng Xue[1], Jianping Lu[1] and Paul Yao[1]**

[1] Department of Child Psychiatry, Kangning Hospital of Shenzhen, Shenzhen Mental Health Center, Shenzhen, China, [2] Department of Biomedical Engineering, Southern University of Science and Technology, Shenzhen, China

OPEN ACCESS

Edited by:
Marco Turi,
Fondazione Stella Maris Mediterraneo Onlus, Italy

Reviewed by:
William Kenkel,
University of Delaware, United States
Yi-Rong Peng,
University of California, Los Angeles, United States

***Correspondence:**
Jianping Lu
szlujianping@126.com
Paul Yao
vasilis112@yahoo.com

[†] These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: 28 November 2020
Accepted: 18 January 2021
Published: 09 February 2021

Citation:
Liu J, Liang Y, Jiang X, Xu J, Sun Y, Wang Z, Lin L, Niu Y, Song S, Zhang H, Xue Z, Lu J and Yao P (2021) Maternal Diabetes-Induced Suppression of Oxytocin Receptor Contributes to Social Deficits in Offspring. Front. Neurosci. 15:634781. doi: 10.3389/fnins.2021.634781

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders characterized by impaired skills in social interaction and communication in addition to restricted and repetitive behaviors. Many different factors may contribute to ASD development; in particular, oxytocin receptor (OXTR) deficiency has been reported to be associated with ASD, although the detailed mechanism has remained largely unknown. Epidemiological study has shown that maternal diabetes is associated with ASD development. In this study, we aim to investigate the potential role of OXTR on maternal diabetes-mediated social deficits in offspring. Our *in vitro* study of human neuron progenitor cells showed that hyperglycemia induces OXTR suppression and that this suppression remains during subsequent normoglycemia. Further investigation showed that OXTR suppression is due to hyperglycemia-induced persistent oxidative stress and epigenetic methylation in addition to the subsequent dissociation of estrogen receptor β (ERβ) from the OXTR promoter. Furthermore, our *in vivo* mouse study showed that maternal diabetes induces OXTR suppression; prenatal OXTR deficiency mimics and potentiates maternal diabetes-mediated anxiety-like behaviors, while there is less of an effect on autism-like behaviors. Additionally, postnatal infusion of OXTR partly, while infusion of ERβ completely, reverses maternal diabetes-induced social deficits. We conclude that OXTR may be an important factor for ASD development and that maternal diabetes-induced suppression of oxytocin receptor contributes to social deficits in offspring.

Keywords: autism spectrum disorders, maternal diabetes, oxidative stress, oxytocin receptor, social deficit

Abbreviations: ALB, autism-like behavior; ASD, autism spectrum disorders; ChIP, chromatin immunoprecipitation; ERE, estrogen response element; ERβ, estrogen receptor β; $O_2.^-$, superoxide anions; ROS, reactive oxygen species; OXT, oxytocin; OXTR, oxytocin receptor; PVN, paraventricular nuclei; SOD2, superoxide dismutase 2; STZ, streptozocin.

INTRODUCTION

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders characterized by deficits in social interaction and communication in addition to restricted and repetitive behaviors (Rossignol and Frye, 2012; Baron-Cohen et al., 2019). Many factors, including genetics/epigenetics, sex and environmental factors have been reported to be associated with ASD development (Rossignol and Frye, 2012; Bralten et al., 2018). We have previously reported that prenatal hormone exposure (Zou et al., 2017; Li et al., 2018; Xie et al., 2018; Xiang et al., 2020) and maternal diabetes (Xiang et al., 2018; Wang et al., 2019) contribute to ASD development, although the detailed mechanism for the etiology of ASD remains largely unknown and various other factors may still need to be investigated.

Oxytocin is a central nervous neuropeptide that is involved in a variety of physiological processes (Marotta et al., 2020) and is mainly synthesized in neurons of the PVN and supraoptic nuclei (SON) in the hypothalamus (Tang et al., 2020). OXTR is widely expressed in human tissues, with particularly high levels being located in limbic brain regions (Kudwa et al., 2014). In conjunction with OXT, OXTR has been reported to regulate diverse social behaviors (Maejima et al., 2018; Gulliver et al., 2019; Resendez et al., 2020; Soltys et al., 2020) and play a role in ASD etiology (Jacob et al., 2007; LoParo and Waldman, 2015; Uzefovsky et al., 2019), although there has been some controversy with these conclusions (Tansey et al., 2010). Epigenetic modification of OXTR has been widely reported to be associated with ASD development (Jack et al., 2012; Maud et al., 2018; Krol et al., 2019; Tops et al., 2019), although the detailed mechanism remains unclear.

Estrogen receptor β (ERβ), together with estrogen receptor α (ERα), is widely expressed in various areas of the brain (Bodo and Rissman, 2006; Phan et al., 2015), and ERβ specifically has been reported to be associated with ASD development and anxiety-related behaviors (Krezel et al., 2001; Crider et al., 2014; Zou et al., 2017). Additionally, ERβ is responsible for the basal expression of superoxide dismutase 2 (SOD2) and estrogen-related receptor α (ERRα) through ERE, subsequently regulating oxidative stress and mitochondria function (Li et al., 2015; Kong et al., 2016). ERβ is colocalized within the PVN and highly expressed in OXT-containing neurons located in hypothalamic regions. Both OXT (Acevedo-Rodriguez et al., 2015) and OXTR (Murata et al., 2014) have been reported to be regulated by ERβ either directly or indirectly; and our recent work showed that maternal diabetes suppresses ERβ expression in brain (Wang et al., 2019), thus, ERβ may play a role in modulating maternal diabetes-mediated social behaviors (Clipperton-Allen et al., 2012; Kudwa et al., 2014).

In this study, we aim to investigate the potential role of OXTR on maternal diabetes-mediated social deficits. Our *in vitro* study in human neuron progenitor cells showed that OXTR expression was suppressed by transient high glucose levels and remained low during subsequent normoglycemia through hyperglycemia-mediated consistent oxidative stress. Further investigation found that OXTR suppression is due to hyperglycemia-mediated epigenetic changes on the OXTR promoter and subsequent dissociation of ERβ from the OXTR promoter. *In vivo* mouse study showed that prenatal OXTR deficiency potentiates maternal diabetes-mediated anxiety-like behavior, while it has little effect on ALB. In addition, postnatal infusion of OXTR reversed maternal diabetes-mediated anxiety-like behavior, while it had little effect on ALB; on the other hand, postnatal infusion of ERβ completely reversed maternal diabetes-mediated social deficits. We conclude that maternal diabetes-induced suppression of oxytocin receptor contributes to social deficits in offspring.

MATERIALS AND METHODS

A detailed description can be found in **Supplementary Data 1**, and the related primers used in this study were shown in **Supplementary Table 1**.

Reagents and Materials

Human neural progenitor cells (NPC, #ACS-5003) were obtained from ATCC and were cultured in NPC medium as described previously (Wang et al., 2019). The mouse primary amygdala neurons were isolated and cultured in DMEM medium plus 10% fetal bovine serum (FBS), 10% heat-inactivated defined horse serum, 20 mM D-glucose and 100 U/ml Pen/Strep (from Invitrogen). All cells were maintained in a humidified incubator with 5% CO_2 at 37°C. In some experiments, the cells were conditionally immortalized using a hTERT lentivirus vector with an extended life span to achieve higher transfection efficiency and experimental stability (Bodnar et al., 1998; Kong et al., 2016).

The antibodies for β-actin (sc-47778), C/EBPα (sc-365318), GATA1 (sc-266), SOD2 (sc-30080), Sp1 (sc-17824) and YY1 (sc-7341) were obtained from Santa Cruz Biotechnology. Antibodies for OXTR (#BS-1314R) was purchased from Fisher; OXT (#AB911) was purchased from Sigma; 8-oxo-dG (4354-MC-050) was purchased from Novus Biologicals; NeuN (#24307) was purchased from Cell Signaling. Antibodies for acetyl-histone H4 K5, K8, K12, and K16 (H4K5,8,12,16ac, #PA5-40084) were obtained from Invitrogen. Antibodies for ERα (ab3575), ERβ (ab3576), anti-histone H3 acetyl K9, K14, K18, K23, K27(H3K9,14,18,23,27ac, ab47915), H4K20me1 (ab9051), H4K20me3 (ab9053), H4R3me1 (ab17339), H3K9me2 (ab1220), H3K9me3 (ab8898), H3K27me2 (ab24684), and H3K27me3 (ab6002) were obtained from Abcam. 3-nitrotyrosine (3-NT) was measured using the 3-Nitrotyrosine ELISA Kit (ab116691 from Abcam) per manufacturers' instructions. The mitochondrial fraction was isolated using a Pierce Mitochondria Isolation Kit (Pierce Biotechnology) per manufacturers' instructions. Protein concentration was measured using the Coomassie Protein Assay Kit (Pierce Biotechnology). Luciferase activity assay was carried out using the Dual-Luciferase™ Assay System (Promega) and the transfection efficiency was normalized using a cotransfected renilla plasmid

(Zhang et al., 2017). Streptozocin (STZ, #18883-66-4) were obtained from Sigma.

Construction of OXT/OXTR Reporter Plasmid

Human genomic DNA was prepared from NPC cells. In order to construct OXT/OXTR reporter plasmids, the gene promoter (2 kb upstream of the transcription start site plus first exon) was amplified from Ensembl gene ID: OXT-201 ENST00000217386.2 (for OXT) and OXTR-201 ENST00000316793.7 (for OXTR) by PCR and subcloned into the pGL3-basic vector (# E1751, Promega) using underlined restriction sites with the following primers: OXT forward: 5′-gcgc-acgcgt- ttg gat gcg ggc cac ctg gga -3′ (*Mlu*I) and OXT reverse: 5′- gtac- aagctt- ctt gcg cac gtc gag gtc cgg -3′ (*Hin*dIII); OXTR forward: 5′-gcgc- ggtacc - tgg aac ttt gag gat ttt ttt -3′ (*Kpn*I) and OXTR reverse: 5′- gtac- aagctt - ctg cac cga gtc cgc agg cga -3′ (*Hin*dIII). To map OXTR promoter activity, the related deletion promoter constructs were generated by PCR methods and subcloned into the pGL3-basic vector. All the vectors were verified by sequencing, and detailed information on these plasmids is available upon request (Zhang et al., 2017).

Generation of Expression Lentivirus

The lentivirus for human ERβ and SOD2 was prepared as described previously in our lab (Wang et al., 2019). The cDNA for mouse ERβ and OXTR was obtained from Open Biosystems and subcloned into the pLVX-Puro vector (from Clontech) using underlined restriction sites with the following primers: mouse ERβ forward primer: 5′- gtac- ctcgag- atg tcc atc tgt gcc tct tct -3′ (Xho1) and mouse ERβ reverse primer: 5′- gtac- tctaga- tca ctg tga ctg gag gtt ctg -3′ (Xba1); mouse OXTR forward primer: 5′- gtac - gaattc- atg gag ggc acg ccc gca gcc -3′ (EcoR1) and mouse OXTR reverse primer: 5′- gtac - tctaga- tca tgc cga gga tgg ttg aga -3′ (Xba1). The lentivirus for ERβ, OXTR, or empty control (CTL) was expressed through Lenti-X^{TM} Lentiviral Expression Systems (from Clontech) per manufacturers' instructions (Wang et al., 2019).

Gene Knockdown by shRNA Lentivirus Particles

The shRNA lentivirus particles for human ERβ and SOD2 were prepared as described previously in our lab (Wang et al., 2019). The shRNA lentivirus plasmids for human SOD2 (sc-41655-SH), ERβ (sc-35325-SH) or non-target control (sc-108060) were purchased from Santa Cruz Biotechnology, and the related lentivirus for either ERβ and SOD2 or empty control (CTL) were expressed through Lenti-X^{TM} Lentiviral Expression Systems (from Clontech) per manufacturers' instructions. The purified and condensed lentivirus were used for *in vitro* gene knockdown. The knockdown efficiency was confirmed by more than 65% of mRNA reduction compared to the control group in cells using real time PCR (see **Supplementary Table 1**).

In vivo Mouse Experiments

The animal protocol conformed to US NIH guidelines (Guide for the Care and Use of Laboratory Animals, No. 85-23, revised 1996), and was reviewed and approved by the Institutional Animal Care and Use Committee from Kangning Hospital of Shenzhen. All the experimental mice were either OXTR wild type (WT) or OXTR null (OXTR$^{-/-}$) mice with a C57BL/6J mixed genetic background (a kind gift from Dr. Haimou Zhang from Hubei University, China). In the generation of diabetic mice, adult (3-month-old) female mice with either WT or OXTR$^{-/-}$ backgrounds were monitored for estrous cycles with daily vaginal smears. Only mice with at least two regular 4- to 5-day estrous cycles were included in the studies. Chronic diabetic female mice were induced by injection of 35 mg/kg streptozocin (STZ, 0.05 M sodium citrate, pH 5.5) after an 8-h fasting period. Animals with blood glucose >250 mg/dl were considered positive with the success rate of ∼90%, while control (CTL) mice received only vehicle injection (Williams et al., 2017).

Mouse Protocol 1 for Prenatal Treatment of Diabetes or OXTR Deficiency

Verified pregnant dams were randomly assigned to the following four groups: Group 1: CTL group mice with OXTR WT background (CTL/WT); Group 2: STZ mice with OXTR WT background (STZ/WT); Group 3: CTL group mice with OXTR null background (CTL/OXTR$^{-/-}$); Group 4: STZ mice with OXTR null background (STZ/OXTR$^{-/-}$). Neurons from the amygdala were isolated on embryonic day 18 (E18) as described below. The male offspring were separated from the dams on day 21 and fed with normal chow until 7–8 weeks old for behavior tests. Then, the offspring were sacrificed and various brain tissues, including the amygdala, hypothalamus and hippocampus, were isolated, flash frozen in dry ice, and then stored in a −80°C freezer for analysis of gene expression and oxidative stress.

Mouse Protocol 2 for Postnatal Manipulation of OXTR/ERβ Expression

The male offspring (6 weeks old) from either the CTL or STZ group in Mouse Protocol 1 were anesthetized with a mixture of ketamine (90 mg/kg) and xylazine (2.7 mg/kg) and implanted with a guide cannula targeting the amygdala (26 gauge; Plastics One) (Neal-Perry et al., 2014). The following stereotaxic coordinates from the bregma were used for the amygdala: anteroposterior (AP) = −1.4, mediolateral (ML) = ±3.5, dorsoventral (DV) = −5.1. Dorsoventral coordinates, which were based on the mouse brain atlas (Heldt and Ressler, 2006), were measured from the skull surface with the internal cannula extending 2 mm beyond the end of the guide cannula. The cannula was attached to the skull with dental acrylic and jeweler's screws and closed with an obturator (Hu et al., 2015). An osmotic minipump (Alzet model 2002; flow rate 0.5 μl/h; Cupertino, CA, United States) connected to a 26-gauge internal cannula that extended 1 mm below the guide was implanted and used to deliver ORTR overexpression (↑OXTR), ERβ overexpression (↑ERβ), or vehicle (VEH) lentivirus. Vehicle consisting of artificial cerebrospinal fluid (aCSF; 140 mM NaCl, 3 mM KCl, 1.2 mM Na2HPO4, 1 mM MgCl2, 0.27 mM NaH2PO4, 1.2 mMCaCl2, and 7.2 mM dextrose, pH 7.4) was used for the infusion of the lentivirus. Infusion (flow rate 0.5 μl/h) begun immediately after placement of the minipump. 0.5 μl of total

2×10^3 cfu of lentivirus was infused for 1 h. The experimental mice were separated into four groups, with 10 in each group. Group 1: CTL offspring with vehicle control lentivirus infusion (CTL/P-VEH); Group 2: STZ offspring with vehicle control lentivirus infusion (STZ/P-VEH); Group 3: STZ offspring with OXTR expression lentivirus infusion (STZ/P-↑OXTR); Group 4: STZ offspring with ERβ expression lentivirus infusion (STZ/P-↑ERβ). Cannula placement was verified histologically postmortem by the injection of 0.5 μl of India ink (volume matching that of drug delivery in the experiments). Mice whose dye injections were not located in the amygdala were excluded from the data analysis. Two weeks after lentivirus infusion, the offspring were used for behavior tests followed by biomedical analysis, as indicated in Mouse Protocol 1 (Zou et al., 2017).

DNA Methylation Analysis

We developed a real-time PCR-based method for methylation-specific PCR (MSP) analysis on the human OXTR promoter according to the previously described method with some modifications (Eads et al., 2000; Ogino et al., 2006; Nosho et al., 2008). The genomic DNA from human #ACS-5003 cells was extracted and purified before then being treated by bisulfite modification using the EpiJET Bisulfite Conversion Kit (#K1461, Fisher). The modified DNA was then amplified using methylated and unmethylated primers for MSP that were designed using the Methprimer software[1] with the below details: Methylated primer: forward 5′- ttt gag ttt att gtt aaa gtc gt -3′, reverse 5′- aaa taa taa tat tct tcc ccg aa -3′; Unmethylated primer: forward 5′- ttt gag ttt att gtt aaa gtt gt -3′; reverse 5′- aaa taa taa tat tct tcc cca aa -3′. Product size: 147 bp (methylated) and 147 bp (unmethylated); CpG island size: 134 bp; Tm: 64.2°C. The final methylation readout was normalized by unmethylated input PCR (Zou et al., 2017).

Animal Behavior Test

The animal behavior test of offspring was carried out at 7–8 weeks of age. Anxiety-like behavior was evaluated using the marbles burying tests (MBT) and the elevated plus maze (EPM) tests (Zou et al., 2017; Xie et al., 2018). ALB was evaluated using ultrasonic vocalization (USV), social interaction (SI) tests and a three-chambered social test as described below (Moy et al., 2004; Silverman et al., 2010; Schaafsma et al., 2017).

Statistical Analysis

The data was given as mean ± SEM and all the experiments were performed at least in quadruplicate unless indicated otherwise. The one-way analysis of variance (ANOVA) followed by the Turkey–Kramer test was used to determine statistical significance of different groups, and the two-way ANOVA followed by the Bonferroni *post hoc* test was used to determine the differences of two factors (e.g., OXTR deficiency and maternal diabetes) using SPSS 22 software, and a P value of <0.05 was considered significant (Li et al., 2019; Zhou et al., 2019).

[1]http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi

RESULTS

Transient High Glucose Causes Persistent OXTR Suppression During Subsequent Normoglycemia Through Hyperglycemia-Mediated Consistent Oxidative Stress

We first evaluated the potential effect of glucose memory on the gene expression of OXTR and OXT. Human ACS-5003 neurons were first treated by high glucose (25 mM HG) for 4 days before remaining in low glucose levels (5 mM LG) for another 4 days. The results showed that 4-day high glucose treatment significantly suppressed the gene expression of both OXTR (see **Figure 1A**) and OXT (see **Figure 1B**); when the cells switched into low glucose, OXTR expression remained low, while OXT expression returned to normal; SOD2 expression (↑SOD2) on day 5 completely reversed the HG-mediated effect; and SOD2 knockdown (shSOD2) on day 5 mimicked the HG-mediated effect. Furthermore, the mRNA levels for OXTR and OXT on day 8 were presented in **Figure 1C** in addition to mRNA levels of SOD2, indicating that SOD2 mRNA expression was suppressed in HG and remained low during subsequent LG. In addition, the manipulation of SOD2 using lentivirus was successful; SOD2 expression lentivirus (↑SOD2) significantly increased, while SOD2 knockdown lentivirus (shSOD2) significantly decreased, SOD2 mRNA levels (see **Figure 1C**). We also measured the protein levels for SOD2 and OXTR, and an expression pattern similar to that of the mRNA was observed (see **Figures 1D,E** and **Supplementary Figure 1a**). On the other hand, we could not detect the presence of OXT proteins by western blotting, indicating that OXT protein is not expressed in ACS-5003 neurons. We then measured the SOD2 activity, and the results showed a pattern similar to that of SOD2 mRNA (see **Figure 1F**). Finally, we evaluated oxidative stress, and the results showed that ROS formation significantly increased in the HG(4d) + LG(4d)/CTL group (see **Figure 1G**). 3-nitrotyrosine formation (see **Figure 1H**) also increased compared to the LG(4d) + LG(4d)/CTL group, and SOD2 expression HG(4d) + LG(4d)/↑SOD2 completely reversed, while SOD2 knockdown LG(4d) + LG(4d)/shSOD2 mimicked, the high glucose-mediated effect. Our results indicate that transient high glucose causes persistent OXTR suppression during subsequent normoglycemia through hyperglycemia-mediated consistent oxidative stress.

Hyperglycemia Induces OXTR Suppression Through Epigenetic Modification and the Subsequent Dissociation of ERβ From the OXTR Promoter

We investigated the possible molecular mechanism for hyperglycemia-mediated OXTR suppression. A series of progressive 5′-promoter deletion constructs for the OXTR promoter were generated, and these constructs were transfected into conditional immortalized neurons for the analysis of OXTR

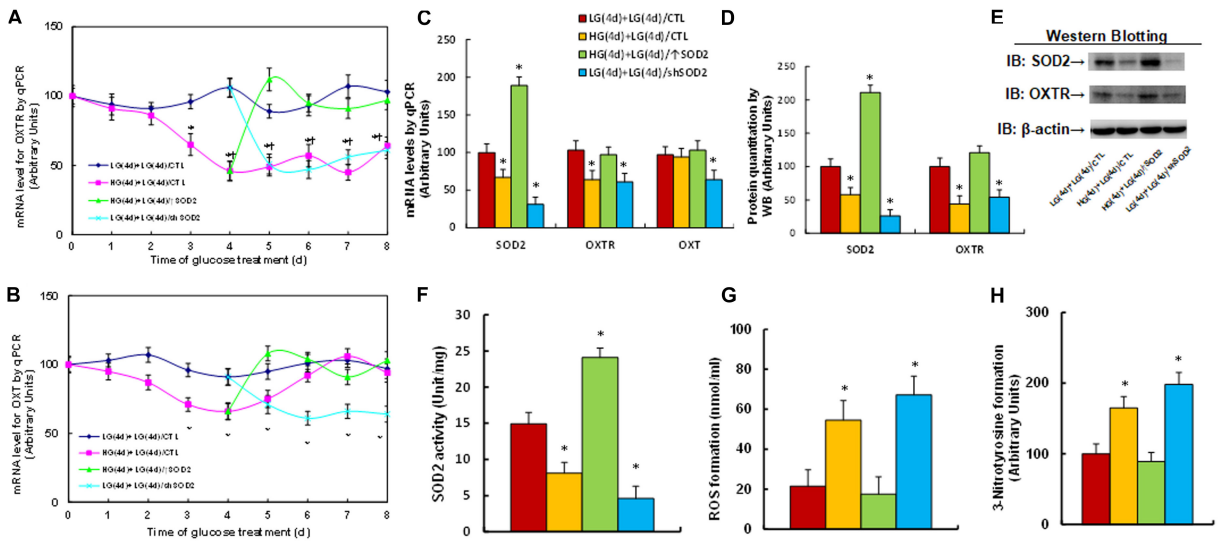

FIGURE 1 | Transient high glucose causes persistent OXTR suppression during subsequent normoglycemia through hyperglycemia-mediated consistent oxidative stress. Human ACS-5003 neurons were treated with either 5 mM low glucose (LG) or 25 mM high glucose (HG) for 4 days. The cells were then infected by empty (CTL), SOD2 overexpression (↑SOD2), or SOD2 knockdown (shSOD2) lentivirus for 1 day before they were then treated by LG for another 4 days in the presence of 1% serum; the cells were then harvested for further analysis. **(A,B)** Cells were harvested at different time points for analysis of mRNA levels. **(A)** OXTR levels; **(B)** OXT levels; $n = 4$, $^*P < 0.05$, vs. day 0 group; ¶ $P < 0.05$, vs. day 3 group. **(C–H)** Cell were harvested on day 8 for biomedical analysis. **(C)** mRNA levels, $n = 4$. **(D)** Quantitation of protein levels, $n = 5$. **(E)** Representative western blotting pictures for **(D)**. **(F)** SOD2 activity, $n = 5$. **(G)** ROS formation, $n = 5$. **(H)** 3-nitrotyrosine formation, $n = 5$. $^*P < 0.05$, vs. LG(4d) + LG(4d)/CTL group. Data were expressed as mean ± SEM.

reporter activity in the presence of either 5 mM LG or 25 mM HG for 24 h. We found that hyperglycemia-induced OXTR reporter suppression occurred among the −2000, −1600, −1400, −1200, −1100, −800, −400 and −200 deletion constructs (numbered according to Ensembl gene ID: OXTR-201 ENST00000316793.7; transcription start site was marked as 0), while suppression was significantly restored in the −1000, and −900 deletion reporter constructs, indicating that hyperglycemia-responsive transcriptional element is located in the range of −1100∼−900 on the OXTR promoter (see **Figure 2A**). The transcription factor database revealed many potential binding motifs, including one of the GATA1, Sp1 and YY1 and two of the C/EBPα and ERE (marked in red) binding sites located in the range of −1100∼−900 on the OXTR promoter (see **Figure 2B**). We then mutated these potential binding motifs in the OXTR full length (pOXTR-2000) reporter construct, and the reporter assay showed that hyperglycemia-induced reporter activation disappeared in two of the ERE mutation constructs (located at −1005 and −944, respectively, marked in green, see **Figure 2B**), indicating that hyperglycemia mediates OXTR suppression through the ERE binding motif on the OXTR promoter (see **Figure 2C**). We then made both single and double mutations on both of the ERE binding sites (located at −1005 and −944) in the pOXTR full length construct, and the reporter assay showed that ERE single mutants (M-1005/ERE, M-944/ERE) significantly decreased OXTR reporter activity in the LG treatment group compared to the wild type full length (pOXTR-2000/LG), while ERE double mutants (M-1005/-944/ERE) further decreased reporter activity, mimicking the reporter activity of the full length reporter construct (pOXTR-2000) in the HG treatment (see **Figure 2D**). Our results indicate that hyperglycemia induces OXTR suppression through decreased association of ERE on the OXTR promoter. We then conducted DNA methylation analysis on the OXTR promoter, and the results showed that there was no significant difference across the treatments (see **Supplementary Figure 2**). We then conducted ChIP analysis using antibodies for transcription factors GATA1, ERα, ERβ, C/EBPα, YY1 and Sp1 as indicated in **Figure 2B**. The results showed that the binding ability of ERβ on the OXTR promoter was significantly decreased in the HG(4d) + LG(4d)/CTL group compared to the LG(4d) + LG(4d)/CTL group, and this effect was completely restored by infection of SOD2 in HG(4d) + LG(4d)/↑SOD2 group; on the other hand, other transcription factors, including ERα, showed no significant difference (see **Figure 2E**), indicating that ERβ is responsible for hyperglycemia-induced OXTR suppression. We then evaluated the epigenetic changes in the range of −1100∼−900 on the OXTR promoter. We first evaluated the effect of hyperglycemia on histone H3 methylation. The results showed that hyperglycemia treatment had no effect on the methylation of H3K9me2 and H3K9me3, while methylation of H3K27me2 and H3K27me3 displayed a significant increase as a result of HG(4d) + LG(4d)/CTL treatment compared to the LG(4d) + LG(4d)/CTL treatment. On the other hand, infection of SOD2 in HG(4d) + LG(4d)/↑SOD2 treatment completely restored this effect (see **Figure 2F**). We also evaluated histone H4 methylation on the OXTR promoter (see **Supplementary Figure 3a**) and found that hyperglycemia did not have any effect on histone H4 methylation. We then evaluated histone acetylation on the OXTR promoter using the acetyl-histone H4 (K5, K8, K12, K16) antibody that

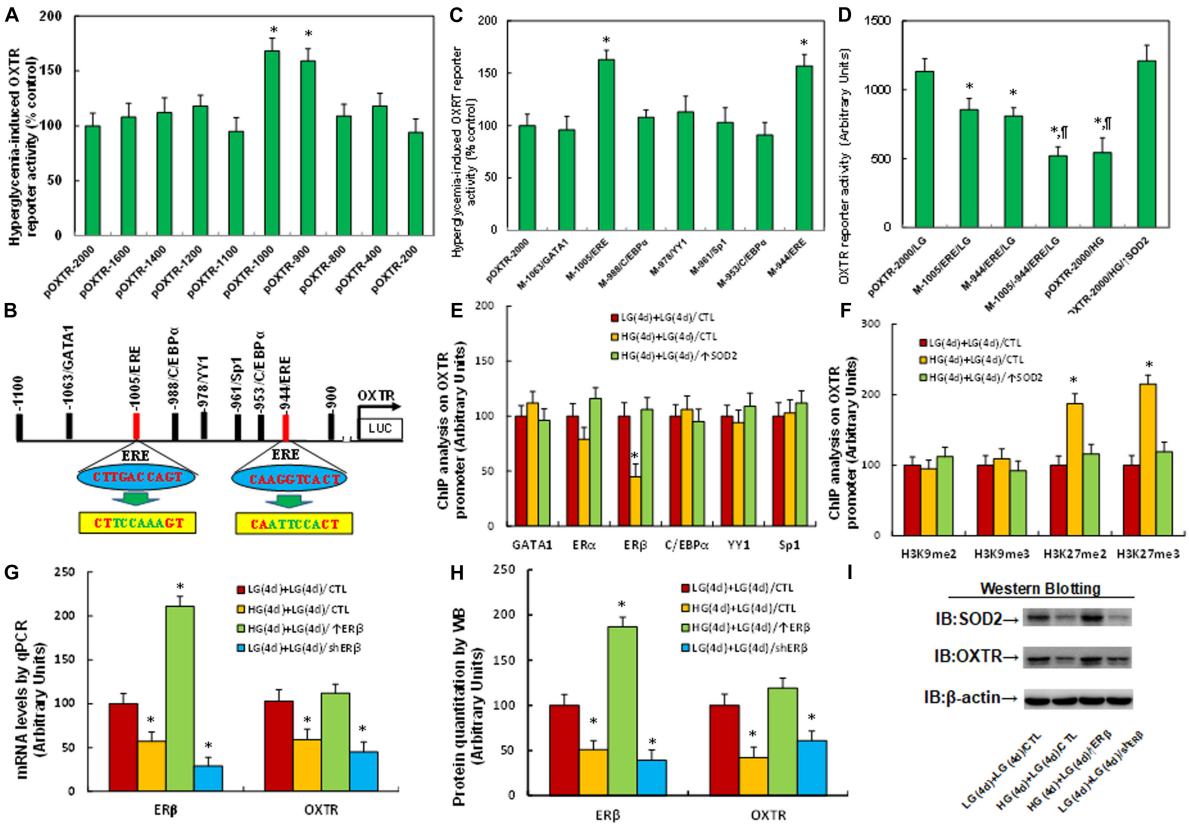

FIGURE 2 | Hyperglycemia induces OXTR suppression through epigenetic modification and the subsequent dissociation of ERβ from the OXTR promoter. **(A)** The conditional immortalized ACS-5003 neurons were transiently transfected with either OXTR full length (pOXTR-2000) or deletion reporter plasmids. After 24 h, the cells were treated with either 5 mM low glucose (LG) or 25 mM high glucose (HG) for 3 days and the OXTR reporter activities were calculated, $n = 5$. $^*P < 0.05$, vs. pOXTR-2000 group. **(B)** The schematic picture for the potential transcriptional binding motif in the range of –900∼1100 (from transcription start site) on the OXTR promoter with two potential ERE binding sites marked in red as well as related mutation sites marked in green. **(C)** The cells were transiently transfected by either a wild type OXTR reporter construct (pOXTR-2000) or single point mutation at the site shown in panel **(B)**, and then treated with either LG or HG for 3 days, and the OXTR reporter activities were calculated, $n = 5$. $^*P < 0.05$, vs. pOXTR-2000 group. **(D)** The cells were transiently transfected by OXTR full length (pOXTR-2000), single mutant, or double mutations as indicated, or infected by SOD2 lentivirus (↑SOD2), and then treated with either LG or HG for 3 days; the OXTR reporter activities were then calculated, $n = 5$. $^*P < 0.05$, vs. pOXTR-2000/LG group; ¶ $P < 0.05$, vs. M-1005/ERE/LG group. **(E,F)** Cells were treated by either 4-day LG plus 4-day LG [LG(4d) + LG(4d)], or 4-day HG plus 4-day LG [HG(4d) + LG(4d)], or the cells were infected on day 4 by SOD2 lentivirus [HG(4d) + LG(4d)/↑SOD2]; the cells were then used for ChIP analysis: **(E)** ChIP analysis by potential transcription factors on OXTR promoter, $n = 4$; **(F)** ChIP analysis by potential histone methylation, $n = 4$. $^*P < 0.05$, vs. LG(4d) + LG(4d)/CTL group. **(G–I)** Cells were treated by either LG(4d) + LG(4d)/CTL or HG(4d) + LG(4d)/CTL, or the cells were infected on day 4 by either ERβ expression lentivirus [HG(4d) + LG(4d)/↑ERβ] or ERβ lentivirus knockdown [LG(4d) + LG(4d)/shERβ]; the cells were then harvested for biomedical analysis: **(G)** mRNA analysis, $n = 4$. **(H)** Protein quantitation, $n = 5$. **(I)** Representative western blotting pictures for **(H)**. $^*P < 0.05$, vs. LG(4d) + LG(4d)/CTL group. Data were expressed as mean ± SEM.

recognizes histone H4 acetylated at lysines 5, 8, 12, or 16 and the acetyl-histone H3 (K9, K14, K18, K23, K27) antibody that recognizes histone H3 acetylated at lysines 9, 14, 18, 23 or 27 by ChIP analysis, and the results showed that there was no significant difference in either histone H3 or H4 acetylation (see **Supplementary Figure 3b**). We proceeded to evaluate the potential effect of ERβ on OXTR expression. The cells were infected by either ERβ expression lentivirus after HG exposure [HG(4d) + LG(4d)/↑ERβ] or ERβ knockdown lentivirus after LG exposure [LG(4d) + LG(4d)/shERβ]. The results showed that ERβ lentivirus manipulation was successful and that ERβ expression completely reversed, while ERβ knockdown mimicked hyperglycemia [HG(4d) + LG(4d)/CTL group]-induced OXTR suppression, compared to the LG(4d) + LG(4d)/CTL control group (see **Figures 2G–I** and **Supplementary Figure 1b**). Our results indicate that hyperglycemia induces OXTR suppression through epigenetic modification and the subsequent dissociation of ERβ from the OXTR promoter.

Prenatal OXTR Deficiency Potentiates Maternal Diabetes-Mediated Oxidative Stress

We evaluated the potential effect of OXTR deficiency on maternal diabetes-mediated oxidative stress. The OXTR null ($OXTR^{-/-}$) mice were used to generate diabetic dams through streptozocin (STZ) injection, and the brain tissues, including the amygdala, hypothalamus and hippocampus, were isolated

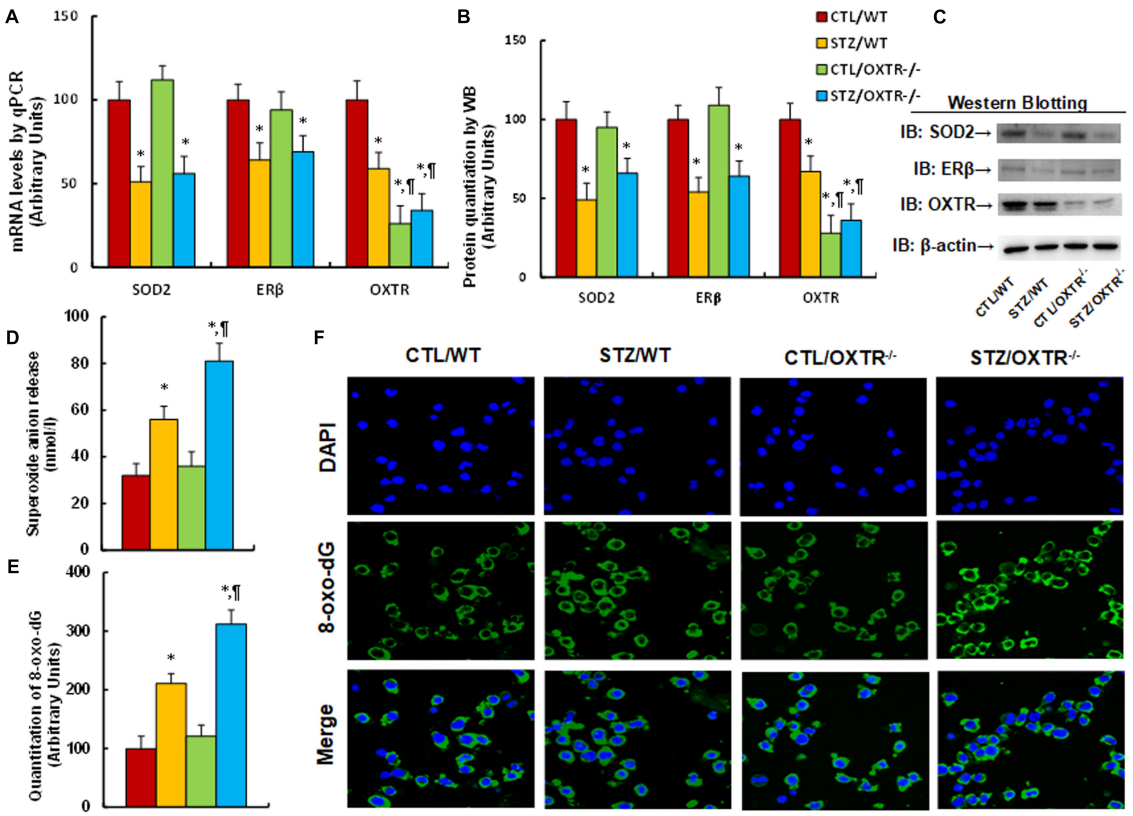

FIGURE 3 | Prenatal OXTR deficiency potentiates maternal diabetes-mediated oxidative stress. The OXTR wild type (WT) or OXTR null ($OXTR^{-/-}$) backgrounds were used to generate either control (CTL) or STZ-induced diabetic (STZ) pregnant dams, and the amygdala neurons or tissues from subsequent male offspring were isolated for further analysis. **(A–D)** The amygdala tissues were isolated from 7- to 8-week-old male offspring for analysis. **(A)** The mRNA levels by qPCR, $n = 4$. **(B)** The quantitation of protein levels, $n = 5$. **(C)** The representative pictures for western blotting for **(B)**. **(D)** *In vivo* superoxide anion release, $n = 5$. **(E,F)** The amygdala neurons were isolated on embryonic day (E18) from the above treatment for immunostaining. **(E)** Quantitation of 8-oxox-dG staining, $n = 5$. **(F)** Representative pictures for 8-oxo-dG staining (green) and DAPI staining for nuclei (blue). Two-way ANOVA was used for the statistical analysis, and each group contained nine mice. $^*P < 0.05$, vs. CTL/WT group; ¶ $P < 0.05$, vs. STZ/WT group. Data were expressed as mean ± SEM.

from subsequent male offspring for further analysis. We first measured the gene expression in amygdala tissues. The results showed that gene expression of SOD2, ERβ and OXTR were significantly decreased in the maternal diabetes (STZ/WT) group compared to the control (CTL/WT) group; OXTR knockout ($OXTR^{-/-}$) mice significantly decreased OXTR expression, but showed no effect on the expression of SOD2 and ERβ in either the control ($CTL/OXTR^{-/-}$) or diabetic ($STZ/OXTR^{-/-}$) groups (see **Figures 3A–C** and **Supplementary Figure 1c**). We then evaluated mRNA expression for those genes from the hypothalamus (see **Supplementary Figure 4a**) and hippocampus (see **Supplementary Figure 4b**). The results showed that the maternal diabetic (STZ/WT) group displayed significantly decreased OXTR expression levels compared to the control (CTL/WT) group, while there was no effect on the expression of SOD2 and ERβ; furthermore, OXTR expression was successfully decreased in OXTR knockout ($OXTR^{-/-}$) mice, but there was no effect on the expression of SOD2 and ERβ. In addition, we measured OXT mRNA levels from the amygdala, hypothalamus and hippocampus, and the results showed that there was no significant difference in OXT expression across any of the treatments (see **Supplementary Figure 4c**). Finally, we evaluated the oxidative stress in amygdala tissues from the mice, and the results showed that maternal diabetic (STZ/WT) group displayed significantly increased superoxide anion release (see **Figure 3D**) and 8-oxo-dG formation (see **Figures 3E,F**) compared to control (CTL/WT) group; there was no effect in OXTR knockout ($OXTR^{-/-}$) mice compared to the control ($CTL/OXTR^{-/-}$) group, but the OXTR knockout further potentiated maternal diabetes ($STZ/OXTR^{-/-}$) -mediated oxidative stress compared to STZ/WT group. Our results indicate that prenatal OXTR deficiency potentiates maternal diabetes-mediated oxidative stress.

Prenatal OXTR Deficiency Potentiates Maternal Diabetes-Mediated Anxiety-Like Behavior, While It Has Little Effect on Autism-Like Behavior in Offspring

We evaluated the potential effect of OXTR deficiency on maternal diabetes-mediated social deficits in male offspring.

We first evaluated anxiety-like behavior in these animals. The results showed that the maternal diabetic (STZ/WT) group buried significantly fewer marbles (see **Figure 4A**) and spent less time in the Open Arm while spending more time in the Closed Arm in EPM tests (see **Figure 4B**) compared to the control (CTL/WT) group. OXTR knockout mice displayed an effect mimicking that of the maternal diabetes group as compared to the control (CTL/OXTR$^{-/-}$) group, and interestingly, it further potentiated the maternal diabetes-mediated anxiety-like behavior in diabetic (STZ/OXTR$^{-/-}$) group compared to STZ/WT group. We then evaluated the effect of OXTR deficiency on ALBs. The results showed that maternal diabetic (STZ/WT) group had significantly fewer ultrasonic vocalizations compared to the control (CTL/WT) group. OXTR knockout mice slightly but significantly mimicked the effect of maternal diabetes in the control (CTL/OXTR$^{-/-}$) group, while there was no further effect in the diabetic (STZ/OXTR$^{-/-}$) group (see **Figure 4C**). In addition, our results showed that mice from the maternal diabetic (STZ/WT) group spent significantly less time in Sniffing, Mounting and interacting in Total, but not in Grooming their partner in the Social Interaction tests (see **Figure 4D**). Additionally, they spent significantly more time in the Empty side for sociability (see **Figure 4E**), and less time for social novelty (see **Figure 4F**) in three-chambered social tests, compared to the control (CTL/WT) group. However, there was no significant effect in the OXTR knockout (OXTR$^{-/-}$) group. Our results indicate that prenatal OXTR deficiency potentiates maternal diabetes-mediated anxiety-like behavior, while it has little effect on ALB in male offspring.

Increasing Postnatal Expression of ERβ Completely Reverses Maternal Diabetes-Induced Oxidative Stress in Offspring, While Expression of OXTR Has no Effect

We evaluated the effect of postnatal expression of ERβ and OXTR on maternal diabetes-mediated oxidative stress. The male offspring from diabetic dams received expression lentivirus infusion for either ERβ or OXTR in the amygdala, and then the brain tissues, including the amygdala, hypothalamus and hippocampus, were isolated for further analysis. We first measured the gene expression in amygdala tissues. The results showed that gene expression of SOD2, ERβ and OXTR was significantly decreased in the maternal diabetes (STZ/P-VEH) group compared to the control (CTL/P-VEH) group; increasing postnatal expression of OXTR (STZ/P-↑OXTR) had no effect on SOD2 and ERβ, while increasing postnatal expression of ERβ (STZ/P-↑ERβ) completely reversed maternal

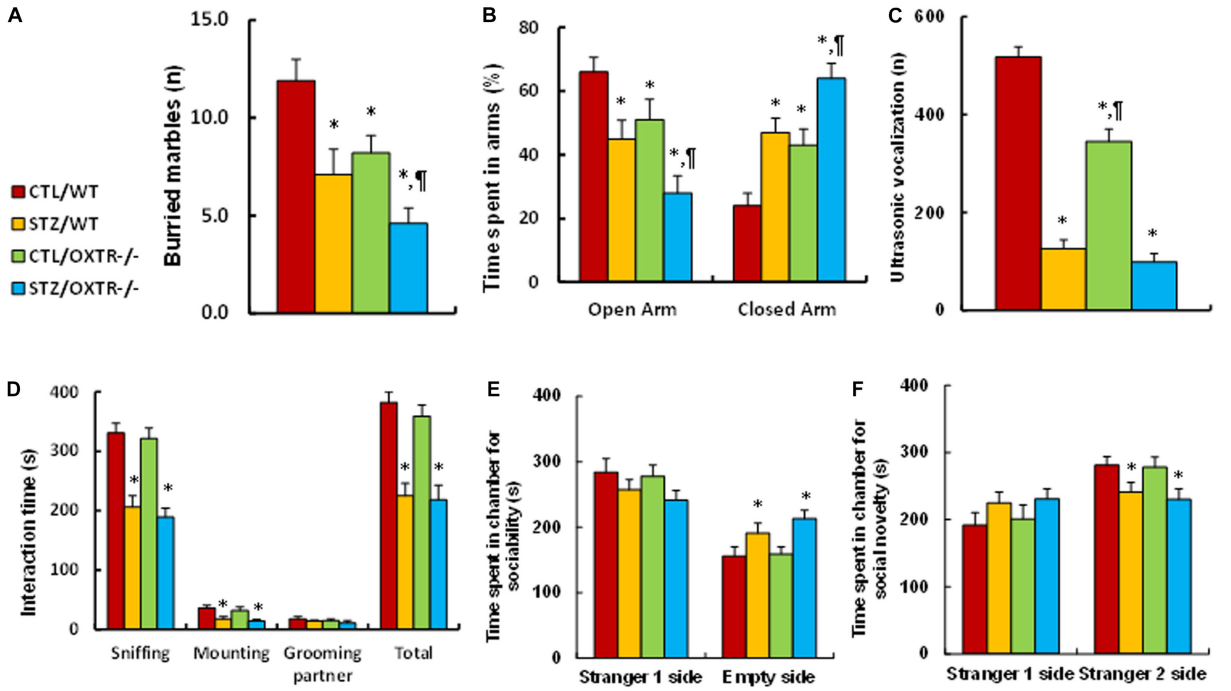

FIGURE 4 | Prenatal OXTR deficiency potentiates maternal diabetes-mediated anxiety-like behavior, while it has little effect on autism-like behavior in offspring. The OXTR wild type (WT) or OXTR null (OXTR$^{-/-}$) background were used to generate either control (CTL) or STZ-induced diabetic (STZ) pregnant dams, and the subsequent 7- to 8-week-old male offspring were used for animal behavior analysis. **(A)** Marbles burying tests (MBT), $n = 9$. **(B)** Time spent in Open Arm and Closed Arms in EPM test, $n = 9$. **(C)** Ultrasonic vocalization, $n = 9$. **(D)** Social interaction (SI) test, the time spent in following, mounting, grooming, and sniffing any body parts of the other mouse was calculated, $n = 9$. **(E,F)** Three-chambered social tests, $n = 9$. **(E)** Time spent in chamber for sociability. **(F)** Time spent in chamber for social novelty. Two-way ANOVA was used for the statistical analysis, and each group contained nine mice. *$P < 0.05$, vs. CTL/WT group; ¶ $P < 0.05$, vs. STZ/WT group. Data were expressed as mean ± SEM.

diabetes-mediated gene suppression of SOD2 and OXTR (see **Figures 5A–C** and **Supplementary Figure 1d**). We then evaluated mRNA expression for these genes in both the hypothalamus and hippocampus. The results showed that OXTR expression was significantly decreased in the maternal diabetic (STZ/P-VEH) group based on analysis from both the hypothalamus (see **Supplementary Figure 5a**) and hippocampus (see **Supplementary Figure 5b**) compared to the control (CTL/P-VEH) group, while there was no significant effect on the expression of SOD2 and ERβ; additionally, postnatal infusion of either OXTR (STZ/P-↑OXTR) or ERβ (STZ/P-↑ERβ) in the amygdala had no effect on gene expression. Furthermore, we measured OXT mRNA from the amygdala, hypothalamus and hippocampus; the results showed that there was no difference on OXT expression in both the amygdala and hippocampus across all treatments, while OXT expression was significantly decreased in the maternal diabetes (STZ/P-VEH) group compared to the control (CTL/P-VEH) group in the hypothalamus, and increasing postnatal expression of either OXTR or ERβ had no effect (see **Supplementary Figure 5c**). Finally, we evaluated oxidative stress in the mice. The results showed that mice from the maternal diabetic (STZ/P-VEH) group had significantly increased superoxide anion release (see **Figure 5D**) and 8-oxo-dG formation (see **Figure 5E**) compared to the control (CTL/P-VEH) group, and amygdala infusion of OXTR (STZ/P-↑OXTR) had no effect, while amygdala infusion of ERβ (STZ/P-↑ERβ) completely reversed the diabetes-mediated effect. Our results indicate that increasing postnatal expression of ERβ completely reverses maternal diabetes-induced oxidative stress in offspring, while expression of OXTR has no effect.

Increasing Postnatal Expression of OXTR Reverses Maternal Diabetes-Induced Anxiety-Like Behavior and Has Little Effect on Autism-Like Behavior, While Expression of ERβ Completely Reverses Maternal-Diabetes-Induced Social Deficits in Offspring

We evaluated the effect of postnatal expression of ERβ and OXTR on maternal diabetes-mediated social deficits in male

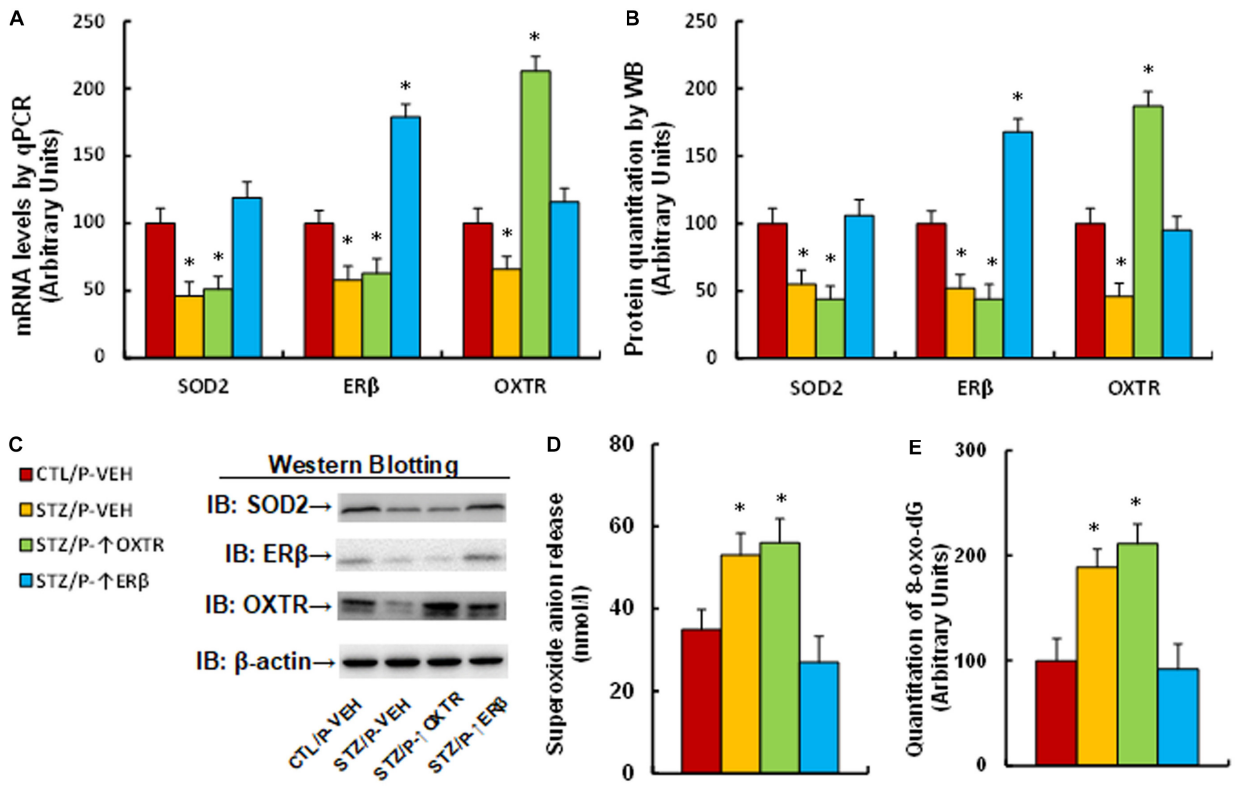

FIGURE 5 | Postnatal expression of ERβ completely reverses maternal diabetes-induced oxidative stress in offspring, while expression of OXTR has no effect. The male offspring from either control (CTL) or maternal diabetes (STZ) groups received either vehicle (P-VEH), or lentivirus infusion for expression of either OXTR (P-↑OXTR) or (P-↑ERβ) at 6 weeks old, and the male offspring were sacrificed for further biomedical analysis at 8 weeks old. **(A–D)** The amygdala tissues were isolated for further analysis as below: **(A)** mRNA levels by qPCR, $n = 4$. **(B)** The quantitation of protein levels, $n = 5$. **(C)** The representative pictures for western blotting. **(D)** *In vivo* superoxide anion release, $n = 5$. **(E)** The amygdala neurons were isolated at embryonic day (E18) from the above treatment for quantitation of 8-oxox-dG staining, $n = 5$. One-way ANOVA was used for the statistical analysis, and each group contained nine mice. $^*P < 0.05$, vs. CTL/P-VEH group. Data were expressed as mean ± SEM.

offspring. We first evaluated anxiety-like behaviors in these animals. The results showed that mice from the maternal diabetic (STZ/P-VEH) group buried significantly fewer marbles (see **Figure 6A**) and spent less time in the Open Arm while spent more time in the Closed Arm in EPM tests (see **Figure 6B**) compared to the control (CTL/P-VEH) group; amygdala infusion of either OXTR (STZ/P-↑OXTR) or ERβ (STZ/P-↑ERβ) completely reversed the maternal diabetes-mediated effect. We then evaluated the effect of postnatal expression in the amygdala on ALB. The results showed that mice from the maternal diabetic (STZ/P-VEH) group had significantly fewer ultrasonic vocalizations compared to the control (CTL/P-VEH) group; amygdala infusion of OXTR (STZ/P-↑OXTR) partly, while amygdala infusion of ERβ (STZ/P-↑ERβ) completely, reversed the maternal diabetes-mediated effect (see **Figure 6C**). In addition, our results showed that maternal diabetic (STZ/P-VEH) group spent significantly less time in Sniffing, Mounting and socially interacting in Total, but not in Grooming their partner in Social Interaction tests (see **Figure 6D**). Furthermore, mice from this group spent significantly more time in the Empty side for sociability (see **Figure 6E**) and less time for social novelty (see **Figure 6F**) in the three-chambered social tests compared to the control (CTL/P-VEH) group; amygdala infusion of OXTR (STZ/P-↑OXTR) showed no effect, while amygdala infusion of ERβ (STZ/P-↑ERβ) completely reversed the maternal diabetes-mediated effect. Our results indicate that increasing postnatal expression of OXTR in amygdala reverses maternal diabetes-induced anxiety-like behavior but has little effect on ALB, while expression of ERβ completely reverses maternal-diabetes-induced social deficits in offspring.

DISCUSSION

In this study, we found that OXTR is suppressed by hyperglycemia-mediated epigenetic changes and the subsequent dissociation of ERβ from the OXTR promoter. Prenatal OXTR deficiency potentiates maternal diabetes-mediated anxiety-like behavior but has little effect on ALB; additionally, postnatal OXTR expression partly, while postnatal ERβ expression completely, reversed maternal diabetes-mediated social deficits.

Maternal Diabetes-Mediated OXTR Suppression

We found that hyperglycemia suppresses the expression of both OXT and OXTR, and OXTR expression remains low, while OXT expression returns to normal during subsequent normoglycemia. This effect can be completely reversed by

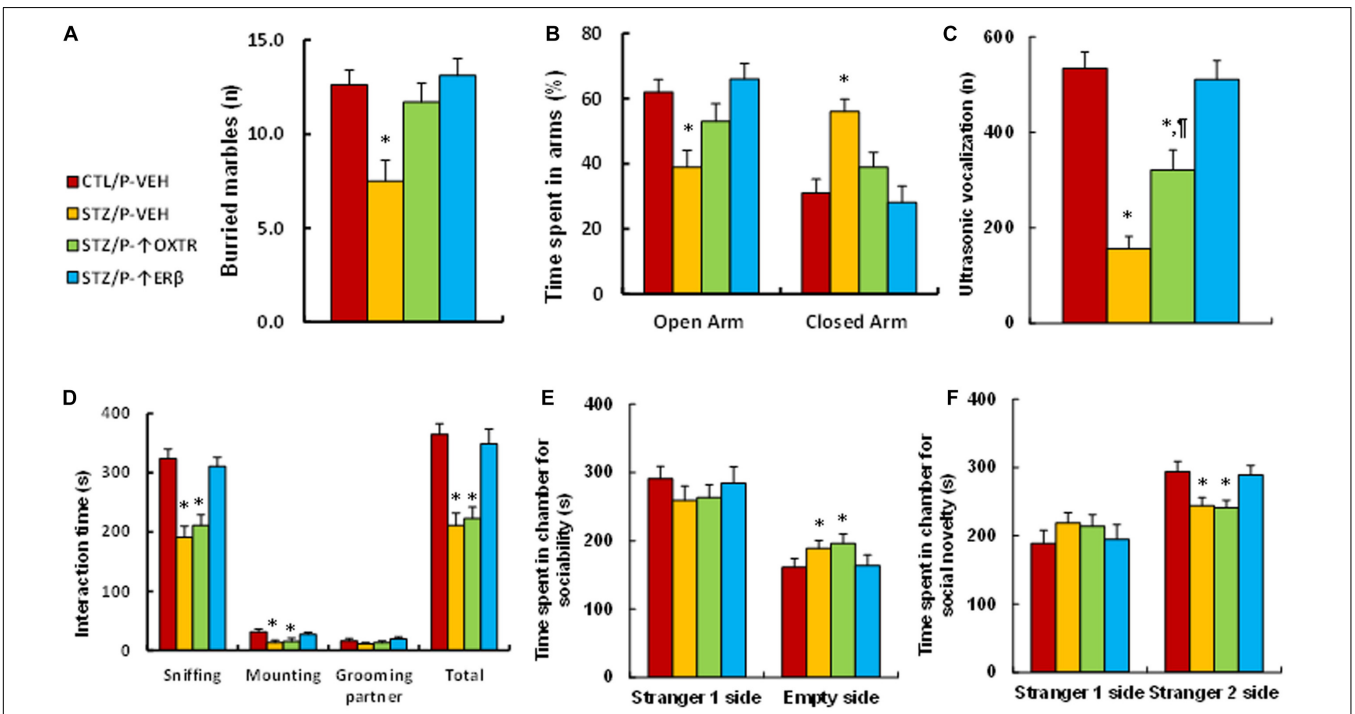

FIGURE 6 | Postnatal expression of OXTR reverses maternal diabetes-induced anxiety-like behavior and has little effect on autism-like behavior, while expression of ERβ completely reverses maternal-diabetes-induced social deficits in offspring. The male offspring from either control (CTL) or maternal diabetes (STZ) groups received either vehicle (P-VEH), or lentivirus infusion for expression of either OXTR (P-↑OXTR) or (P-↑ERβ) at 6 weeks old, and the male offspring were used for animal behavior analysis at 8 weeks old. **(A)** Marbles burying tests (MBT), $n = 9$. **(B)** Time spent in Open Arm and Closed Arms in EPM test, $n = 9$. **(C)** Ultrasonic vocalization, $n = 9$. **(D)** Social interaction (SI) test, the time spent following, mounting, grooming, and sniffing any body parts of the other mouse was calculated, $n = 9$. **(E,F)** Three-chambered social tests, $n = 9$. **(E)** Time spent in chamber for sociability. **(F)** Time spent in chamber for social novelty. One-way ANOVA was used for the statistical analysis, and each group contained nine mice. $^*P < 0.05$, vs. CTL/WT group; ¶ $P < 0.05$, vs. STZ/WT group. Data were expressed as mean ± SEM.

SOD2 expression, indicating that hyperglycemia-induced OXTR suppression is due to hyperglycemia-induced consistent oxidative stress, which has been termed "hyperglycemia memory" (El-Osta et al., 2008; Lu et al., 2020). Further investigation showed that hyperglycemia-induced OXTR suppression is due to oxidative stress-mediated consistent histone methylation on the OXTR promoter, indicating that these types of epigenetic changes can be inherited in offspring as a result of maternal diabetes. This conclusion has been further supported by the results from our *in vivo* study, which showed that OXTR expression was suppressed in many brain tissues, including the amygdala, hypothalamus and hippocampus, in prenatal diabetes exposure-induced offspring. In addition, we found that high glucose suppresses OXT expression, even though this cannot be inherited in offspring, indicating that diabetes may suppress OXT-mediated physiological processes, which is consistent with previous findings (Lippert et al., 2003; Gutkowska et al., 2009; Dai et al., 2018; Ding et al., 2019).

Role of OXTR in Maternal Diabetes-Mediated Social Deficits

We found that prenatal OXTR deficiency induces many social deficits in offspring, it mimics the effects of maternal diabetes-induced anxiety-like behavior and ultrasonic vocalization (Tsuji et al., 2020), while has little effect on ALB. Very interestingly, prenatal OXTR deficiency potentiates maternal diabetes-mediated anxiety-like behavior while again having little effect on ALB, which is consistent with previous findings that OXT is associated with anxiety, but not necessarily with ALB (Yoshida et al., 2009; Puglia et al., 2015, 2018; Duque-Wilckens et al., 2020). In addition, our results showed that prenatal OXTR deficiency does not directly trigger oxidative stress in offspring, while we have previously found that maternal diabetes-induces ALB through persistent oxidative stress and SOD2 suppression (Wang et al., 2019). Taken altogether, we suggest that OXTR may contribute to ALB through other mechanisms, such as serotonergic or glutamatergic neurons, instead of triggering oxidative stress alone (Yoshida et al., 2009; Tan et al., 2019; Wang et al., 2019). On the other hand, this study has a potential limitation due to the lack of OXTR transgenic mice, and our conclusions are made using the lack of an effect of increased OXTR expression in the amygdala, however, OXTR changes were observed in several brain regions beyond the amygdala. In this case, OXTR expression in other regions of brain may also contribute to the animal behaviors, and this needs to be further investigated.

Role of ERβ and Epigenetic Modifications on OXTR Expression

It has been reported that genetic and epigenetic change-mediated OXTR deficiency is associated with ASD (Gregory et al., 2009), and DNA methylation (Behnia et al., 2015; Maud et al., 2018; Puglia et al., 2018) on the OXTR promoter contributes to OXTR deficiency and subsequent social deficits (Puglia et al., 2015, 2018). In this study, we found that maternal diabetes-mediated OXTR suppression is due to oxidative stress-mediated histone methylation on the OXTR promoter as opposed to DNA methylation, indicating that many different factors may contribute to ASD through different mechanisms. In addition, our study has shown that hyperglycemia-induced histone methylation dissociates ERβ from the OXTR promoter and subsequently resulting in OXTR down-regulation (Kudwa et al., 2014). Additionally, we have previously reported that maternal diabetes induces suppression of both SOD2 and ERβ, subsequently contributing to ALBs (Wang et al., 2019). In this study, maternal diabetes-mediated OXTR suppression may be partly due to histone methylation and partly due to suppressed ERβ expression, supporting our previous conclusions that ERβ may play an important role in ASD development (Zou et al., 2017; Xie et al., 2018). In addition, our preliminary study showed that maternal diabetes induces significantly decreased expression of SOD2 and ERβ in brain, resulting in more severe ALBs in male offspring compared to female offspring since male offspring have relatively much lower basal ERβ expression in brain, making male offspring more susceptible to hyperglycemia-induced damage. Furthermore, the presence of high levels of estrogen in female offspring ameliorates maternal diabetes-induced ALBs by estrogen-mediated ERβ activation (Zou et al., 2017; Wang et al., 2019). In this case, the male offspring were chosen in this study to evaluate the potential effect of maternal diabetes on animal behaviors.

CONCLUSION

Oxytocin receptor is suppressed by hyperglycemia-induced persistent oxidative stress and epigenetic changes, which can be inherited during subsequent normoglycemia. Maternal diabetes-induced OXTR suppression contributes to anxiety-like behavior, while it has less of an effect on ALB; moreover, prenatal OXTR deficiency potentiates maternal diabetes-mediated social deficits. We conclude that maternal diabetes-induced suppression of oxytocin receptor contributes to social deficits in offspring.

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.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee from Kangning Hospital of Shenzhen.

AUTHOR CONTRIBUTIONS

PY wrote the manuscript. PY and JLu designed, analyzed the data, and interpreted the experiments. YL, JX, YS, LL, SS, and ZX performed vector constructions and gene expression analysis. XJ, ZW, YN, and HZ performed statistical analysis and part of the mouse experiments. JLi and YLi performed the remaining experiments. All authors read and approved the final manuscript.

FUNDING

This study was supported by the Key Realm R&D Program of Guangdong Province # 2019B030335001, Shenzhen Fund for Guangdong Provincial High-Level Clinical Key Specialty # SZGSP013, Shenzhen Key Medical Discipline Construction Fund # SZXK042, Sanming Project of Medicine in Shenzhen # SZSM201612079, Shenzhen Double Chain Grant # (2018)256, and Natural Science Foundation of Guangdong Province # 2020A1515011581.

ACKNOWLEDGMENTS

The authors thank Dr. Haimou Zhang (from Hubei University, China) for his generosity in providing the transgenic OXTR null mice.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.634781/full#supplementary-material

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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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ORIGINAL RESEARCH
published: 17 March 2021
doi: 10.3389/fnins.2021.637079

Dysfunction of the Auditory Brainstem as a Neurophysiology Subtype of Autism Spectrum Disorder

Jierong Chen[1], Zhen Wei[1], Chun Liang[1], Binguang Liu[2], Jimin Guo[2], Xuejun Kong[3], Minshi Huang[1], Ziwen Peng[4]* and Guobin Wan[1]**

[1] Department of Child Psychiatry and Rehabilitation, Affiliated Shenzhen Maternity and Child Healthcare Hospital, Southern Medical University, Shenzhen, China, [2] Department of Radiology, Affiliated Shenzhen Maternity and Child Healthcare Hospital, Southern Medical University, Shenzhen, China, [3] Martinos Center, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA, United States, [4] Center for Studies of Psychological Application, School of Psychology, South China Normal University, Guangzhou, China

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***Correspondence:**
Guobin Wan
gbw1978@aliyun.com
Ziwen Peng
pengzw@email.szu.edu.cn
Jierong Chen
leoandxm@foxmail.com

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: *02 December 2020*
Accepted: *19 February 2021*
Published: *17 March 2021*

Citation:
Chen J, Wei Z, Liang C, Liu B, Guo J, Kong X, Huang M, Peng Z and Wan G (2021) Dysfunction of the Auditory Brainstem as a Neurophysiology Subtype of Autism Spectrum Disorder. Front. Neurosci. 15:637079. doi: 10.3389/fnins.2021.637079

Autism spectrum disorder (ASD) is very heterogeneous, particularly in language. Studies have suggested that language impairment is linked to auditory-brainstem dysfunction in ASD. However, not all ASD children have these deficits, which suggests potential subtypes of ASD. We classified ASD children into two subtypes according to their speech-evoked auditory brainstem response (speech-ABR) and explored the neural substrates for possible subtypes. Twenty-nine children with ASD and 25 typically developing (TD) peers were enrolled to undergo speech-ABR testing and structural magnetic resonance imaging (sMRI). There were significant differences between the ASD group and TD group in surface area, cortical volume and cortical thickness. According to speech-ABR results, ASD participants were divided into the ASD-typical (ASD-T) group and ASD-atypical (ASD-A) group. Compared with the ASD-T group, the ASD-A group had a lower score in language of the Gesell Developmental Diagnosis Scale (GDDS), increased left rostral middle frontal gyrus (lRMFG) area and decreased local gyrification index of the right superior temporal gyrus. GDDS-language and surface area of lRMFG were correlated to the wave-A amplitude in ASD. Surface area of lRMFG had an indirect effect on language performance *via* alteration of the wave-V amplitude. Thus, cortical deficits may impair language ability in children with ASD by causing subcortical dysfunction at preschool age. These evidences support dysfunction of the auditory brainstem as a potential subtype of ASD. Besides, this subtype-based method may be useful for various clinical applications.

Keywords: autism spectrum disorder, subtype, speech-ABR, neuroimaging, mediation, neurophysiology

Abbreviations: ASD, autism spectrum disorder; ABR, auditory brainstem response; speech-ABR, speech-evoked auditory brainstem response; MRI, magnetic resonance imaging; ADOS, Autism Diagnostic Observational Schedule; CARS, Childhood Autism Rating Scale; GDDS, Gesell Developmental Diagnosis Scale; DQ, developmental quotient; BioMARK, Biological Marker of Auditory Processing; lRMFG, left rostral middle frontal gyrus; rSTG, right superior temporal gyrus.

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder involving deficits in social communication and restricted and repetitive behaviors, with an onset prior to 3 years of age (American Psychiatric Association, 2013). The heterogeneity of individuals with ASD in clinical presentations poses a significant challenge for the interpretability and replicability of research studies (Grzadzinski et al., 2013). Identifying potential subtypes may provide insight into the pathogenesis and/or neurologic mechanism of ASD.

Over the past several decades, researchers have made great efforts to find ways to categorize ASD heterogeneity (Grzadzinski et al., 2013). Classifying individuals with ASD into different groups based on clinical symptoms is one of most widely used methods (Heaton et al., 2008; Deboth and Reynolds, 2017). Besides, numerous studies have used clustering analyses to distinguish different subtypes based on cognitive-behavior and neuropsychology characteristics (Bitsika et al., 2008; Sacco et al., 2012). However, attempts to assign subtypes to individuals with ASD have been largely unsuccessful because distinct, empirically defined subgroups have yet to be identified reliably.

There is an absence of neurobiological evidence for why individuals with ASD present symptoms of varying severity. However, paying attention to the phenotypic behavioral heterogeneity of language in early development is important (Anderson et al., 2007; Pickles et al., 2014). One important reason is that, even though the language trajectories of ASD cases in the first years of life are highly unstable, these trajectories in early childhood are relatively stable and predictive of long-term outcome (Lombardo et al., 2015). Numerous neuroimaging studies have found that abnormalities in the cortex and/or subcortex may be neural mechanisms causing language deficits in ASD (Russo et al., 2008; De Fossé et al., 2010). Nevertheless, the degree of language impairment of individuals with ASD is vastly heterogeneous (Kjelgaard and Tager-Flusberg, 2001). Thus, better understanding of the language-related subtype of ASD in early childhood may help to reveal the neural mechanism that underlies language impairment in such children.

The auditory brainstem response (ABR) is a far-field electric potential measured from the scalp reflecting the electrophysiological activity of the subcortical auditory pathway from the distal portion of the auditory nerve to higher midbrain structures (Skoe et al., 2015). The speech-evoked auditory brainstem response (speech-ABR) is a response to complex sound, and can provide cues as to how temporal and spectral features are preserved in the brainstem (Anderson et al., 2010). Compared with use of questionnaires, the speech-ABR (as an electrophysiological indicator) is more objective, quantitative, non-invasive, precise, and appropriate for varying levels of function. Studies have shown that an abnormal pattern of ABR waveforms in ASD children indicates dysfunction at the subcortical level (Russo et al., 2010a; Miron et al., 2018). More importantly, auditory brainstem function has been linked to language impairment (Banai et al., 2005; Wible et al., 2005; Johnson et al., 2007). However, those results have been contradictory, involving prolongation, shortening, and no abnormalities in the latency and/or amplitude of waveforms (Klin, 1993; Miron et al., 2017). Besides, not all ASD children have subcortical auditory deficits (Russo et al., 2010a), and the exact percentage needs to be obtained by large-sample studies. These findings suggest there are potential auditory subcortical processing-related subtypes of ASD children linked to language impairment.

Functional neuroimaging studies in the past decade have provided evidence that the cortico–subcortical network contributes to auditory, speech and language functions (Dick et al., 2014). According to the theory of corticofugal systems (Winer, 2005), the cortex may provide an inevitable contribution to the subcortex during auditory processing (Yan and Ehret, 2002; Chandrasekaran and Kraus, 2010). Furthermore, numerous studies have found abnormalities in the cortex and subcortex levels of ASD cases, and these abnormalities have been related to language impairment (Brambilla et al., 2003; Bauman and Kemper, 2005). Taken together, those findings suggest the possibility of interactions among the cortex, subcortex, and language in ASD children, but little empirical evidence is available.

In the present study, we subcategorized participants with ASD by the speech-ABR. Then, we used structural magnetic resonance imaging (sMRI) to find the neural substrates for a possible subtype of the subcortical auditory function in ASD. Finally, we explored the interactive relationship among the cortex, subcortex, and language. We hypothesized that: (i) two distinct subtypes in a population of ASD cases aged 3–6 years would have significant differences in language scores; (ii) there would be significant differences in brain structure among ASD subtypes and typically developing (TD) children; and (iii) structural differences of ASD subgroups would interact with the function of the subcortex and language in ASD children (hypothesized model see **Supplementary Figure 1**).

MATERIALS AND METHODS

Ethical Approval of the Study Protocol

The study protocol was approved by the Research Ethics Board of Affiliated Shenzhen Maternity & Child Healthcare Hospital (ASMCHH; Shenzhen, China). For ASD children who met the inclusion criteria, relevant researchers informed the parents of the research content, and invited them to participate in the present study. Written informed consent was obtained from the parents of children enrolled in our study.

Participants

Twenty-five TD and thirty ASD children between 3 and 6 years of age were recruited from department of Child Psychiatry and Rehabilitation, Affiliated Shenzhen Maternity & Child Healthcare Hospital (Guangdong, China). All ASD participants were diagnosed by child psychiatrists with extensive experience at ASMCHH, and met the criteria for ASD using the Autism Diagnostic Observational Schedule (ADOS) and Diagnostic and Statistical Manual, Fifth Edition (DSM-5) of the American Psychiatric Association (2013). Child psychiatrists recommended

these children undergo magnetic resonance imaging (MRI) to exclude organic brain disorders.

All participants passed hearing screenings at birth and had good hearing sensitivity bilaterally (<25 dB HL from 500 to 4,000 Hz). Besides, all participants were screened to ensure that they met the inclusion criteria: (i) met criteria of autistic disorder using both the ADOS and DSM-5 criteria; (ii) age of 3–6 years; (iii) no diseases of the acoustic meatus or hearing disorders; (iv) no history of epilepsy or head injury; and (v) no combined other neurodevelopmental disorders. Age-matched TD participants were screened with questionnaires. Individuals with a family history of any neuropsychiatric disorder (e.g., autism, learning disability, affective disorders, schizophrenia, and epilepsy) were excluded. All parents of participants were informed of the need to add a three-dimensional (3D) scan sequence, speech-ABR, and relevant clinical evaluation.

Clinical Evaluation

ADOS

The ADOS is a semi-structured, standardized interaction-and-observation tool that measures autism symptoms for individuals with possible autism or other pervasive developmental disorders (Lord, 1989). Each module contains standard activities and materials that allow examiners to assess the developmental and language levels of participants (Stacy et al., 2012).

Childhood Autism Rating Scale

The Childhood Autism Rating Scale (CARS) is a behavior-observation instrument which differentiates people with ASD from those with other developmental disorders (Schopler et al., 1980; Chlebowski et al., 2010). The instrument consists of 15 domains (14 domains assessing behaviors associated with autism and one domain assessing the general impressions of autism) rated on a seven-point scale from "normal" to "severely abnormal." The total score of all domains is evaluated, which is useful as a continuous measure of autism severity.

Gesell Developmental Diagnosis Scale

The Gesell Developmental Diagnosis Scale (GDDS) can be used to evaluate the developmental status of infants and young children from the age of 0 to 72 months (Westman, 1976; Raheli et al., 2009). GDDS assesses different aspects of developmental (including adaptive, gross motor, fine motor, language, and personal–social) abilities (Meinzen et al., 2010). Each participant was assigned a developmental quotient (DQ). This is the ratio between the developmental age and chronological age in each of the five specific domains. DQ ≥ 76 was considered "normal"; 55–75 denoted "mild retardation"; 40–54 indicated "moderate retardation"; ≤39 reflected "severe retardation."

Speech-ABR

The stimulus comprised the five first formants of the syllable /da/, including consonant /d/ and vowel /a/. The /da/ was 40 ms in duration, and synthesized by a Klatt (1980) synthesizer at a rate of 10 kHz. Laterality can affect the speech-ABR, so the stimulus was presented monaurally (right ear) (Hornickel and Skoe, 2009). The stimulus intensity was 80 dB sound pressure level with a presentation rate of 10.9 stimuli/s through headphones. The default stimulus was provided using Biological Marker of Auditory Processing (BioMARK) software (Boise, ID, United States). Recording of the speech-ABR took place in an electrophysiology room at ASMCHH. The Navigator PRO system (Bio-logic Systems, Mundelein, IL, United States) and BioMARK software were used to record the speech-ABR. Three sweeps of 3000 stimuli were recorded and grand-averaged for each participant to improve the signal-to-noise ratio. The parameters used to obtain the speech-ABR have been used in our previous study (Chen et al., 2019).

Criteria for subtypes

Biological Marker of Auditory Processing is a testing protocol developed by Auditory Neuroscience Laboratory of Northwestern University (Evanston, IL, USA). The parameters evaluated for the speech-ABR were the: latency of wave-V; latency of wave-A; V/A slope; first formant frequencies; higher frequencies (Billiet and Bellis, 2011; Sanfins et al., 2015). The BioMARK score was obtained as a composite score derived from all five parameters that it assessed. There were different scoring criteria in different age groups. At the age of 3–4 years, a score of ≤3 was considered to be "normal" and a score from 4 to 22 was considered "abnormal." At the age of 5–12 years, a score of ≤7 was considered "normal" and a score from 8 to 22 was considered "abnormal." According to whether the speech-ABR was normal or abnormal, ASD participants were divided into the ASD-atypical (ASD-A) group or ASD-typical (ASD-T) group. According to BioMARK scores, ASD participants were divided into the ASD-A group (n = 15) and ASD-T group (n = 15). The BioMARK score was normal for all TD children. The ASD-T, ASD-A, and TD groups were matched on age. Relevant demographic information is shown in **Table 1**. The grand-average waveforms of the speech-ABR between the two subgroups were illustrated in **Figure 1**.

Acquisition of MRI Data

For ASD participants who could cooperate to undergo MRI, 10% chloral hydrate (0.75 mL/kg, p.o.) was used for sedation. All ASD children were sedated for MRI according to the sedation protocol set by the Department of Radiology of ASMCHH. The imaging data of TD children were collected during natural sleep at night without use of sedation. High-resolution T1-weighted MRI sequences were obtained on a 1.5-T Magnetom Symphony Maestro Class Syngo MR 2002B scanner (Siemens, Munich, Germany) with echo time = 3.30 ms, repetition time = 10 ms, flip angle = 15°, 180 slices, and in-plane voxel size = 1 mm × 1 mm.

TABLE 1 | Demographic information of ASD and TD participants.

	ASD	ASD-T	ASD-A	TD	*F*	*p*
Number	29	15	14	25		
Male:female	27:2	13:2	14:0	17:8		
Age (years)	4.51 ± 1.20	4.17 ± 1.00	4.71 ± 1.22	4.63 ± 0.56	1.332	0.270

Data are the mean ± SD.

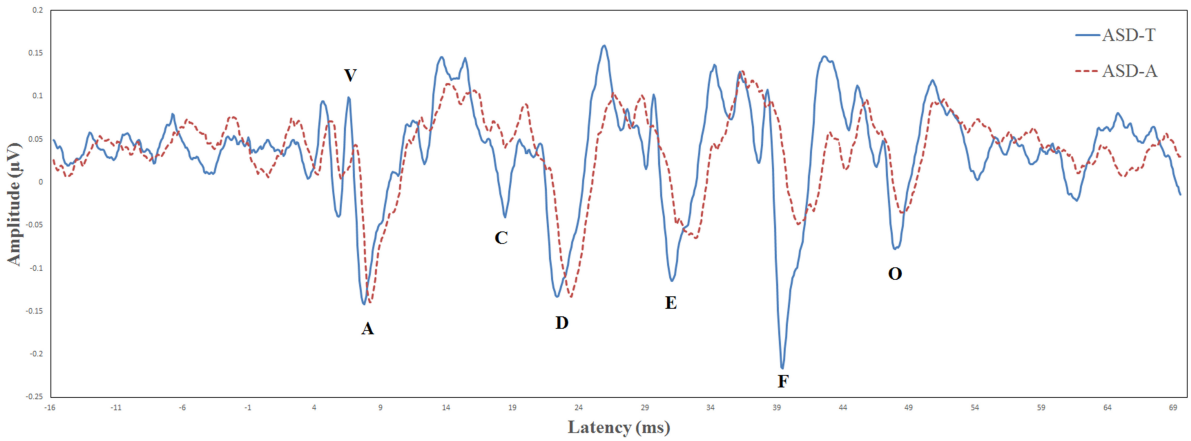

FIGURE 1 | Comparison of the brainstem response to speech in the ASD-T group (solid line) and ASD-A group (dotted line).

Following visual assessment of MRI data and output from the FreeSurfer image analysis suite[1], the raw data from one ASD participant which were of insufficient quality were excluded. Hence, the final analysis comprised data from 29 ASD cases and 25 TD participants.

Processing of MRI Data

T1-weighted images were processed using FreeSurfer v6.0.0. Cortical reconstruction for each participant was examined slice-by-slice to identify inaccuracies in surface placement. Inaccuracies were corrected or excluded by a single experienced FreeSurfer user. The details of these procedures are described elsewhere (Wallace et al., 2013). Surface area, cortical thickness, and cortical volume were measured automatically at each FreeSurfer surface vertex. The local gyrification index is a 3D surface-based measure of the degree of cortical folding (which is the ratio of the cortical surface area within the sulcal folds relative to the amount of cortex on the outer visible cortex). The local gyrification index was measured using an added flag to the FreeSurfer reconstruction-processing stream (Schaer et al., 2008).

Statistical Analyses

Group differences in the latency and amplitude of speech-ABR were assessed using one-way analysis of variance (ANOVA) with the diagnostic group as a three-level (ASD-A, ASD-T and TD) factor adjusted for multiple comparisons. If the main effect of the group was significant, *post hoc* pairwise comparisons were conducted using the Bonferroni correction. The complete characterization and compare of speech-ABR among three group were reported in **Table 2**. A between-group analysis using a two-sample *t*-test was undertaken to detect differences in scores for the ADOS, CARS, GDDS, and speech-ABR between the ASD-A group and ASD-T group. A two-step general linear model was used to estimate the difference in surface area, cortical thickness, cortical volume and the local gyrification index between two groups, including ASD and TD, ASD-A and TD, ASD-T and TD, and ASD-A and ASD-T. Surface area, cortical thickness, and cortical volume were smoothed using a 10-mm full-width at half maximum (FWHM) 2D Gaussian kernel. The local gyrification index measure is intrinsically smooth, so data were smoothed at 5-mm FWHM. To provide stringent criteria to minimize false-positive results, all analyses were set at $p < 0.01$ (corrected for multiple comparisons using Monte Carlo stimulations) (Hagler et al., 2006). Clusters identified in the Monte Carlo stimulations were used as regions of interest which the value of the brain structure was extracted from. A Fisher transformation was applied to improve the normality of the correlation coefficient (Lowe et al., 1998). Based on all ASD participants having different degrees of language impairment, Pearson correlations were undertaken in all ASD participants to investigate the relationship among brain structure, speech-ABR waveforms, and clinical assessments.

Mediation Effect

According to the result of correlation analyses, we further explored whether the auditory brainstem function moderated the relationship between the brain structure and language ability of all ASD participants controlled for age and sex. The PROCESS macro program within SPSS (IBM, Armonk, NY, United States) designed by Hayes (2013) was used to measure the mediating or moderating effect. Within PROCESS, "model 4" was selected and the confidence interval was set to 95%. In the moderation model, the surface area of the left rostral middle frontal gyrus (lRMFG) was entered as the predictor (X), language score of the GDDS as the outcome (Y), and the amplitude of wave-V as the moderator (M). Statistical tests were evaluated at $p < 0.05$ (two-tailed).

RESULTS

Clinical Evaluation of ASD Subgroups

As shown in **Table 3**, ASD subgroups did not differ in scores for the ADOS or CARS (**Table 3**). Two subgroups of ASD

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

TABLE 2 | Latency and amplitude of speech-ABR waveforms recorded in ASD subgroups and the TD group.

Wave		ASD-T	ASD-A	TD	ANOVA		*Post hoc*		
					F	*p*	ASD-T vs. ASD-A	ASD-T vs. TD	ASD-A vs. TD
V	Latency (ms)	6.57 ± 0.24	7.23 ± 0.46	6.70 ± 2.45	16.07	0.000*	0.000**	0.434	0.000**
	Amplitude (μv)	0.11 ± 0.07	0.04 ± 0.06	0.11 ± 0.06	6.74	0.003*	0.009**	1.000	0.005**
A	Latency (ms)	7.52 ± 0.34	8.42 ± 0.57	7.67 ± 0.27	20.18	0.000*	0.000**	0.739	0.000**
	Amplitude (μv)	−0.21 ± 0.05	−0.22 ± 0.08	−0.22 ± 0.08	0.03	0.966	1.000	1.000	1.000
C	Latency (ms)	18.27 ± 0.31	18.64 ± 1.05	18.45 ± 0.50	1.11	0.338	0.433	1.000	1.000
	Amplitude (μv)	−0.09 ± 0.05	−0.03 ± 0.05	−0.13 ± 0.21	1.97	0.151	1.000	1.000	0.163
D	Latency (ms)	22.27 ± 0.41	23.17 ± 0.52	22.49 ± 0.63	8.41	0.001*	0.001**	0.467	0.010**
	Amplitude (μv)	−0.18 ± 0.07	−0.21 ± 0.06	−0.18 ± 0.08	1.80	0.176	1.000	0.855	0.214
E	Latency (ms)	31.10 ± 0.55	31.36 ± 0.80	31.16 ± 0.77	0.51	0.602	1.000	1.000	1.000
	Amplitude (μv)	−0.20 ± 0.09	−0.14 ± 0.05	−0.22 ± 0.11	2.39	0.102	0.370	1.000	0.109
F	Latency (ms)	39.57 ± 0.59	40.56 ± 0.88	39.54 ± 0.59	11.00	0.000*	0.001**	1.000	0.000**
	Amplitude (μv)	−0.27 ± 0.09	−0.15 ± 0.05	−0.22 ± 0.14	4.50	0.016	0.013	0.522	0.149
O	Latency (ms)	47.67 ± 0.86	48.86 ± 1.27	48.06 ± 0.39	7.52	0.001*	0.001**	0.452	0.019**
	Amplitude (μv)	−0.14 ± 0.06	−0.13 ± 0.04	−0.18 ± 0.17	1.11	0.338	1.000	0.787	0.598

Data are the mean ± SD.
SD, standard deviation.
**Significant using the threshold 0.05/14 = 0.004, adjusted for multiple comparisons.*
***Significant using the threshold 0.05, adjusted for the Bonferroni correction.*

participants were mildly retarded and did not differ in gross-motor, fine-motor, or adaptive functions. In GDDS-language, the score of the ASD-A group was lower than that of the ASD-N group ($t = 2.425$, $p = 0.025$).

Differences in Brain Structure Among the Groups

There were significant (cluster-corrected $p < 0.01$) differences between the TD group and ASD group for cortical thickness, cortical volume and surface area (**Supplementary Table 1** and **Figure 2**). Compared with the TD group, the ASD-T group and ASD-A group showed a significant difference in surface area, cortical volume, cortical thickness, and the local gyrification index (**Table 4**). Surface area of lRMFG in ASD-A group was larger than that in the ASD-T group and TD group (**Figure 2** and **Table 4**). The local gyrification index of the right superior temporal gyrus (rSTG) in the ASD-A group was decreased significantly compared with that in the ASD-T group (**Figure 3** and **Table 4**).

TABLE 3 | Clinical characteristics of ASD subgroups.

	ASD-T	ASD-A	*t*	*p*
CARS-score	31.58 ± 1.52	31.50 ± 1.30	0.114	0.910
ADOS-communication	5.50 ± 0.97	5.11 ± 2.26	0.497	0.626
ADOS-social interaction	7.80 ± 1.69	6.78 ± 1.48	1.396	0.181
ADOS-score	13.30 ± 2.45	11.89 ± 3.41	1.044	0.311
GDDS-adaptive	65.07 ± 17.59	59.55 ± 14.64	0.847	0.406
GDDS-gross motor	72.07 ± 9.55	69.22 ± 10.26	0.679	0.505
GDDS-fine motor	64.07 ± 13.18	61.22 ± 5.97	0.606	0.551
GDDS-language	59.43 ± 17.67	45.71 ± 10.37	2.425	0.025*
GDDS-social	61.50 ± 13.18	56.22 ± 9.62	1.034	0.313

Data are the mean ± SD.
CARS, Child Autism Rating Scale; ADOS, Autism Diagnostic Observation Scale; GDDS, Gesell Developmental Diagnosis Scale; SD, standard deviation.
**$p < 0.05$.*

Correlations Among Brain Structure, Speech-ABR and Clinical Scores in ASD Group

We also analyzed the potential link among the speech-ABR, brain-structural and clinical-assessment data. Surface area of lRMFG was related to the latency of waves V and A, and amplitude of wave-V (**Table 5**). Local gyrification index of rSTG was related to the latency of waves V and A. GDDS-language scores were related to the amplitude of wave-V and latency of wave-A.

Mediation Effect Among Regional Surface Area, Language Scores and Amplitude of Wave

Table 6 presents the results of the mediation model. **Figure 4** shows the regression coefficient for each pathway from surface area of lRMFG to language-GDDS in the mediation model. There was a significant negative association between surface area of lRMFG and the wave-V amplitude ($\beta = -0.514$, $p = 0.006$). There was a significant positive association between the wave-V amplitude and GDDS-language ($\beta = 0.566$, $p = 0.008$). The bootstrap procedure revealed significant indirect effects between surface area of lRMFG and GDDS-language controlled for age and sex [$\beta = -0.013$, SE = 0.007, BC 95%CI (-0.028, -0.002)].

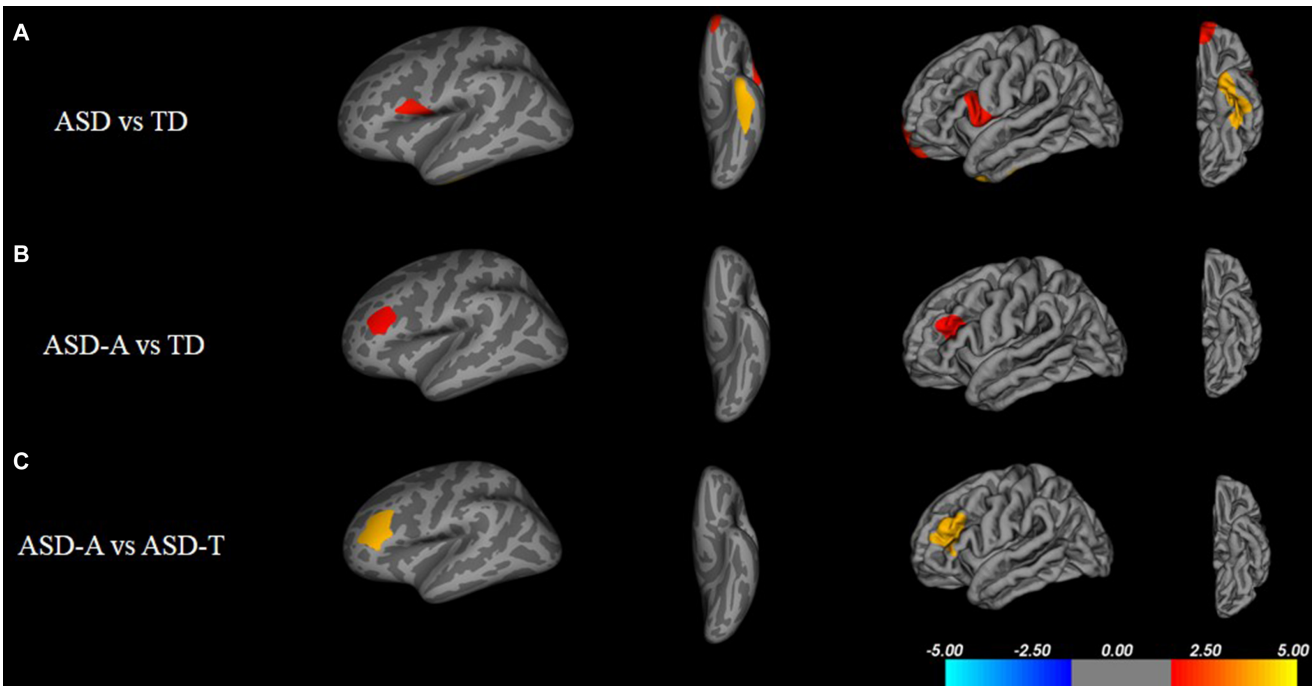

FIGURE 2 | Inflated and pial surface maps (dark gray, sulci; light gray, gyri) of the left hemispheres showing increasing surface area in **(A)** fusiform, frontal pole and pars opercularis in the ASD group compared with that in the TD group, **(B,C)** rostral middle frontal gyrus in the ASD-A group compared with that in the TD group and ASD-T group. Significance threshold was set at $p < 0.01$ (cluster-corrected).

DISCUSSION

This is the first study to classify ASD children into two subtypes based on function of auditory brainstem and to discover neural substrates for the possible subtypes.

Our study elicited four main findings. First, the language score of the ASD-A group was lower than that of the ASD-T group. Second, compared with the TD group, the ASD subgroups showed significant differences in brain structure. Surface area of lRMFG in the ASD-A group was larger, and the local gyrification index of rSTG in the ASD-A group was decreased significantly, compared with those in the ASD-T group. Third, GDDS-language and surface area of lRMFG were correlated to the wave-A amplitude in ASD. Finally, analysis revealed surface area of lRMFG to have an indirect effect on language *via* the wave-V amplitude in ASD children.

Autism spectrum disorder subgroups defined in the present study showed a significant difference in the speech-ABR at preschool age. The ASD-T group exhibited age-appropriate subcortical auditory processing function. In contrast, the ASD-A group showed the latency of speech-ABR waves to be longer and/or the amplitude to be smaller compared with those in the TD group and ASD-T group. Besides, ASD subtypes showed a significant difference in language but not in other aspects or symptom severity. Consistent with our previous research showing that the speech-ABR is related to language ability (Chen et al., 2019), children with an efficient speech-ABR had better language ability. Our study indicated that the speech-ABR could be a clinical-assessment tool to predict the language ability of ASD children at preschool age. Combined with findings that early language ability is an important predictive factor for later outcomes of ASD (Szatmari et al., 2010; Tager-Flusberg and Kasari, 2013), ASD children with a normal speech-ABR may have a better outcome compared with abnormal ones. Taken together, these data suggest that auditory brainstem function not only has a crucial role in language, but also in the long-term outcome of ASD children.

We found differences in brain structure not only between TD children and ASD children, but also among ASD subtypes. Consistent with other studies, we found an aberrant cortical structure in ASD children, including cortical thickness, surface area, and cortical volume (Blackmon et al., 2016; Patriquin et al., 2016). Besides, differences in the local gyrification index were observed only between ASD subtypes and TD, and not ASD and TD. Combined with contradictory results in the neuroimaging of ASD (Pua et al., 2017), ASD may be composed of different subtypes which exist differences in brain anatomy. Hence, comparison of ASD and TD before correct differentiation of subtypes will lead to unstable and unrepeatable research results.

Moreover, we found a significant difference in brain structure between ASD subtypes, which may provide neuroimaging evidence for subtype classification. We found a larger regional surface area (lRMFG) in the ASD-A group compared with that in the ASD-T group and TD group. This result indicated that an increase in surface area of lRMFG was a characteristic structural change in the ASD-A subtype. The RMFG, as part of the

TABLE 4 | Clusters of significant differences in cortical morphometry.

Group	Measure	Size (mm^2)	*X*	*Y*	*Z*	Number of vertices	Peak region
ASD-A vs. ASD-T	Area	1067.46	−38.4	36.1	28.7	1747	L rostral middle frontal
	Local gyrification index	859.3	47.5	5	−27.2	1271	R superior temporal
ASD-T vs. TD	Thickness	5114.47	−26.3	23.8	−6	9550	L lateral orbitofrontal
		769.09	−19.9	−88	−7.4	789	L lateral occipital
		1122.41	44	−12.8	20.7	3031	R postcentral
		1098.71	27.7	57.8	−9.5	1551	R rostral middle frontal
		1056.15	8.3	37	−3.9	1711	R rostral anterior cingulate
		1015.37	31	−41.1	−9	1822	R parahippocampal
		602.63	37	−84	15.9	830	R inferior parietal
		550.12	44	−67.7	7	1079	R inferior parietal
		548.19	45.1	−37.2	17.7	1110	R supramarginal
	Area	728.78	−55.3	−12.4	31.3	1665	L postcentral
	Volume	458.44	−39.8	−68.1	0	783	L lateral occipital
		407.34	−55.3	−12.4	31.3	888	L postcentral
		1231	1231	−67.7	7	1901	R inferior parietal
		540.54	29.7	−45.7	−15.4	943	R fusiform
	Local gyrification index	1593.53	−12	−67.1	34.7	2723	L precuneus
ASD-A vs. TD	Thickness	1595.18	−26.3	23.8	−6	4144	L lateral orbitofrontal
		1022.82	−12.8	−11.1	67.7	2159	L superior frontal
		693.74	−7.3	37.5	13.5	1199	L rostral anterior cingulate
		528.3	−38.1	50	−3.4	732	L rostral middle frontal
		509.2	−4.1	−33.4	30.5	1282	L isthmus cingulate
		456.39	−28.8	−47.9	−5.4	925	L lingual
		1378.09	14.2	−65.9	−3	2041	R lingual
		1151.45	37.1	−13	1.8	3134	R insula
		834.91	3.3	−32.8	65.1	1974	R paracentral
		733.2	12.8	−52.3	41.4	1846	R precuneus
		564.84	8.3	37	−3.9	1067	R rostral anterior cingulate
		558.42	32.5	35.1	−6.7	844	R lateral orbitofrontal
	Area	760.35	−37.3	23.6	24.8	1337	L rostral middle frontal
	Volume	1289.21	−37.3	23.6	24.8	2007	L rostral middle frontal
		940.3	−13.4	−91.2	3.8	1497	L pericalcarine
		675.25	−58.1	−20.9	−14.8	1398	L middle temporal
		847.58	44	−67.7	7	1395	R inferior parietal
	Local gyrification index	1091.79	45	−60.1	21.3	1869	R inferior parietal
		1030.32	55.1	−38.4	27.5	2179	R supramarginal

dorsolateral prefrontal cortex (DLPFC), is related to the function of language, as discovered in various lesion studies (Chapman et al., 1992, 1998). Patients with left-DLPFC lesions have been found to use fewer complex sentences and to perseverate on the first proposition (Carl et al., 2012). These symptoms can also occur in some ASD children who use simple sentences and "chatter" on a topic. Besides, a lower local gyrification index of the right STG was found in the ASD-A group. The traditional view is that the STG is involved primarily in auditory processing, including language (Gernsbacher and Kaschak, 2003; Martin, 2003). However, recent studies have reported that the STG is not only involved in auditory processing, but is also implicated in social cognition (Bigler et al., 2007; Ethofer et al., 2013). Taken together, the differences in brain structure between ASD subtypes are involved in the cortex related to auditory and language, but also in the high-order cortex related to society. The differences in altered subtypes in the brain may be one of the important reasons for ASD heterogeneity.

Further analyses revealed that surface area of lRMFG had an indirect effect on language by altering the wave-V amplitude in ASD children. Combined with the previous findings showing that the neural generators of waves V and A may be the inferior colliculus (Song et al., 2008), one parsimonious explanation is that the lRMFG targeting the inferior colliculus mediates the language of ASD children through corticofugal neurons. As a crucial mechanism of neuromodulation, physiological studies have demonstrated that these descending pathways can affect several aspects of subcortical function (Villa et al., 1991; Diamond et al., 1992; Ma and Suga, 2001). Studies have demonstrated language impairment to be related to cortical and/or subcortical dysfunctions in ASD children (Groen et al., 2008; Russo et al., 2010a). Our findings suggest that cortical deficits may impair

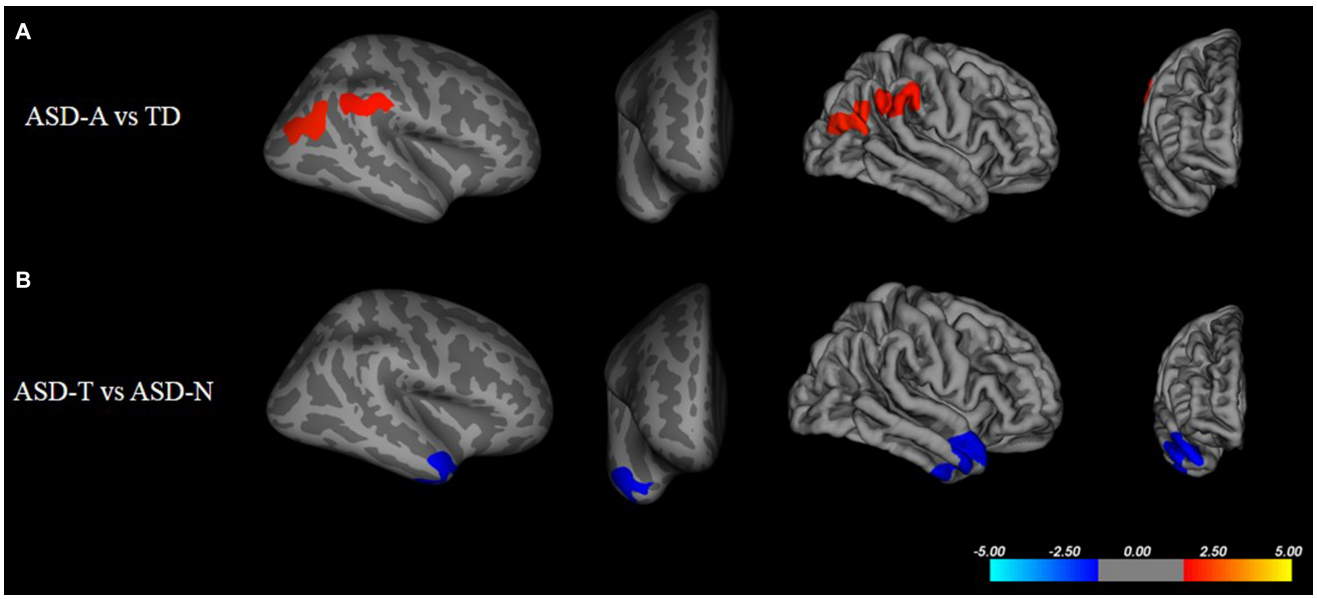

FIGURE 3 | Inflated and pial surface maps (dark gray, sulci; light gray, gyri) of the right hemispheres showing a decreasing local gyrification index in **(A)** inferior parietal and supramarginal areas in the TD group compared with that in the ASD-A group, **(B)** superior temporal gyrus (STG) in the ASD-A group compared with that in the ASD-T group. Significance threshold was set at $p < 0.01$ (cluster-corrected).

language ability in children with ASD by causing subcortical dysfunction at preschool age.

Using subtypes may be the most useful method for specific intervention in ASD. Experience-dependent mechanisms drive the plasticity of the auditory brainstem, which can be improved by auditory training (Hayes et al., 2003; Russo et al., 2005) and language experience (Krishnan et al., 2004, 2005). Skoe et al. (2015) proposed the corticofugal system nears maturation stabilization around the age of 8–11 years. Therefore, we postulate that ASD children with an abnormal speech-ABR should undergo related auditory and language training as soon as possible, especially at preschool age. The Fast ForWord language-training program has been found to improve auditory-brainstem and cortical responses in ASD children (Russo et al., 2010b). Few intervention studies on ASD have been done. However, based on behavioral and genetic similarities (Herbert and Kenet, 2007; Smith, 2007) in language impairment between ASD and language-based learning disorders (Rapin and Dunn, 2003; Cardy et al., 2005), intervention programs of language-based learning disorders to improve auditory-brainstem function could be conducted in ASD children with an abnormal speech-ABR. In addition, the subtype-based method may be useful as a prognostic biomarker in ASD children. A stable ABR is associated with heightened language abilities (Hornickel and Kraus, 2013). Previously, we found the auditory brainstem to be impaired and immature in preschool children with ASD (Chen et al., 2019). We speculated the ASD children with an abnormal speech-ABR indicates impairment of the related brain region and poor language ability, which may lead to a poor prognosis. In the future, we will examine if an abnormal speech-ABR is associated with a worse long-term outcome compared

TABLE 5 | Association between speech-ABR waveforms and brain structure and GDDS-language characteristics in ASD participants.

	Surface area-lRMFG		Local gyrification index-rSTG		GDDS -language	
	r	*p*	*r*	*p*	*r*	*p*
Wave-V latency	0.452	0.014*	−0.428	0.023*	−0.351	0.067
Wave-V amplitude	−0.533	0.003*	0.019	0.922	0.551	0.002*
Wave-A latency	0.402	0.031*	−0.533	0.003*	−0.479	0.010*
Wave-A amplitude	−0.067	0.729	0.003	0.989	0.066	0.738
GDDS-language	−0.262	0.178	−0.102	0.612	–	–

IRMFG, left rostral middle frontal gyrus; rSTG, right superior temporal gyrus; GDDS, Gesell Developmental Diagnosis Scale.
*$p < 0.05$.

TABLE 6 | Direct and indirect effects between surface area of lRMFG and GDDS-language mediated by the amplitude of wave-V.

	Product of coefficients		BC 95% bootstrap CI	
	β	SE	Boot LL CI	Boot UL CI
Direct effect	−0.004	0.009	−0.0184	0.0180
Indirect effect	−0.013	0.007	−0.0280	−0.0016

Mediation model controlled for age and sex.
"BC 95% CI" refers to the bias-corrected confidence intervals. The mediate effect is significant at the 0.05 level of significance.
β, standardized regression coefficient; SE, standard error; CI, confidence interval; lRMFG, left rostral middle frontal gyrus; GDDS-language, language scores for the Gesell Developmental Diagnosis Scale.

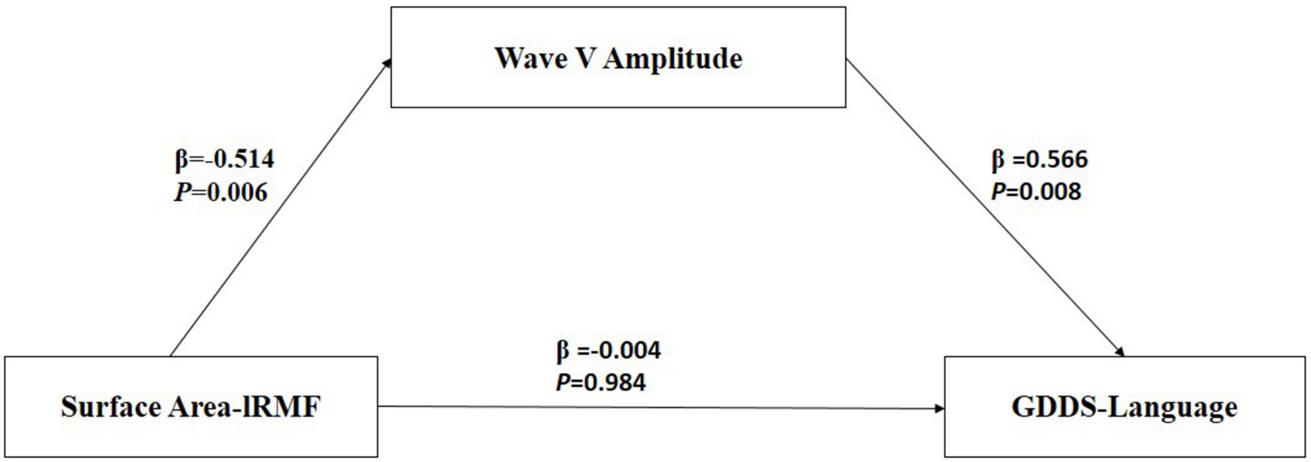

FIGURE 4 | Mediation model among surface area-lRMFG (predictor), Wave-V amplitude (mediation), and GDDS-language (outcome). β, standardized regression coefficient. Surface area-lRMFG, surface area of left rostral middle frontal gyrus; GDDS-language, language scores of the Gesell Developmental Diagnosis Scale.

with normal ones. Overall, ASD children should undergo a speech-ABR after 3 years of age for use as a subtype and prognostic biomarker, and a subtype-specific intervention should be employed for a better outcome.

In interpreting our results, some limitations should be considered. First, our study had a relatively small sample size. There were only two female participants in this study, and both were in the ASD-A. This study does not exclude the potential of gender factors for subtypes. Our future studies will recruit more children with ASD who meet the criteria for an appropriate gender ratio. Second, MRI was done on a 1.5-T system, but several recent studies have used 3-T MRI scanners. MRI scanners working at 3 T have a higher field strength and increased signal-to-noise ratio compared with those using a 1.5-T MRI scanner. In future work, we will use a 3.0-T MRI scanner to acquire higher spatial resolution data. Finally, intelligence and language ability are highly correlated. Although there were no significant differences in overall developmental levels between the two subtypes. It is possible that there were significant differences in intelligence levels. Our future studies will investigate whether intellectual factors affect subcortical auditory processing in ASD.

CONCLUSION

This is first study to distinguish ASD children by auditory brainstem function and to explore neural evidence for identification of potential subtypes. There were significant differences between the ASD group and TD group in surface area, cortical volume, and cortical thickness. Compared with the ASD-T group, the ASD-A group showed a lower score in GDDS-language, increased surface area of lRMFG, and decreased local gyrification index of rSTG. GDDS-language and surface area of lRMFG were correlated with the wave-A amplitude. Surface area of lRMFG had an indirect effect on the performance of language by altering the wave-V amplitude. Thus, cortical deficits may impair language ability in children with ASD by causing subcortical dysfunction at preschool age. These evidences support dysfunction of the auditory brainstem as a potential subtype of ASD. Besides, this subtype-based method may be useful for various clinical applications (e.g., prognosis and subtype-specific intervention).

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.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Board of Affiliated Shenzhen Maternity & Child Healthcare Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JC, ZP, and GW conceived the study, prepared the data, analyzed the data, and drafted, and revised the manuscript. BL, JG, and MH helped to analyze the data and helped to draft the manuscript. XK and CL participated in the study design and helped to draft and revise the manuscript. All authors approved the final version of the manuscript.

FUNDING

This study was supported by the Key-Area Research and Development Program of Guangdong Province (2019B030335001), the Shenzhen Key Medical Discipline Construction Fund (SZXK071), Sanming Project of Medicine

of Shenzhen (SZSM201612079), National Natural Science Foundation of China (31871113 and 31900770), and Guangdong Key Project in development of new tools for diagnosis and treatment of autism (2018B030335001).

ACKNOWLEDGMENTS

We thank the children and their families for their participation to make this research possible.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.637079/full#supplementary-material

Supplementary Figure1 | Hypothesized model among cortex, subcortex, and language in ASD preschool children.

Supplementary Table 1 | Cluster of significant differences in cortical morphometry between the TD group and ASD group.

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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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ORIGINAL RESEARCH
published: 22 March 2021
doi: 10.3389/fnins.2021.644543

Alterations of Regional Homogeneity in Preschool Boys With Autism Spectrum Disorders

Zhihong Lan[1†], Shoujun Xu[2†], Yunfan Wu[1], Likun Xia[3], Kelei Hua[1], Meng Li[1], Mengchen Liu[1], Yi Yin[1], Chunlong Li[1], Shumei Huang[1], Ying Feng[1], Guihua Jiang[1] and Tianyue Wang[1]**

[1] Department of Medical Imaging, Guangdong Second Provincial General Hospital, Guangzhou, China, [2] Department of Radiology, Shenzhen Children's Hospital, Shenzhen, China, [3] Department of Magnetic Resonance Imaging, People's Hospital of Yuxi City, Yuxi, China

Objectives: The study was aimed at investigating the alterations of local spontaneous brain activity in preschool boys with autism spectrum disorders (ASD).

Methods: Based on regional homogeneity (ReHo), the acquired resting state functional magnetic resonance imaging (fMRI) data sets, which included 86 boys with ASD and 54 typically developing (TD) boys, were used to detect regional brain activity. Pearson correlation analysis was used to study the relationship between abnormal ReHo value and the Childhood Autism Rating Scale (CARS), Autism Behavior Checklist (ABC), developmental quotient, and age.

Results: In the ASD group, we found increased ReHo in the right calcarine as well as decreased ReHo in the opercular part of the left inferior frontal gyrus, the left middle temporal gyrus, the left angular gyrus, and the right medial orbital frontal cortex ($p < 0.05$, false discovery rate correction). We did not find a correlation between the results of brain regions and the CARS, ABC, and age.

Conclusions: Our study found spontaneous activity changes in multiple brain regions, especially the visual and language-related areas of ASD, that may help to further understand the clinical characteristics of boys with ASD.

Keywords: autism spectrum disorders, regional homogeneity, resting-state fMRI, preschool boys, neural activity

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Edited by:
Eugenia Conti, Fondazione Stella Maris (IRCCS), Italy

Reviewed by:
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***Correspondence:**
Guihua Jiang
GH.jiang2002@163.com
Tianyue Wang
wmefficient@163.com

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: *08 January 2021*
Accepted: *22 February 2021*
Published: *22 March 2021*

Citation:
Lan Z, Xu S, Wu Y, Xia L, Hua K, Li M, Liu M, Yin Y, Li C, Huang S, Feng Y, Jiang G and Wang T (2021) Alterations of Regional Homogeneity in Preschool Boys With Autism Spectrum Disorders. Front. Neurosci. 15:644543. doi: 10.3389/fnins.2021.644543

INTRODUCTION

Autism spectrum disorders (ASD) have social disorders, communication difficulties, repetitive and stereotyped behaviors, and limited and narrow interests as the core symptoms. These neurodevelopmental disorders occur early in childhood. The latest large-scale global survey has estimated that the incidence rate of ASD is about 1–2%, and it has been steadily increasing

Abbreviations: ASD, autism spectrum disorders; ReHo, regional homogeneity; fMRI, functional magnetic resonance imaging; TD, typically developing; CARS, childhood autism rating scale; ABC, autism behavior checklist; DQ, developmental quotient; IFG operc, the opercular part of inferior frontal gyrus; MTG, middle temporal gyrus; AG, angular gyrus; mOFC, medial orbital frontal cortex; FDR, false discovery rate; KCC, kendall's coefficient of concordance; ADOS, autism diagnostic observation schedule; FOV, field-of-view.

(Lai et al., 2014). Although the neurobiological basis of ASD has been known for more than 40 years (Damasio and Maurer, 1978), the heterogeneity and the high proportion of co-occurring symptoms with other mental disorders (Lai et al., 2019), the independent brain basis of ASD, has not been clearly identified in individuals.

In recent years, the development of neuroimaging provides a new platform for the study of the neural changes of ASD. Dajani and Uddin (2016) divided their subjects into three groups—children, adolescents, and adults—for Regional homogeneity (ReHo) analysis. The authors found that, compared with the typically developing (TD) group, all three groups of patients with ASD had abnormal spontaneous neural activity, which changed with age, especially among the children. ReHo is a method that uses Kendall's coefficient of concordance (KCC) to measure the similarity of the time series of a given voxel with its 26 nearest neighbors in the brain. ReHo, which can reflect the strength of local spontaneous neural activity in the brain, was used in the study of ASD (Paakki et al., 2010; Shukla et al., 2010; Itahashi et al., 2015; Li et al., 2018), but the results were heterogeneous. Jao Keehn et al. (2019) also used the ReHo analysis and found that there was spontaneous nerve activity enhancement in the pericalcarine visual cortex in adolescent patients with ASD (8–18 years old). In another ASD study of unlimited age groups, it was found that, in the ASD group, the local spontaneous nerve activity in the occipital and posterior temporal regions was increased, and that in the middle/posterior cingulate gyrus and medial prefrontal lobe was decreased (Maximo et al., 2013). The researchers believed that the heterogeneity in the current results may be attributed to the different age of patients with ASD.

Autism spectrum disorders has always been considered to be a disease characterized by early changes in brain development, as early as 6–12 months of age, where there is excessive growth of the cortical surface area (Hazlett et al., 2017). With the increase of age and treatment intervention, patients with ASD may have compensatory neural functional activity (Mevel et al., 2015). Furthermore, they are more prone to co-occurring insomnia or other mental disorders (Lai et al., 2019). All these factors may lead to heterogeneity in the brain functional changes of ASD patients at different ages. In consequence, narrowing the age span of patients with ASD in younger children seems likely to produce purer, more reliable results. However, there have been few reports of resting-state functional magnetic resonance imaging (fMRI) studies based on ReHo of ASD patients under 7 years old.

In addition, more studies have begun to focus on sex differences in ASD. Although the morbidity of ASD is two to five times more common in men than in women (Ecker et al., 2017), sex differences in ASD (including clinical and genetic/neurobiological aspects) have gone beyond the scope of epidemiological studies (Lai et al., 2015). For example, in clinical manifestations, males exhibit more restrictive, repetitive, and stereotyped behaviors than females although females exhibit more comorbid psychopathology (Retico et al., 2016). Brain structural imaging studies also found differences in neuroanatomic regions (e.g., corpus callosum volume) among ASD patients of different sexes (Lai et al., 2017). However, in previous fMRI studies of ASD, sex was rarely separated.

Therefore, our study intended to acquire resting-state fMRI data based on ReHo analysis from a relatively large sample size of preschool boys (3–6 years old) to specifically explore the alterations of local spontaneous activity in younger children with ASD and analyze its correlation with the severity of symptoms in patients with ASD. Based on a literature review, we speculated that there might be abnormal spontaneous neural activity in the visual cortex and semantic system–related brain areas in young children with ASD.

SUBJECTS AND METHODS

Subjects

We recruited 86 ASD preschool boys and 54 age-matched TD boys from Shenzhen Children's Hospital. Healthy boys were recruited into the TD group through advertisement. All data sets were collected from November 2016 to August 2018. The study was approved by the ethics committee of Shenzhen Children's Hospital. The guardian of each subject was asked to sign informed consent forms on the premise of fully understanding the purpose of the study. Before the scans, all children with ASD and TD who met the following criteria were excluded: neurological disorders (e.g., epilepsy and Tourette's syndrome), genetic disorders (e.g., Fragile X and Rett syndrome), or psychiatric disorders (e.g., childhood disintegrative disorder, selective mutism, obsessive-compulsive disorder, or Asperger's syndrome). Children with a history of severe physical illness, a history of loss of consciousness for more than 5 min, or who were currently taking psychoactive drugs were also excluded.

Methods

Clinical Assessment

All ASD subjects were co-diagnosed by two associate chief physicians of pediatrics and psychiatry, met the ASD DSM-V criteria and cutoffs on all the Childhood Autism Rating Scale (CARS) (Schopler et al., 1980) and Autism Behavior Checklist (ABC) (Krug et al., 1980) domains. CARS and ABC are the main diagnostic and screening tools for ASD in Chinese children. CARS, which is suitable for people over 2 years old, was assessed by a trained doctor. Although the diagnostic sensitivity of CARS for ASD is not as high as the Autism Diagnostic Observation Schedule, which is known as the gold standard, the CARS has a higher specificity (Randall et al., 2018), which can avoid the inclusion of over-diagnosed children in the ASD group. The ABC was completed by parents of the subjects and is applicable to persons aged 8 months to 28 years. We also used the developmental diagnostic scale, which is suitable for children aged 0–6 years to assess the developmental quotient (DQ) (DQ $<$ 70 as a low score).

MRI Data Acquisition

Image data acquisition was performed by two experienced radiographers. Each child received 0.5% chloral hydrate 0.5 ml/kg (maximum dose 10 ml) and was sedated by a certified nurse. During the entire scan, each participant was required to have a caregiver and guardian present.

MRI data were collected by a 3T Siemens Skyra scanner from the radiology department of Shenzhen Children's hospital. Each participant was in the supine position, and the head was fixed firmly on a foam pad to prevent head movement. Adhesive earmuffs were also used to plug the ears to protect hearing. Then the resting-state fMRI data set was obtained using the gradient echo-planar imaging sequence. Resting-state fMRI collection parameters were as follows: repeat time (TR)/echo time (TE): 2000 ms/30 ms; flip angle: 90°; thickness/interval: 3.6 mm/0.72 mm; field of view (FOV): 230 × 230 mm; matrix: 64 × 64, and layers: 35. In 8 min, 240 volumes were obtained. After the MRI scan, images of each participant were examined to ensure that they met the experimental requirements. Meanwhile, a T1-weighted sequence of magnetization-prepared rapid-acquisition gradient echo (MPRAGE) prepared by three-dimensional magnetization covering the whole brain (176 sagittal sections) was obtained. The corresponding acquisition parameters were set as TR: 2300 ms, TE: 2.26 ms, TI: 900 ms, flip angle: 8°, acquisition matrix: 256 × 256, FOV: 256 × 256 mm, and layer thickness was 1 mm.

Data Processing and ReHo Calculations

Image preprocessing was performed using the data processing assistant in the resting state fMRI toolbox (DPARSF 3.0 Advanced Edition,[1]). For each participant, the first 10 time points were discarded due to transient signal changes before magnetization reached a stable state and the participant adapted to the fMRI noise. To minimize the influence of head movement, subjects with maximum displacement greater than 1.5 mm and angle movement greater than 1.5° during the whole fMRI scan were excluded. No subjects were deleted in this step. Then, the resting-state fMRI data were corrected for intra-volume acquisition delay and co-registered together with anatomical scanning. The co-registered anatomical images were divided into gray matter, white matter, and cerebrospinal fluid. Then, all data spaces were normalized to the whole brain template of the Montreal Neurological Institute standard space (age 4.5–8.5 years) with an isotropic voxel size 3 mm × 3 mm × 3 mm (Fonov et al., 2011). Furthermore, a regression analysis was conducted to minimize the influence on head motion (Friston 24 model), cerebrospinal fluid, and white matter, following which the high-frequency physiological noise and low-frequency drift were filtered (0.01–0.08 Hz).

The ReHo calculation process was the same as that reported in previous studies (Zang et al., 2004). In short, ReHo was estimated on a voxel-by-voxel basis by calculating the KCC of a given voxel time series with its nearest 26 adjacent voxel time series. The KCC value was calculated as the voxel, and a separate KCC map was obtained for each subject. The data were then spatially smoothed with an 8-mm full-width at half-maximum Gaussian kernel to reduce the noise and residual in the gyrus anatomy.

Statistical Analysis

Two independent-sample, nonparametric tests were used to assess the age difference between ASD boys and TDs. To explore the differences in ReHo between ASD boys and TDs, the REST-State fMRI Data Analysis Toolkit (REST 1.8) was used to conduct a two-sample *t*-test on a single, normalized ReHo map, and the brain regions that had significant differences were found after the false discovery rate correction with $p < 0.05$. To determine the relationship between the ReHo alteration in different brain regions and the CARS, ABC, DQ, and age, Pearson correlation analysis was conducted in SPSS 20.0 software ($p < 0.05$).

[1]http://rfmri.org/DPARSF

RESULTS

Demographic and Clinical Characteristics

All of the data of the ASD and TD groups were retained after processing. There was no significant difference in age between the two groups. The values of the ABC, CARS, and DQ of the ASD group were consistent with the ASD standard as shown in **Table 1**. TD subjects were not measured by the corresponding scale.

Regional Spontaneous Activity Changes

Resting-state fMRI analysis showed that, compared with the TD group, ASD boys had increased ReHo in the right calcarine as well as decreased ReHo in the opercular part of the left inferior frontal gyrus (IFG operc), left middle temporal gyrus (MTG), left angular gyrus (AG), and right medial orbital frontal cortex (mOFC) (**Table 2** and **Figures 1**, **2**).

Correlation Between ReHo Values in Abnormal Regions and CARS, ABC, DQ, and Age

The ReHo values of the five brain regions and the values of the ABC, CARS, DQ, and age were in accordance with normal distribution; hence, Pearson correlation analysis was used. However, we did not find a significant correlation between any of the five brain regions and ASD-related scales or age.

DISCUSSION

In this study, based on a large sample size of resting-state fMRI, we used ReHo analysis to explore the alterations of spontaneous neural activity in the brain of ASD preschool boys. The results show that the ReHo value in the right calcarine of the primary

TABLE 1 | Demographic and clinical characteristics of ASD boys and TDs.

	ASD group (*n* = 86)	TD group (*n* = 54)	*Z*	*P*
Age	3.92 ± 0.95	4.09 ± 0.96	−1.693	0.09
ABC	68.12 ± 15.15			
CARS	34.17 ± 2.08			
DQ	53.44 ± 7.90			

Data are mean ± standard deviation. ABC, Autism Behavior Checklist; CARS, Childhood Autism Rating Scale; DQ, developmental quotient.

TABLE 2 | Brain regions with abnormal ReHo in ASD boys.

Brain region	Cluster size	MNI coordinates			AAL	Peak *T*-value
		X	*Y*	*Z*		
R calcarine	36	9	−90	3	Calcarine_R	5.1821
L IFG operc	22	−39	3	21	Frontal_Inf_Oper_L	−5.1215
L MTG	49	−42	−48	9	Temporal_Mid_L	−6.2140
L AG	27	−42	−57	27	Angular_L	−4.7905
R mOFC	21	6	51	−9	Frontal_Med_Orb_R	−4.8747

L, left; R, right; MNI, Montreal Neurological Institute; ReHo, regional homogeneity. IFG operc, opercular part of inferior frontal gyrus; MTG, middle temporal gyrus; AG, angular gyrus; mOFC, medial orbital frontal cortex; AAL, anatomical automatic labeling.

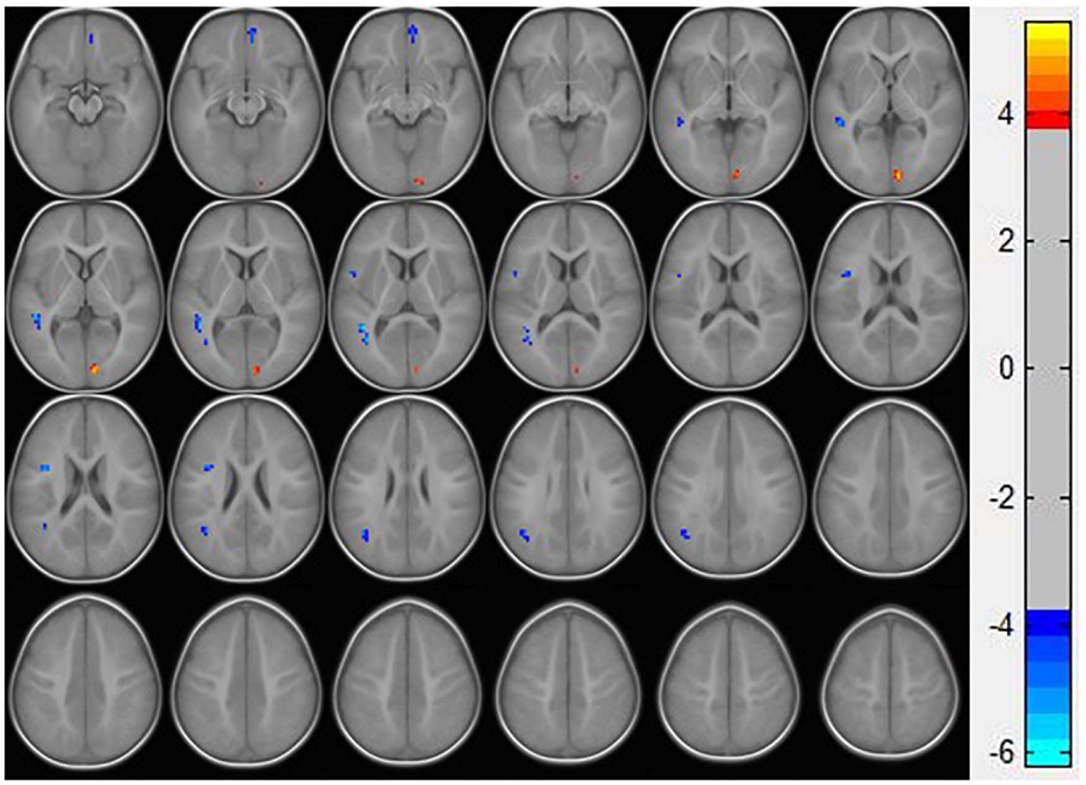

FIGURE 1 | Statistically significant differences between ASD boys and TDs are shown in a ReHo map of the whole brain with MRI. The ASD boys showed a significant ReHo increase in the right calcarine (warm colors) as well as a decrease in the opercular part of the left inferior frontal gyrus, left middle temporal gyrus, left angular gyrus, and right medial orbital frontal cortex with decreased ReHo values (cold colors). A T-score bar is shown on the right. Warm and cold colors denote increases and decreases in ReHo, respectively.

visual cortex was increased in preschool boys with ASD, and the ReHo value in the language-related brain region (left IFG operc, left MTG, and left AG) and right mOFC was decreased. No significant correlation was found between the abnormal brain regions and the score on the ASD scale.

We found that the ReHo value of the right calcarine increased, suggesting that there was an increase in local spontaneous neural activity in the right calcarine. The calcarine is an important part of the primary visual cortex, and abnormal visual processing in patients with ASD has been the focus of the research on a mechanism of ASD. Among them, many recent studies attributed the enhancement of the primary visual cortex of ASD patients to the functional compensation of semantic dysfunction (Samson et al., 2012; Chen et al., 2016); in fact, abnormal activity in the primary visual cortex of ASD patients at different ages has been frequently reported in previous studies. Peiker et al. (2015) found that the primary visual cortex of 24- to 45-year-old ASD patients had an abnormally strong neuromodulation to the intensity of visual stimuli by using magnetoencephalography. Based on ReHo analysis, Nair et al. (2018) also found local over-connectivity of the posterior visual region in the resting-state fMRI data of ASD patients aged 7–18 years. Similarly, the increase in spontaneous activity in the primary visual cortex was found in preschool-age boys with ASD in our study. Even before the onset of symptoms

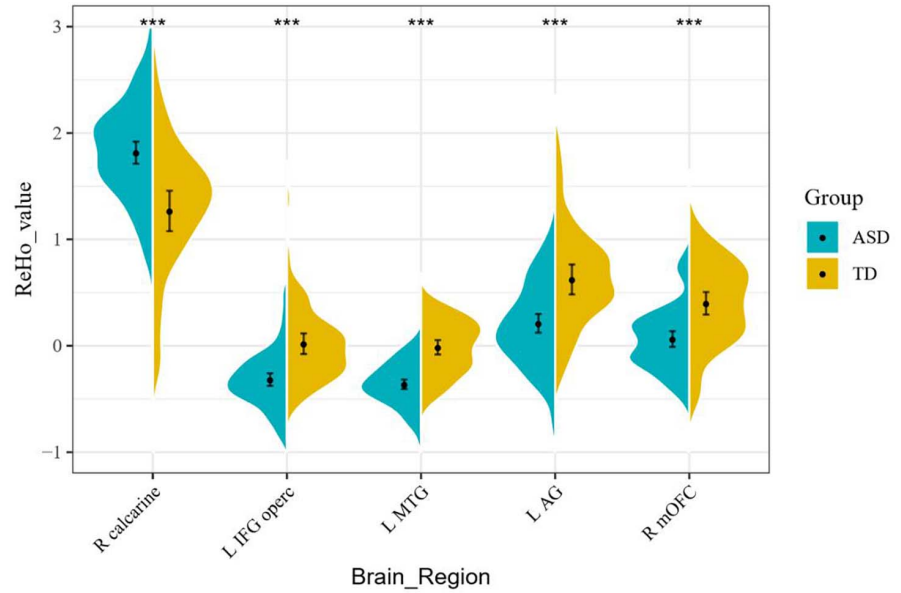

FIGURE 2 | ReHo signal values for altered regional brain regions between ASD boys and TDs.

in ASD, 9-month-old children with a high risk of ASD who developed ASD subsequently also had an enhanced visual-search ability related to the severity of ASD that subsequently was identified (Gliga et al., 2015). In consequence, it is reasonable to assume that abnormal visual processing in patients with ASD is not the secondary result of possible functional compensation. In contrast to the compensatory viewpoint, using a dense-array electroencephalography method, Ronconi et al. (2018) found that individuals with ASD demonstrated an advantage in detail-focused tasks, which tended to suggest that the imbalance between neuroenhancement and inhibitory mechanisms in the primary visual cortex in patients with ASD contributes to this phenomenon. Thus, we suggest that increased spontaneous activity of the primary visual cortex may be one correlative variable of ASD itself.

Simultaneously, we found that there was a decrease of spontaneous neural activity in the left IFG operc and left MTG in preschool boys with ASD. The former is a part of Broca's area, which is an important area related to semantic expression and comprehension (Bednarz et al., 2017), whereas the latter is an important part of the semantic system in Wernicke's brain area, which is mainly responsible for semantic representation and vocabulary storage (Binder et al., 2009). The left MTG can assist the left IFG in semantic understanding and retrieval from vocabulary storage to participate in the selection process of lexical association degree (Wong et al., 2019). Previous studies have found that, in ASD patients, the left MTG and left IFG present simultaneous decrease activation (Ogawa et al., 2019) and reduce connectivity between them (Sahyoun et al., 2010). Moreover, with the improvement of language comprehension in ASD patients, after reading intervention, the reduced connectivity between the left MTG and the left IFG can be restored (Murdaugh et al., 2016). The results that we found showed a decrease in spontaneous neural activity in the left IFG operc and left MTG, which is consistent with previous studies. Therefore, although the decrease of spontaneous neural activity in the left IFG operc and left MTG we found has no significant correlation with the score on CARS and ABC, we still have reason to speculate that these decreases are closely related to the early language development delay of ASD.

In this study, decreased local spontaneous neural activity in the left AG was also found. The AG is considered an important brain region responsible for episodic memory. The AG may play a particular role in behaviors requiring fluent conceptual combination, such as sentence comprehension, discourse, problem solving, and planning (Binder et al., 2009). Some researchers have found that inhibiting AG activity can reduce subjects' retrieval of episodic details in past and future events (such as time, place, and person), thus interrupting or weakening episodic simulation and memory (Thakral et al., 2017). Thus, we speculate that the decreased local spontaneous neural activity of the left AG may be related to the symptom of episodic prediction and episodic memory as well as the inversion of words and poor intelligibility in language expression of ASD children. In addition, the abnormal AG was also found in previous studies of ASD magnetoencephalography (Lajiness-O'Neill et al., 2018), and the impairment of episodic memory in ASD patients has been repeatedly replicated (Hutchins and Prelock, 2018), which further demonstrates our view.

The decreased local spontaneous neural activity in the right mOFC was observed in this study as well. The mOFC, as a part of the prefrontal cortex, is thought to be associated with complex emotions and reward and punishment processing, which are essential for social behavior (Kandilarova et al., 2019). Based on fMRI analysis of patients with obsessive-compulsive disorder (OCD), Fan et al. (2017) found that the abnormality

of the right mOFC may cause patients with OCD to lose the ability to judge the value of compulsive behaviors, resulting in compulsive behaviors. Another fMRI neuro-basic study of ASD in adolescents also found that reduced OFC activation was associated with abnormal reward decision making in patients with ASD (Carlisi et al., 2017). We replicated this result, which may indicate that ASD patients have altered their reward decision making since childhood, resulting in a failure to make value judgments about repetitive and stereotyped behaviors. This is also consistent with the finding that males with ASD exhibit more stereotypical behaviors than females (Retico et al., 2016).

We did not find a correlation between the ASD preschool boys' abnormal brain regions and the ASD score scale. Possible reasons may be that (Lai et al., 2014) our study did not further subdivide the ASD group into high- or low-functioning autism or (Damasio and Maurer, 1978) the boys with ASD in our study were too young to score by themselves, so the CARS (scored by a doctor) and ABC (scored by a parent) are subjective. Therefore, we still cannot rule out that these abnormal brain regions do correlate with the severity of ASD.

There are some limitations in our study. First, we only investigated the ReHo of preschool boys with ASD although ASD has atypical brain development trajectories (Mevel et al., 2015) and sex differences (Ecker et al., 2017). As such, the interpretation of our results cannot be extended to ASD boys of other ages or females. Second, the results we observed still cannot completely explain that secondary changes in brain development of ASD have been excluded. In fact, 9-month-old high-risk ASD infants (later diagnosed with ASD) have been shown to have enhanced visual search ability (Cheung et al., 2018). Therefore, our next research analysis should focus on the prospective study of brain function alterations in high-risk ASD children.

Finally, based on resting-state fMRI data, the brain regions with functional abnormalities in preschool boys with ASD were found by using ReHo analysis. We suggest that increased spontaneous activity of the primary visual cortex might be a potential primary disorder of boys with ASD. In addition, some brain regions (e.g., the left IFG operc, left MTG, and left AG and right mOFC) may partly explain the core symptoms of ASD, such as social dysfunction and stereotyped behavior(s). Given that more than 60% of children with ASD are prescribed for any clinical indication and that more than 41% of them are taking more than one psychotropic drug with limited effect (Lamy et al., 2020), these results may help us to provide new targets for treatment and better understand the clinical features of preschool boys with ASD.

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 Ethics Committee of Shenzhen Children's Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

ZL: validation, formal analysis, investigation, writing – original draft, and visualization. SX: software, investigation, resources, and funding acquisition. YW: methodology and software. LX, KH, and McL: methodology. ML and YY: software. CL, SH, and YF: validation. GJ: conceptualization, data curation, writing – review and editing, supervision, project administration, and funding acquisition. TW: formal analysis, data curation, writing – review and editing, and funding acquisition. All authors contributed to the article and approved the submitted version.

FUNDING

This study has received funding by the National Natural Science Foundation of China (Grant Nos. U1903120 and 81771807), the Guangzhou Science and Technology Plan Project (Grant No. 202002030234), the Science Foundation of Guangdong Second Provincial General Hospital (grant no. YQ2016-010) and the Sanming Project of Medicine in Shenzhen (Grant Nos. SZSM202011005 and SZSM201612036).

ACKNOWLEDGMENTS

We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript. The authors wish to express their thanks to all the critics who have made great improvements to the article. We also thank all the volunteers who participated in this study.

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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.

frontiers in Neuroscience

BRIEF RESEARCH REPORT
published: 26 May 2021
doi: 10.3389/fnins.2021.660330

Relationship Between Social Motivation in Children With Autism Spectrum Disorder and Their Parents

Mirko Uljarević[1,2], Thomas W. Frazier[3], Booil Jo[4], Jennifer M. Phillips[4], Wesley Billingham[5], Matthew N. Cooper[5] and Antonio Y. Hardan[4]*

[1] Melbourne School of Psychological Sciences, University of Melbourne, Melbourne, VIC, Australia, [2] School of Psychology and Public Health, La Trobe University, Melbourne, VIC, Australia, [3] Department of Psychology, John Carroll University, University Heights, OH, United States, [4] Department of Psychiatry and Behavioral Sciences, Stanford University, Stanford, CA, United States, [5] Telethon Kids Institute, University of Western Australia, Perth, WA, Australia

Impairment in social motivation (SM) has been suggested as a key mechanism underlying social communication deficits observed in autism spectrum disorder (ASD). However, the factors accounting for variability in SM remain poorly described and understood. The current study aimed to characterize the relationship between parental and proband SM. Data from 2,759 children with ASD (M_{age} = 9.03 years, SD_{age} = 3.57, 375 females) and their parents from the Simons Simplex Collection (SSC) project was included in this study. Parental and proband SM was assessed using previously identified item sets from the Social Responsiveness Scale (SRS). Children who had parents with low SM scores (less impairments) showed significantly lower impairments in SM compared to children who had either one or both parents with elevated SM scores. No parent-of-origin effect was identified. No significant interactions were found involving proband sex or intellectual disability (ID) status (presence/absence of ID) with paternal or maternal SM. This study establishes that low SM in children with ASD may be driven, in part, by lower SM in one or both parents. Future investigations should utilize larger family pedigrees, including simplex and multiplex families, evaluate other measures of SM, and include other related, yet distinct constructs, such as social inhibition and anhedonia. This will help to gain finer-grained insights into the factors and mechanisms accounting for individual differences in sociability among typically developing children as well as those with, or at risk, for developing ASD.

Keywords: social motivation, familiality, broader autism phenotype, autism spectral disorder, heterogeneity

OPEN ACCESS

Edited by:
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Emory University, United States

****Correspondence:***
Mirko Uljarević
mirko.uljarevic@unimelb.edu.au

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: *29 January 2021*
Accepted: *14 April 2021*
Published: *26 May 2021*

Citation:
Uljarević M, Frazier TW, Jo B, Phillips JM, Billingham W, Cooper MN and Hardan AY (2021) Relationship Between Social Motivation in Children With Autism Spectrum Disorder and Their Parents. Front. Neurosci. 15:660330. doi: 10.3389/fnins.2021.660330

INTRODUCTION

Social motivation (SM), or the drive to engage, affiliate, and interact with others, has been proposed as a crucial factor for human adaptation and survival throughout evolution (Boyd et al., 2011; Tomasello et al., 2012). Lack or low levels of SM during very early development has been suggested as a key mechanism behind the subsequent social interaction and communication impairments that characterize autism spectrum disorder (ASD) (Chevallier et al., 2012; Kohls et al., 2012). More specifically, it has been hypothesized that due to low SM, children with ASD are less likely to orient to socially salient stimuli that provide key information for learning and the development and

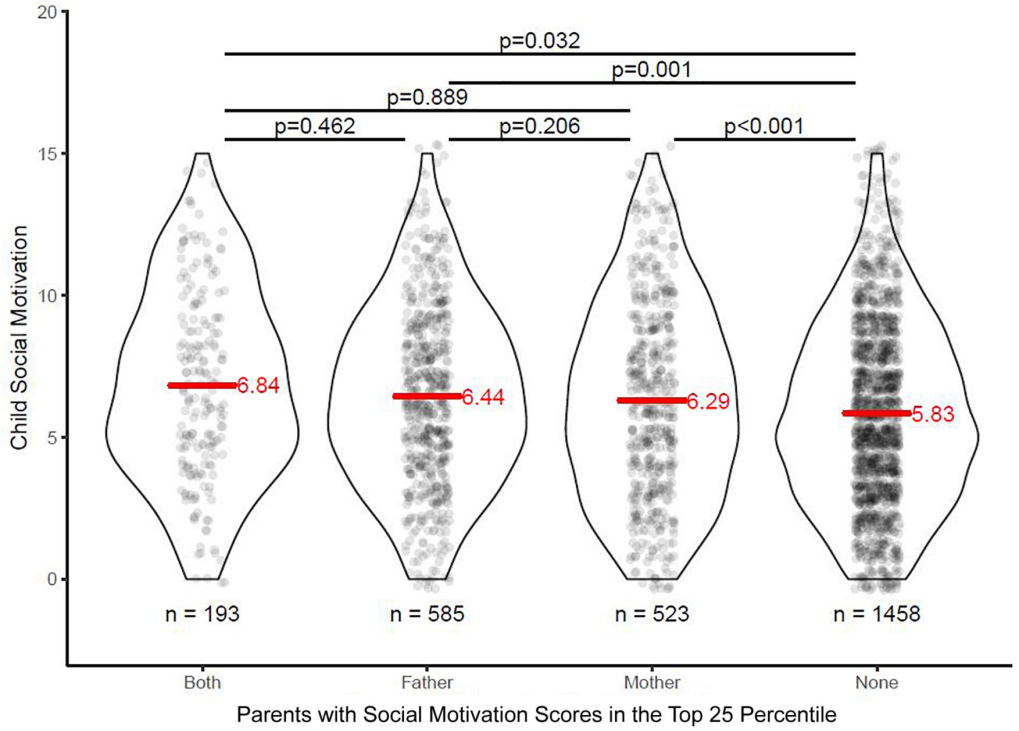

FIGURE 1 | Children's social motivation scores as a function of parental social motivation status. Both, both mother and father had SM scores in the top 25 percentile of the respective score distribution; neither, neither mother or father had SM scores in the top 25 percentile of the respective score distribution; SM, social motivation.

specialization of brain circuits underpinning processes crucial for the ability to successfully navigate the complexities of the social world (Mundy, 1995; Dawson et al., 2005). Although the described causal pathway is yet to be confirmed through longer-term longitudinal studies, several lines of evidence provide some support for the SM theory. Firstly, lack of orienting to, and preference for, visual and auditory social stimuli, have been found during early development (Dawson et al., 1998; Osterling et al., 2002; Klin et al., 2009; Falck-Ytter et al., 2013) and throughout later childhood and adolescence (Klin et al., 2002; Sasson et al., 2011; Chevallier et al., 2015; Wright et al., 2016). Secondly, both structural and functional neuroimaging studies have provided consistent evidence for atypicality in key brain regions within the reward processing circuitry (Scott-Van Zeeland et al., 2010; Delmonte et al., 2012; Herrington et al., 2017; Kohls et al., 2018), although it is still unclear whether noted deficits are constrained to social rewards or extend across other reward types (Clements et al., 2018). Importantly, Naturalistic Developmental Behavioral Interventions such as the Early Start Denver Model (ESDM) (Rogers and Dawson, 2010) and Pivotal Response Treatment (PRT) (Koegel et al., 1999) that focus, among other aspects, on SM as a treatment target, have been shown to be effective in improving a range of skills and domains and to result in the need for fewer services later in life (Cidev et al., 2017; Sandbank et al., 2020).

There is pronounced variability in SM among individuals with ASD, with some individuals lacking social interest and awareness of others or actively avoiding social interactions, and others showing the strong drive to form and sustain friendships and romantic relationships and often experiencing loneliness (Wing and Gould, 1979; Bauminger et al., 2008; Calder et al., 2012; Mendelson et al., 2016; Uljarević et al., 2020a). However, despite the centrality of SM in ASD, the factors accounting for large individual differences in this domain remain poorly characterized and understood. Across a range of neurodevelopmental disorders, even in cases of deleterious *de novo* mutations, parental traits have been shown to provide a substantial contribution to the phenotypic variability in children's morphological, behavioral and cognitive characteristics (Hanson et al., 2014; Moreno De Luca et al., 2015; Klaassen et al., 2016;

TABLE 1 | Summary of *post hoc* comparisons.

Contrast	Estimate	95% CI	Adjusted *p*-value
Only father-both	−0.40	−1.11 to 0.3	0.462
Only mother-both	−0.54	−1.26 to 0.17	0.206
Only mother-only father	−0.14	−0.66 to 0.37	0.889
Neither-both	−1.01	−1.66 to −0.36	0.000
Neither-only father	−0.61	−1.02 to −0.19	0.001
Neither-only mother	−0.46	−0.9 to −0.03	0.032

Both parents, both mother and father had SM scores in the top 25 percentile of the respective score distribution; neither, neither mother or father had SM scores in the top 25 percentile of the respective score distribution; SM, social motivation.

Evans and Uljarević, 2018). Therefore, consideration of SM among parents of children with ASD might provide a potentially promising means for understanding the sources of individual variability in SM among their children.

The presence of the broader autism phenotype (BAP) among parents and family members of individuals with ASD has been recognized since original clinical descriptions by Kanner (1943). Subsequent studies have provided robust empirical evidence that parents of children with ASD tend to show higher levels of difficulties in language, communication, social interaction, and cognition as well as the presence of certain higher-order repetitive behaviors when compared to the general population (Gerdts and Bernier, 2011; Sucksmith et al., 2011). Importantly, evidence of familiality and inter-generational transmission of these traits has also been reported (Virkud et al., 2009; De la Marche et al., 2012; Taylor et al., 2013; Lyall et al., 2014; Uljarević et al., 2016). Both clinical observations by Kanner (1943) and several studies that focused on personality characteristics (e.g., Bolton et al., 1994; Piven et al., 1997; Bailey et al., 1998) have reported traits indicative of lower levels of SM among parents of children with ASD; however, the pattern of relationship between SM in children with ASD and their parents remains largely unexplored. The only exceptions are a study by Sung et al. (2005) that demonstrated high heritability of SM in a sample of 201 families with a child with ASD and a study by Jones et al. (2017) that reported an association between lower levels of parental SM with shorter peak look at faces in their infant children. However, Sung et al. (2005) used the SM subscale of the Broader Autism Phenotype Scale (Dawson et al., 2007) which consists of only two items, therefore providing limited range. Similarly, Jones et al. (2017) used the Social Competence Questionnaire (Sarason et al., 1985) and the Social Avoidance and Distress Scale (Watson and Friend, 1969) that assess social comfort and social anxiety, respectively, rather than directly assessing SM. In addition to measurement limitations, both studies were limited by small sample size.

The current study aimed to characterize the relationship between parental and proband SM. It was hypothesized that higher levels of SM impairments in parents would be associated with higher levels of SM impairment in their children with ASD. Given the well established sex differences in SM across normative development and neurodevelopmental disorders, including ASD (Sedgewick et al., 2016; Uljarević et al., 2020b,c), we aimed to explore the possibility of sex-specific transmission of SM. Recent findings suggest that familial risk and heritability may vary depending on the presence or absence of intellectual disability (ID) in probands (Xie et al., 2019), therefore, the familiality pattern of SM depending on the IQ status of the child with ASD was investigated. In this study, parent and proband SM was measured by the SM factor derived in our

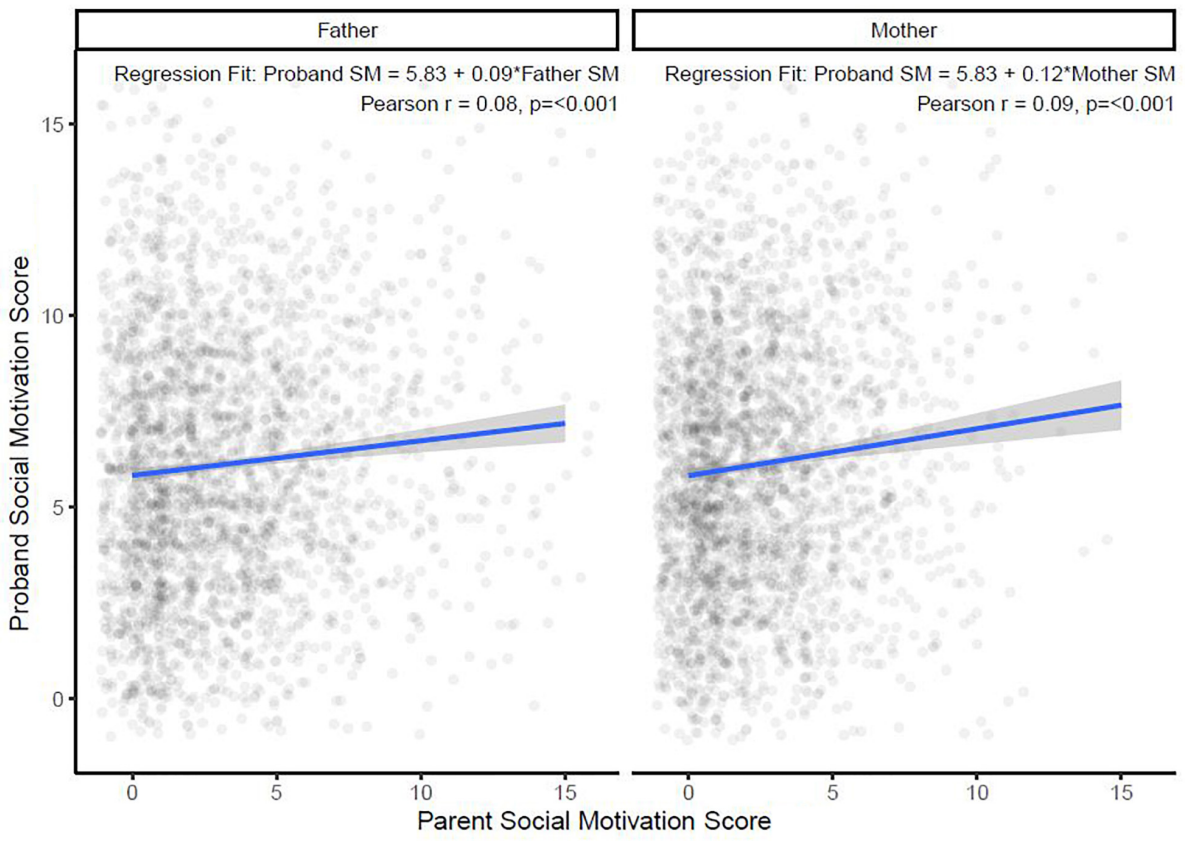

FIGURE 2 | Relationship between paternal and maternal social motivation scores with autistic children's with social motivation scores. SM, social motivation.

recent analysis of the Social Responsiveness Scale (SRS-2; Constantino and Gruber, 2005, 2012). The SM factor utilized here was derived in a large sample of N = 27,953 individuals spanning normative and atypical development, including ASD (Uljarević et al., 2020b). We have opted for this specific SRS-2 subscale over the original SM subscale proposed by Constantino and Gruber (2005, 2012) given that the latter was not supported by any of the SRS/SRS-2 factor analytic investigations (e.g., Frazier et al., 2014; Uljarević et al., 2020b). Factor analysis by Frazier et al. (2014) derived a social avoidance factor that included several items related to SM, however, this factor also contained several items that do not readily map onto the construct of SM (e.g., "Expressions on his/her face don't match what he/she is saying", and "Is too tense in social situations"). Therefore, to ensure that several distinct constructs are not conflated within a single factor, we have chosen to focus on the SM scale derived in our work given that it was specifically optimized to capture only that specific construct and excluded any other broad/not-related items.

METHODS

Participants

Data was obtained from the Simons Simplex Collection (SSC) project. The SSC consisted of a sample of clinically referred individuals with a diagnosis of ASD but without any other medical conditions and their families. Participants were recruited from 12 university-based sites (Fischbach and Lord, 2010). No age restrictions were applied. Data from 2,759 children with ASD (M_{age} = 9.03 years, SD_{age} = 3.57, range: 4–18 years; 375 females) and their parents [N = 2,747 fathers (M_{age} = 42.5 years, SD_{age} = 6.4, range: 22–55 years); N = 2,752 mothers (M_{age} = 40.4 years, SD_{age} = 5.7, range: 21–58 years)] was included in this study.

Procedures and Measures

This study was approved by the Stanford University Institutional Review Board. All participants or their parent/legal guardian have provided informed consent for participation as part of SSC.

The Social Responsiveness Scale (*SRS*; Constantino and Gruber, 2005, 2012). The SRS is a 65-item measure designed to index autism trait severity. Each item is rated on a 4-point Likert scale (from 1 = Not True to 4 = Almost Always True) with higher scores indicating higher trait severity/atypicality. Mothers and fathers rated their own traits and behaviors using the adult SRS form, and mothers completed a parent-report version of the SRS for their child with ASD. As noted, in this study we utilized the subscale derived in our previous work (Uljarević et al., 2020b) that contains five items and captures SM. Although originally labeled as Attachment and Affiliation to be aligned with the Research Domain Criteria nomenclature that does not specifically highlight SM as a distinct construct, all five items within this factor map onto the SM construct and do not include attachment-related aspects. In this sample, the SM subscale derived in our recent study (Uljarević et al., 2020b) showed good internal consistency in fathers (α = 0.81) and acceptable internal consistency in mothers (α = 0.74) and children with ASD (α = 0.74). We have chosen a five-item SM factor derived in our previous work over the originally proposed, theoretically derived SRS Social Motivation Scale (Constantino and Gruber, 2005, 2012) which has not been replicated in the subsequent factorizations of the SRS and over the Social Avoidance SRS factor derived by Frazier et al. (2014) given that this factor included several items that do not readily map onto SM (e.g., "Expressions on his/her face don't match what he/she is saying", and "Is too tense in social situations").

TABLE 2 | Summary of regression models.

	Estimate	95% CI	*F*	*t*	*p*	R^2
Model 1			22.87			0.008
SM mother	0.12	0.07 to 0.017		4.78	<0.001	
Model 2			19.65			0.007
SM father	0.09	0.05 to 0.13		4.43	< 0.001	
Model 3			14.55			0.015
SM mother	0.13	0.06 to 0.20		3.62	<0.001	
SM father	0.10	0.04 to 0.15		3.53	<0.001	
SM mother × SM father	−0.00	−0.02 to 0.01		−0.31	0.757	
Model 4			21.79			0.015
SM mother	0.12	0.07 to 0.18		4.85	<0.001	
SM father	0.09	0.05 to 0.13		4.58	<0.001	
Model 5			7.77			0.007
SM mother	0.13	−0.00 to 0.26		1.96	0.051	
Proband sex	−0.09	−0.61 to 0.42		−0.35	0.728	
SM Mother × proband sex	−0.01	−0.15 to 0.13		−0.16	0.876	
Model 6			7.21			0.007
SM father	0.03	−0.08 to 0.14		0.55	0.582	
Proband sex	−0.37	−0.89 to 0.15		−1.39	0.165	
SM father × proband sex	0.07	−0.05 to 0.19		1.17	0.244	
Model 7			29.19			0.030
SM mother	0.10	0.01 to 0.20		2.11	0.035	
Proband ID	−1.17	−1.54 to −0.80		−6.16	<0.001	
SM mother × proband ID	0.03	−0.08 to 0.15		0.59	0.553	
Model 8			29.96			0.031
SM father	0.05	−0.03 to 0.13		1.18	0.238	
Proband ID	−1.35	−1.73 to −0.97		−6.98	<0.001	
SM father × proband ID	0.08	−0.01 to 0.17		1.71	0.088	
Model 9			28.72			0.039
SM mother	0.13	0.08 to 0.18		5.14	<0.001	
SM father	0.11	0.07 to 0.15		5.45	<0.001	
Proband sex	0.02	−0.34 to 0.38		0.13	0.9	
Proband ID	−1.14	−1.41 to −0.88		−8.33	<0.001	

CI, confidence intervals; ID, presence/absence of intellectual disability; SM, social motivation.

RESULTS

Effects of parental SM on children's SM was firstly investigated by conducting a comparison between children whose mother or father had elevated SM scores. Elevated parental SM score was defined as the top 25th percentile of the score distribution for mothers and fathers, respectively, and the remaining distribution was used as the referent group. Children whose parents both reported low personal SM scores (lower impairment) showed significantly lower impairment in SM compared to children who had either one or both parents with elevated SM scores (**Figure 1**). A cross-tabulation of these dichotomous SM impairment factors for mothers and fathers resulted in four groups (neither parent with elevated SM scores, only mother with elevated SM scores, only father with elevated SM scores, both parents with elevated SM scores). An analysis of variance (ANOVA) on child SM scores showed a significant difference between these groups, $F(3,2743) = 9.01$, $p < 0.001$, and a subsequent Tukey's *post hoc* test showed that child had significantly poorer SM when either one or both parents had elevated SM scores. However, child SM was not significantly exasperated when both parents had elevated SM scores compared to just one parent. Please see **Figure 1** for the score distribution and **Table 1** for a detailed overview of the *post hoc* comparisons.

Next, a linear regression model was used to investigate the relationship between SM scores of parents and their child with ASD (**Figure 2**). An increase of 1 unit in mother SM score was significantly associated with a small increase (0.12; 95% CI: 0.07, 0.17; $p < 0.001$; Model 1, **Table 2**) in child SM, and the same 1 unit increase in father SM was significantly associated with a similarly small increase (0.09; 95% CI: 0.05, 0.13; $p < 0.001$; Model 2 in **Table 2**) in child SM. A multivariate regression model was then fitted with child SM as the outcome and both mother and father SM included in the model with an interaction term (Model 3, **Table 2**). The interaction term was non-significant and therefore dropped from the final model, which showed a cumulative effect of maternal SM (0.12; 95% CI: 0.07, 0.18; $p < 0.001$) and paternal SM (0.09; 95% CI: 0.05, 0.13; $p < 0.001$; Model 4, **Table 2**) on child SM. Full regression models are presented in **Table 2**.

Further multiple regression models were used to examine whether the effect of each parent's SM on the child's SM depended on the child's sex [**Figure 3**; models 5 (effect of maternal SM) and 6 (effect of paternal SM) in **Table 2**] and/or on the presence/absence of ID in the child [**Figure 4**; models 7 (effect of maternal SM) and 8 (effect of paternal SM) in **Table 2**]. No significant sex interaction with paternal or maternal SM was found. The observed paternal effect on a male child was over threefold higher than on a female child, however, it was

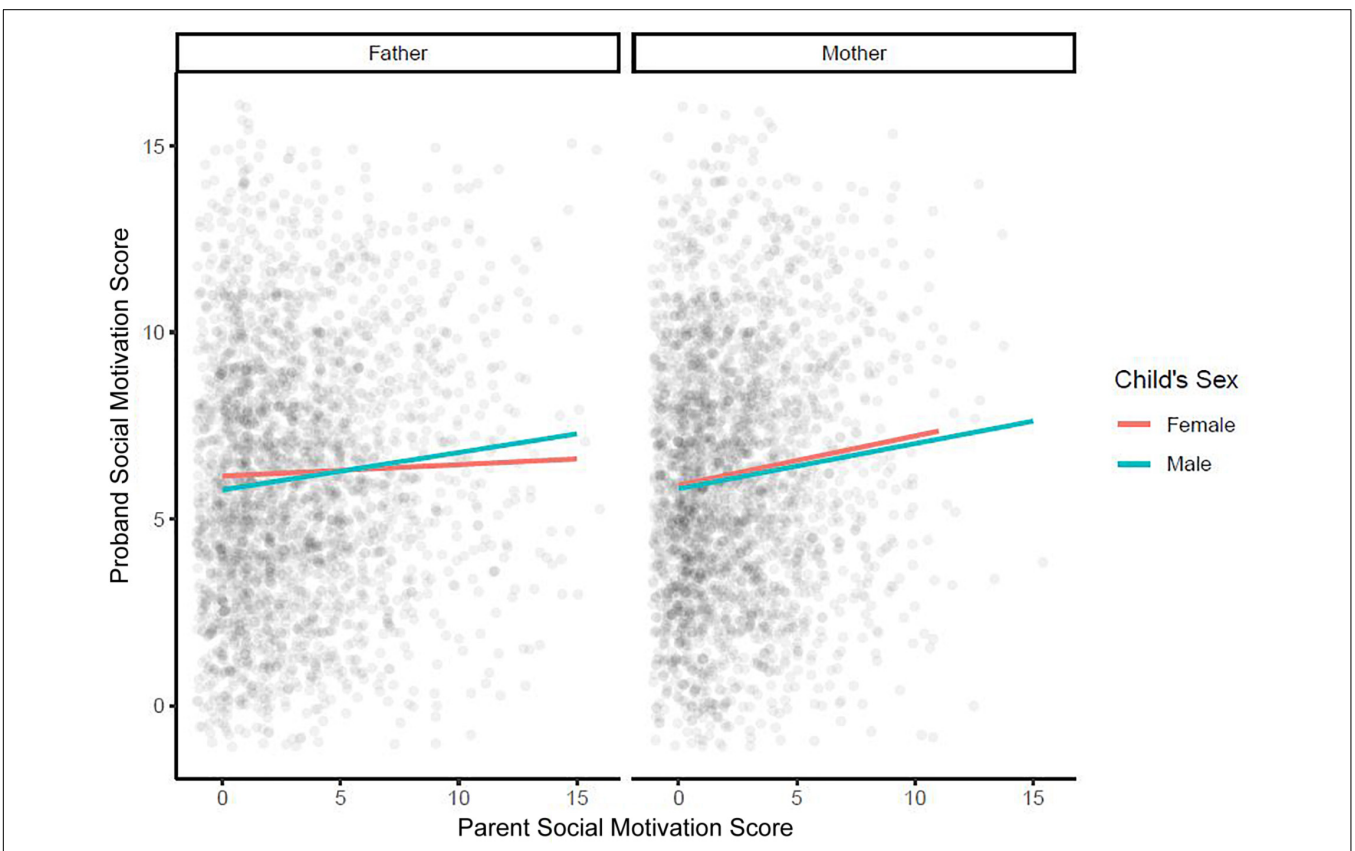

FIGURE 3 | Multiple regression model examining whether the effect of each parent's social motivation on a child's social motivation depends on a child's sex. SM, social motivation.

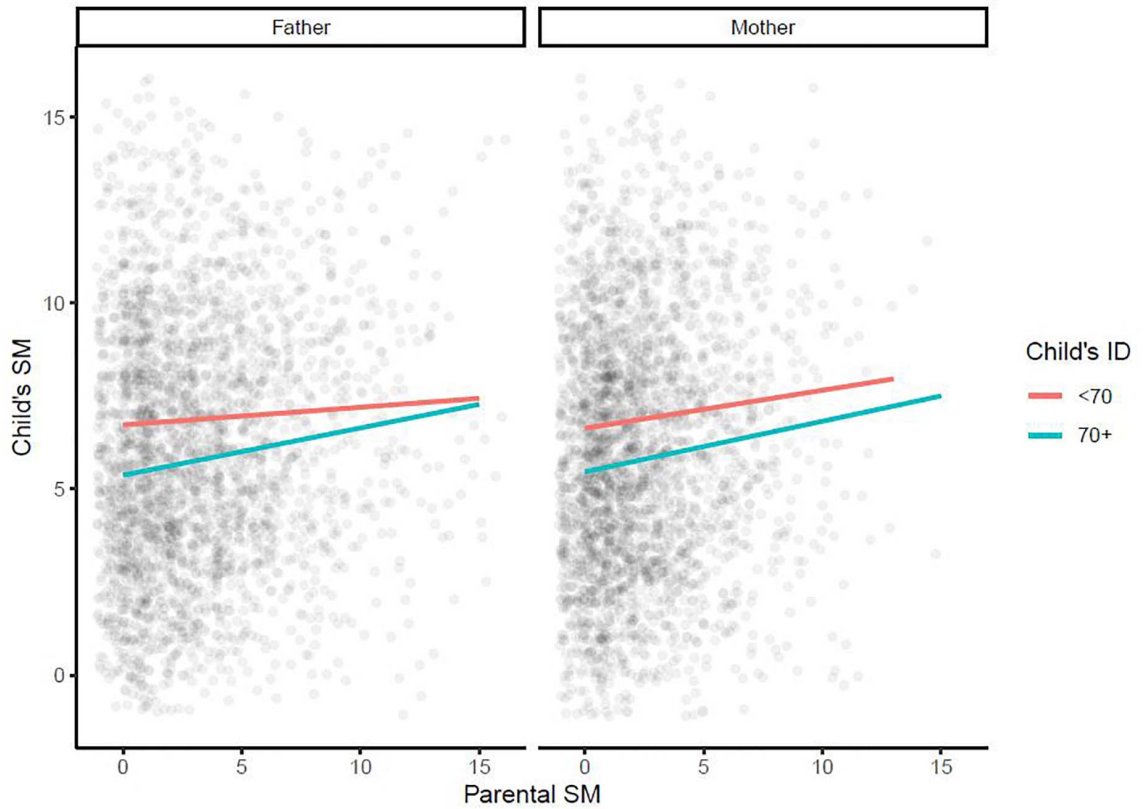

FIGURE 4 | Multiple regression model examining whether the effect of each parent's social motivation on a child's social motivation depends on the presence/absence of intellectual disability in children. ID, intellectual disability; SM, social motivation.

not statistically significant. No significant IQ interaction with paternal or maternal SM was found. A final multiple regression model was run with mother SM, father SM, child sex, and child ID as covariates (Model 9, **Table 2**). Mother SM (0.13; 95% CI: 0.08, 0.18; $p < 0.001$), father SM (0.11; 95% CI: 0.07, 0.15; $p < 0.001$) and ID (−1.14; 95% CI: −1.41, −0.88, $p < 0.001$) were all significant predictors of child SM, while sex (male: 0.02, 95% CI: −0.34, 0.38, $p = 0.9$) was not significant as a predictor. Full regression models are presented in **Table 2**.

DISCUSSION

The current study aimed to examine the familiality of SM by exploring the link between parental and proband the social responsiveness scale (SRS-2) SM scores. Our analysis demonstrated that low levels of paternal and maternal SM were associated with a significant deficit in SM in children with ASD. Importantly, these effects were independent and cumulative, and no parent-of-origin effect was found. This finding is in line with two previous studies that have investigated familiality and heritability of SM in small samples of families of children with ASD (Sung et al., 2005) and those with typically developing youth (Jones et al., 2017). While indications for potential sex-specific transmission of SM were observed as paternal effect on a male child with ASD was over three-fold higher than the effect on a female child with ASD, this effect was no statistically significant and these findings should therefore be interpreted as very preliminary and warrant further replication.

The present study used the SSC data which is a relatively large and well-characterized sample of mother-father-child with ASD triads. In contrast to previous studies by Sung et al. (2005) and Jones et al. (2017) who used a two-item subscale and constructs of social discomfort and anxiety to capture SM, respectively, our investigation utilized SM items derived from the SRS in our recent SRS factorization (Uljarević et al., 2020b). The SM scale used here had good conceptual clarity as it encompasses only items directly relating to the drive for social approach/to interact socially. However, the findings reported here should also be considered in light of several limitations. Firstly, we relied on a questionnaire measure of SM and therefore a potential impact of the common method variance will need to be considered. This is particularly relevant in the light of the findings by De la Marche et al. (2015) and Jones et al. (2017) that emphasize potential method-specific (questionnaire versus more objective assessments and experimental protocols) pattern of findings in the studies of similar design as ours. Therefore, it will be crucial to replicate and further refine findings reported here by utilizing multi-method assessment protocols. Secondly, the sample used here only included simplex families

and did not include a general population sample. Given the suggestions that etiologic mechanisms operating within simplex and multiplex families might be somewhat distinct (Virkud et al., 2009; Lyall et al., 2014), it will be important for future studies to better characterize the pattern of transmission of SM depending on simplex versus multiplex status and whether any potential specificities would emerge when compared to the transmission pattern in the general population. Thirdly, although SSC database afforded a significantly larger sample size for female participants. However, given the well established over-representation of ASD in males, the sample used in this study was nevertheless heavily skewed toward male participants, which could have impacted the ability to detect some of the more nuanced sex-specific effects. Therefore, it will be important for future studies to further investigate the possibility of sex-specific transmission of SM.

Importantly, SM is a complex construct and has been suggested to encompass a range of inter-related elements including social orienting, seeking enjoyment in social interactions, and behaviors and actions aimed at maintaining social bonds (Chevallier et al., 2012). The SM scale used here only captures the seeking/enjoyment element, and it is not clear whether the familiality pattern would be continuous with the social orienting and maintenance elements, or whether potential discontinuities might arise. Despite the centrality of the SM construct in ASD, there is a paucity of instruments that can effectively and comprehensively capture individual differences in SM in a sensitive and quantitative manner. The recently developed Stanford Social Dimensions Scale (SSDS) (Phillips et al., 2019) has been specifically designed to capture a broad spectrum of traits and behaviors indicative of the seeking/linking and maintenance components described by Chevallier et al. (2012) and shows promising psychometric properties and ability to capture individual differences in distinct SM subdomains in children and adolescents with ASD (Uljarević et al., 2020a). It will therefore be crucial for future investigations to incorporate the SSDS and other scales capturing related, yet distinct constructs such as social inhibition and anhedonia, to gain an in-depth insight into the factors and mechanisms accounting for the individual differences in key determinants of sociability among children with, and at risk, for developing ASD.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: www.sfari.org.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Stanford University Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MU, TF, and AH designed the study. MU, WB, and MC analyzed the data. MU, TF, BJ, JP, WB, and AH wrote the manuscript. All authors reviewed the manuscript and approved the final version.

FUNDING

This study was supported by the R03MH111846-01 National Institute of Mental Health grant (AH and BJ) by the National Institute of Mental Health. MU was currently supported by the Australian Research Council Discovery Early Career Researcher Award (DE180100632).

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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.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 17 June 2021
doi: 10.3389/fnins.2021.669194

Altered Gray-White Matter Boundary Contrast in Toddlers at Risk for Autism Relates to Later Diagnosis of Autism Spectrum Disorder

Michel Godel[1], Derek S. Andrews[2], David G. Amaral[2], Sally Ozonoff[2], Gregory S. Young[2], Joshua K. Lee[2], Christine Wu Nordahl[2†] and Marie Schaer[1†]*

[1] Department of Psychiatry, University of Geneva School of Medicine, Geneva, Switzerland, [2] Department of Psychiatry and Behavioral Sciences, The Medical Investigation of Neurodevelopmental Disorders (MIND) Institute, UC Davis School of Medicine, University of California, Davis, Sacramento, CA, United States

OPEN ACCESS

Edited by:
Eugenia Conti, Fondazione Stella Maris (IRCCS), Italy

Reviewed by:
Anila Maria D'Mello, Massachusetts Institute of Technology, United States
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Yuta Aoki, Showa University, Japan

***Correspondence:**
Michel Godel
michel.godel@unige.ch

† These authors have contributed equally to this work and share senior authorship

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: *18 February 2021*
Accepted: *18 May 2021*
Published: *17 June 2021*

Citation:
Godel M, Andrews DS, Amaral DG, Ozonoff S, Young GS, Lee JK, Wu Nordahl C and Schaer M (2021) Altered Gray-White Matter Boundary Contrast in Toddlers at Risk for Autism Relates to Later Diagnosis of Autism Spectrum Disorder. Front. Neurosci. 15:669194. doi: 10.3389/fnins.2021.669194

Background: Recent neuroimaging studies have highlighted differences in cerebral maturation in individuals with autism spectrum disorder (ASD) in comparison to typical development. For instance, the contrast of the gray-white matter boundary is decreased in adults with ASD. To determine how gray-white matter boundary integrity relates to early ASD phenotypes, we used a regional structural MRI index of gray-white matter contrast (GWC) on a sample of toddlers with a hereditary high risk for ASD.

Materials and Methods: We used a surface-based approach to compute vertex-wise GWC in a longitudinal cohort of toddlers at high-risk for ASD imaged twice between 12 and 24 months (n = 20). A full clinical assessment of ASD-related symptoms was performed in conjunction with imaging and again at 3 years of age for diagnostic outcome. Three outcome groups were defined (ASD, n = 9; typical development, n = 8; non-typical development, n = 3).

Results: ASD diagnostic outcome at age 3 was associated with widespread increases in GWC between age 12 and 24 months. Many cortical regions were affected, including regions implicated in social processing and language acquisition. In parallel, we found that early onset of ASD symptoms (i.e., prior to 18-months) was specifically associated with slower GWC rates of change during the second year of life. These alterations were found in areas mainly belonging to the central executive network.

Limitations: Our study is the first to measure maturational changes in GWC in toddlers who developed autism, but given the limited size of our sample results should be considered exploratory and warrant further replication in independent and larger samples.

Conclusion: These preliminary results suggest that ASD is linked to early alterations of the gray-white matter boundary in widespread brain regions. Early onset of ASD diagnosis constitutes an independent clinical parameter associated with a specific corresponding neurobiological developmental trajectory. Altered neural migration and/or altered myelination processes potentially explain these findings.

Keywords: autism spectrum disorder, sibling risk, toddlers, neurodevelopment, neuroimaging, FreeSurfer

BACKGROUND

Autism Spectrum Disorder (ASD) is a heterogeneous neurodevelopmental disorder characterized by difficulties in the domains of social interactions and communication, along with repetitive behaviors and restricted interests (American Psychiatric Association, 2013; Lai et al., 2014). ASD affects 1 in 54 individuals with an increasing prevalence over the past decades (Weintraub, 2011; Maenner et al., 2020). Etiological mechanisms of ASD are thought to be mainly due to complex interactions of genetic predisposition and environmental risk factors, but have not been fully elucidated (Huguet et al., 2013). It is now established that early intensive specific intervention can result in lasting positive outcomes (Reichow, 2012). Despite recent improvement in symptom screening tools and procedures, often the age of ASD diagnoses still remains too late to capitalize on a critical therapeutic window for intervention (Daniels et al., 2014; Brett et al., 2016). Even when specific intervention is delivered early, clinical response is highly variable between toddlers for reasons that are not yet fully explained (Howlin et al., 2009). Urgency for earlier diagnosis, intervention and more targeted therapeutic recommendations have led researchers to explore early behavioral and neurobiological markers of ASD.

Siblings of individuals with ASD share common genetic variants and exhibit an estimated risk of 18% to develop the disorder (Ozonoff et al., 2011). Studies on these children at high risk for ASD (HR) have allowed a better characterization of early clinical signs and trajectories of ASD (Szatmari et al., 2016). For example, we now know that the first reliable signs of ASD usually emerge during the second year of life (Sacrey et al., 2018) and are often preceded by less specific atypical behaviors in infancy (Sacrey et al., 2020). At the age of 18 months, approximately one-third of children who ultimately receive an ASD diagnosis get a stable and reliable diagnosis after a standardized assessment, while the other two-third will not yet demonstrate the full clinical presentation at this age (Ozonoff et al., 2015). This reduced clinical sensitivity of the early ASD behavioral phenotype has motivated the exploration of neuroimaging endophenotypes that could precede the emergence of symptoms and thus support clinical investigations (Wolff et al., 2018) as well as earlier identification of risk. Several magnetic resonance imaging (MRI) studies have found that children aged from 6 to 24 months who will later have a diagnosis of ASD exhibited a larger volume of extra-axial cerebrospinal fluid compared to typically developing children (TD) (Shen et al., 2013, 2017). Faster cortical surface expansion in infancy followed by brain volume overgrowth during the second year of life has also been shown to be predictive of later ASD diagnosis (The IBIS Network et al., 2017a) as have higher fractional anisotropy values at the age of 6 months and decreased values after 12 months in various deep white matter tracts such as the corpus callosum and inferior longitudinal fasciculus (Wolff et al., 2012, 2015). Despite these promising results, the imaging literature exploring early brain developmental signatures of later ASD diagnosis remains sparse. Furthermore, all the mentioned studies have focused on global morphological metrics, such as extra-axial cerebrospinal fluid volume or mean fractional anisotropy of major fiber tracts. To our knowledge, no studies have explored regional developmental differences in this population through either vertex-wise or voxel-wise methods to date.

In adults with ASD, alterations of the boundary between cerebral gray and white matter in widespread cortical areas have been identified using various methodological approaches. In histological studies less clear delineation of the transition between gray and white structures has been described in postmortem tissue of adult patients with ASD (Avino and Hutsler, 2010). *In vivo* assessment of the gray-white matter boundary has been conducted using an MRI morphometric based measure of gray-white matter intensity contrast (GWC) (Salat et al., 2009). GWC was first introduced in neurodegenerative imaging studies and has been extensively explored in aging populations (Alzheimer's Disease Neuroimaging Initiative et al., 2015). In neurodevelopmental studies, GWC has been found decreased in many regions in school-aged children and adults with ASD, (Andrews et al., 2017) whereas adolescents with ASD have been found to exhibit similar GWC values compared to their TD peers (Andrews et al., 2017; MRC Aims Consortium et al., 2018). A more recent study reported increased GWC in adults with ASD, mostly in primary cortices (Fouquet et al., 2019). Although still sparse and somewhat inconsistent, this existing literature suggests that GWC is likely decreased in many secondary cortices amongst children and adults with ASD and increased in some primary cortical regions.

The precise biological mechanisms underlying these alterations is unknown. GWC alterations in autism have largely been attributed to neural migration deficits. This interpretation is supported by findings of abnormal migration of neurons in ASD (Packer, 2016). Identification of alterations in GWC very early in life would support this hypothesis, but to date, there have been no studies evaluating GWC in very young infants or toddlers with later ASD outcomes. To our knowledge, only one study in young TD toddlers reported increased GWC rates of change in areas relevant for language development between 12 and 19 months (e.g., the left superior temporal sulcus) (Travis et al., 2014) and GWC trajectories during the first years of life have never been assessed in ASD.

In the current study, we performed exploratory quantitative whole-brain surface-based analyses to test for associations between early GWC values and various ASD-related clinical parameters in a longitudinal cohort of HR infants to evaluate the potential of GWC as an early biomarker of diagnostic outcome

Abbreviations: ADOS, Autism Diagnostic Observation Schedule; ASD, Autism Spectrum Disorder; CEN, central executive network; CSS, calibrated severity score; CT, Cortical thickness; CWP, Cluster-wise *p*-value; DMN, default mode network; DQ, Developmental quotient; EOA, Early onset ASD; FWHM, Full-width at half-maximum; GLM, General linear model; GM, Gray matter; GWC, Gray-white matter contrast; HR, High risk for ASD; HR-non-TD, HR with atypical development; HR-TD, HR with TD; IFG, Inferior frontal gyrus; LOA, Late onset ASD; LR-TD, Low risk for ASD with TD; MCS, Monte-Carlo simulation; MRI, Magnetic resonance imaging; MSEL, Mullen Scales of Early Learning; MTG, Middle temporal gyrus; Pvm, vertex with maximal *p*-value; SPC, Symmetrical percentage of change; TD, Typical development; TPJ, temporo-parietal junction; WM, White matter; ΔCT, CT rate of change; ΔGWC, GWC rate of change.

and symptom severity. Each participant underwent two MRI scans between the age of 12 and 24 months. Given the lack of previous studies using GWC in children with ASD younger than 2, we didn't have any *a priori* hypotheses regarding the location and direction of potential alterations. We assessed whether GWC was correlated with symptom severity at the time of the scan acquisition, and whether this was predictive of clinical diagnostic outcome at 36 months of age. Given the heterogeneity of age at which a stable and reliable diagnosis of ASD can be established (Ozonoff et al., 2015), we performed further *post hoc* exploratory analyses to evaluate the association between the age of first reliable diagnosis and GWC alterations. We hypothesized that if GWC alterations were to be found, they would be more prominent amongst ASD children with an early onset of ASD diagnosis (EOA) compared to children with a later onset of ASD diagnosis (LOA).

MATERIALS AND METHODS

We leveraged a MRI dataset involving participants recruited through the UC Davis MIND Institute between 2009 and 2011. The recruitment process as well as clinical and imaging procedures have been described in detail previously (Shen et al., 2013).

Participants

Between 2009 and 2011, participants from a clinical longitudinal cohort (Ozonoff et al., 2010) were asked to also take part in an MRI acquisition protocol. Invitation was made through phone screening and led to the recruitment of 64 participants. For the current longitudinal analyses, we first selected the 41 participants who were categorized as HR (13 females). In this study, HR was defined as having an older sibling with a confirmed diagnosis of ASD. Having a sample exclusively constituted of HR participants allows to study the continuum of symptom severity and the emergence of ASD amongst participants who share a similar risk to develop the disorder (Ozonoff et al., 2011). For our analysis, we included only the HR participants who underwent 2 MRI scans (first at 12–15 months and second at 18–24 months of age). From the initial 41 HR participants, 6 were excluded (14.6% from initial sample) due to failure of one scan acquisition, 1 (2.4%) due to failure of both scan acquisitions, 4 (9.8%) for not coming to one MRI session, 7 (17.1%) due to dropping out from the study, 1 (2.4%) due to lost data, and 2 (4.9%) because of poor image quality (described below). From the overall 61 attempted scans, there were 9 failures resulting in a success rate of 85.2%. This resulted in a final sample of 20 HR children (5 females).

Demographic characteristics of the sample are displayed in **Table 1**. We divided our sample into three groups according to their diagnostic outcome at age 3: HR with typical development (HR-TD, n = 8, 3 females), HR with ASD (HR-ASD, n = 9, 1 female) and HR with atypical development (HR-non-TD, n = 3, 1 female). One HR-TD child did not undergo the 18-month clinical assessment but was not excluded from the study. As an additional exploratory analysis, we further separated HR-ASD participants

TABLE 1 | Sample characteristics.

	Groups according to diagnostic outcome					*p*-value of Bonferroni's multiple comparison		
	High-risk toddlers (*n* = 20)	**HR-TD (*n* = 8)**	**HR-nonTD (*n* = 3)**	**HR-ASD (*n* = 9)**	***p*-value**	**HR-TD / HR-ASD**	**HR-TD/HR-non-TD**	**HR-non-TD/HR-ASD**
Mean MRI age [months] (n = 20)	16.5 ± 1.6 (14.6-19.8)	**17.7 ± 1.5 (15.6-19.8)**	**15.3 ± 0.5 (14.8-15.7)**	**15.8 ± 1.2 (14.6-18.3)**	**0.001**	**0.020**	**0.041**	
Time interval between scans [months] (n = 20)	6.5 ± 0.9 (5.2-9.0)	6.8 ± 1.4 (5.2-9.0)	6.1 ± 0.5 (5.6-6.6)	6.3 ± 0.5 (5.6-7.2)	0.474			
Gender (female number) (n = 20)	5	3	1	1	0.427			
Age at 1nd clinical assesment (n = 19)	18.1 ± 0.4 (17.6-19.1)	18.1 ± 0.4 (17.7-18.6)	17.8 ± 0.2 (17.6-18)	18.2 ± 0.4 (17.7-19.1)	0.253			
ADOS CSS (n = 19)	3.5 ± 2.7 (1-10)	2.6 ± 2.1 (1-7)	2 ± 1 (1-3)	4.8 ± 3.1 (1-10)	0.161			
DQ (n = 19)	87.1 ± 14.2 (54.1-119.4)	96.4 ± 14.9 (87.3-119.4)	85.8 ± 11.8 (72.6-95.5)	80.4 ± 11.2 (54.1-90.9)	0.073			
Age at 3rd clinical assesment (n = 20)	36.5 ± 1.2 (34.9-39.9)	36.9 ± 1.4 (35.6-39.9)	35.7 ± 0.7 (35.3-36.6)	36.9 ± 1.4 (34.9-38.3)	0.356			
ADOS CSS (n = 20)	3.9 ± 2.6 (1-8)	**1.5 ± 0.5** (1-2)	**2.7 ± 1.5** (1-4)	**6.3 ± 1.3** (5-9)	**<0.001**	**<0.001**		**<0.001**
DQ (n = 20)	87.5 ± 19.1 (70.8-108.0)	**103.2 ± 5.3 (93.3-109.9)**	**76 ± 0.2 (75.9-76.3)**	**77.4 ± 20.8 (32.5-103.2)**	**0.004**	**0.006**	**0.042**	

Sample demographics and behavioral information. P-value of statistical comparisons between groups (One-way ANOVA) displayed as well as post hoc comparison between groups (Student's t-test) with Bonferroni correction for multiple comparison when P-value was < 0.05. Significant differences between groups are highlighted in bold.

into two subgroups according to age of first established diagnosis. HR-ASD who were diagnosed at 18 months or before were classified as early onset autism (EOA; n = 4, 1 female). HR-ASD participants whose diagnosis was established later than 18 months of age were labeled as later onset autism (LOA; n = 5, 0 female). EOA and LOA sample characteristics are detailed in **Supplementary Table 1**.

One could notice that the proportion of ASD in our HR sample (45%) is greater than the prevalence of ∼20% which is reported in the literature (Ozonoff et al., 2011). Nevertheless, one must take into account the fact that our population is constituted by a majority of male HR in which the prevalence of ASD has been reported to be around 32% (Zwaigenbaum et al., 2012). Another explanation could rely in the fact that parents who were more worried about their child's development were more motivated to participate to the scan acquisition, thus leading to a recruitment bias.

It should be noted that HR-TD do not share similar developmental trajectories with low-risk children with a typical development (LR-TD) as HR-TD exhibit higher ASD traits and increased risk for other conditions such as anxiety and attention-deficit/hyperactivity disorders (Charman et al., 2017; Shephard et al., 2017). As a supplementary analysis, we included the 10 LR-TD (2 females) from the cohort who completed two scans at 12–15 and 18–24 months for comparison with HR-TD and HR-ASD. The LR-TD mean age between two scans was 17.4 ± 1.9 months (15.4–20.1). Time interval between both scans was 7.1 ± 1.3 months (5.7–9.5).

Behavioral Measures and Outcome Classification

Clinical assessments were conducted with each participant at 6, 12, 18, 24, and 36 months.

The Mullen Scales of Early Learning (MSEL) was used to assess development in cognitive (expressive and receptive language, visual reception) and motor (fine and gross) areas (Mullen, 1995). Developmental quotient scores (DQ) were used instead of standard scores in order to limit truncation of very low performing participants (Lord et al., 2006). Individual DQs were obtained by dividing age-equivalent developmental age output from MSEL by chronological age and multiplying by 100.

ASD-related symptom severity was quantified with the Autism Diagnostic Observation Schedule (ADOS) (Lord et al., 2002). The ADOS is a semi-structured observational evaluation with cut-offs to guide diagnostic decisions, appropriate for ambulatory children of 12 months and older. Either module 1 (intended for non-verbal children or those using only isolated words) or module 2 (intended for children with phrase speech) was conducted at ages 18, 24, and 36 months. To allow comparison of ADOS total scores across ages and modules, the calibrated severity score (CSS) was used. ADOS CSS ranges from 1 to 10 (with 10 being the most severe) (Gotham et al., 2009; Rutter et al., 2012).

At each visit from 18 months and later, ASD diagnostic outcome was established by a licensed clinician according to ADOS diagnosis cut-offs and DSM-IV criteria (American Psychiatric Association (APA), 2000). Children who did not meet the criteria for a diagnosis of ASD were categorized as having typical development (TD) or non-typical development (non-TD). TD was defined as having an ADOS CSS equal to or less than 2, a total DQ of at least 85, no DQ subtest less than 80 and no more than one DQ subtest less than 85. If one or more of these criteria were not met, participants without ASD were classified as non-TD.

Image Acquisitions

All children were scanned during natural sleep following previously published procedures (Nordahl et al., 2008), at the UC Davis Imaging Research Centre on a 3 Tesla Siemens TIM Trio MRI system with an eight-channel head coil. Structural T1-weighted 3D MP-RAGE images were acquired with 1 mm^3 isometric voxels, repetition time = 3,200 ms, echo time = 5.08 ms, field of view = 176 mm, and 192 sagittal slices. The success rate of these MRI acquisitions was 78%. A 3D image distortion map (Image Owl) was acquired at the end of each scan with a calibration phantom (Phantom Laboratory, Inc.). Distortion correction was carried out as described in Nordahl (2012).

Participants had a first MRI scan at 6–9 months of age which was not evaluated in the present analyses because of the difficulty to obtain accurate 3D white matter surface reconstructions at this age. Accordingly, we utilized the participant's second scan, acquired between 12 and 15 months of age, and third scan, acquired between 18 and 24 months of age. This third scan was acquired an average of 1.5 ± 2.0 months after the 18-months ADOS.

Image Processing and Quality Control

We used the automated pipeline provided by FreeSurfer v6.0 to process the T1-weighted cerebral MRIs[1]. The successive steps of this automated procedure are described in detail elsewhere (Dale et al., 1999; Fischl et al., 1999a,b; Fischl and Dale, 2000). Briefly, non-cerebral tissues are removed, signal intensity is normalized, and the image is segmented using a connected components algorithm. Then, a single filled volume of white matter is generated for each hemisphere. For each volume of white matter, a triangular surface tessellation is created by fitting a deformable template. Through deformation of this tessellated surface, a cortical mesh is created that defines the boundary between white and cortical gray matter (called the outer white matter surface) as well as the boundary between the gray matter and the extra-axial fluid (called the pial surface). This surface deformation process is calculated through an energy minimization function that determines the sharpest shift in intensity between voxels to define the transition between tissue categories. This process is independent of absolute intensity values and can delineate boundaries at a subvoxel resolution. The described pipeline has previously been applied within toddlers (Travis et al., 2014) as well as children with ASD (MRC Aims Consortium et al., 2018). Importantly, FreeSurfer delineation of white matter and gray matter is solely based on intensity shift and does not rely on an age specific template.

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

A trained operator (M.G.), blind to any clinical outcome, visually inspected images obtained with the described automated pipeline. First, he attributed a subjective score ranging from one to ten for every image denoting the level of motion artifact. A motion rating (MR) was then estimated for every participant by averaging these scores between their two scans. Second, he implemented manual corrections when required following recommended procedures described in the FreeSurfer manual[2]. All final cortical surfaces were visually validated by a second trained independent operator (M.S.) who was also blind to all clinical outcomes.

Gray-White Matter Intensity Contrast

We first sampled white matter intensity at each vertex *v* at 1 mm beneath the white matter (WM) outer surface (**Figure 1**). A distance of 1 mm was chosen to facilitate comparisons with previous literature since it is the most commonly used value in GWC studies, including all existing studies exploring GWC in ASD (MRC Aims Consortium et al., 2018; Fouquet et al., 2019). Gray matter (GM) intensity value was sampled at each vertex at a distance of 30% of cortex width (defined as the distance between outer white matter surface and pial surface) starting from the white matter outer surface. The value of 30% was set because it is the most commonly used in the GWC literature (MRC Aims Consortium et al., 2018) and is the default value provided by FreeSurfer. In addition, a previous study of ASD found diagnostic differences to be greatest when GM intensity sampled between 30 and 40% was used to compute GWC (Fouquet et al., 2019). GWC at each vertex *v* was computed by dividing the difference between GM and WM intensities by the mean between GM and WM intensities and multiplying by 100 to get a ratio expressed in [%]. This was performed for each individual scan at time T.

$$\mathrm{GWC}_v T\,[\%] = 100 \times \frac{(WM_v - GM_v)}{(WM_v + GM_v)/2} \quad (1)$$

GWC values were then registered on an average template provided by FreeSurfer to allow vertex-wise inter-participants comparison. During this process, GWC values were smoothed with a full-width at half-maximum (FWHM) surface-based Gaussian kernel of 10 mm.

Then, for each participant two different GWC longitudinal values were computed. Longitudinal neuroimaging designs allow many advantages over cross-sectional designs, including reduction of within-participant variability and the possibility to analyze the effect of time on the variable of interest (Reuter et al., 2012). First, we estimated the individual average GWC*v* values between 12 and 24 months of age by computing the mean of GWC*v* values between the two scans.

$$\mathrm{GWC}_v\,[\%] = \frac{GWC_v1 + GWC_v2}{2} \quad (2)$$

Where GWC*v*1 is GWC*v* at 12–15 months and GWC*v*2 is GWC*v* at 18–24 month.

Second, we computed the individual GWC rate of change between two scans (ΔGWC) at each vertex. ΔGWC represents the effect of time on GWC between the age of 12 and 24 months and was computed through the symmetrical percentage of change (SPC) formula (Reuter et al., 2012). SPC consists of calculating, for each participant at each vertex *v*, the GWC difference between two scans divided by the age difference between the two scans, giving a rate in [%/month]. This rate is divided by mean GWC at each vertex v and multiplied by 100, giving a result expressed in [%]. Using symmetrized measures of change (such as $(B_y - B_x)/B_x$) over absolute differences (such as $B_y - B_x$ that would not be scaled to the mean) is recommended in longitudinal analyses as they allow many advantages such as increased statistical robustness, higher reliability in small samples and balanced consideration of both measures B_x and B_y (Berry and Ayers, 2006). SPC expresses the rate at which GWC changes in each vertex between two scans relative to mean GWC.

$$\mathrm{GWC}_v\,[\%] = 100 \times \frac{(GWC_v2 - GWC_v1)/(age2 - age1)}{GWC_v} \quad (3)$$

Where *age1* is participant's age at 12–15 months scan and *age2* is their age at 18–24 months scan.

Cortical Thickness

Cortical thickness (CT) alterations have been found to influence GWC values (Westlye et al., 2009). CT is defined by the distance in mm between WM outer surface and pial surface and is automatically computed by the standard FreeSurfer processing pipeline (Fischl and Dale, 2000). To control for this possible confound, we sampled CT at each vertex *v*. We then computed the individual average CT*v* across the two scans and the CT*v* rate of change (ΔCT*v*) with the same formulas we described for longitudinal GWC parameters. Spatial overlap between significant effects on GWC and CT were explored.

Motion Rating

Motion artifacts are a significant confound that can influence GWC (Fouquet et al., 2019). While we found no difference in motion rating (MR) across main outcome groups (HR-TD, HR-ASD and HR-non-TD), we also performed vertex-wise analyses to explore potential local correlations between MR and GWC. We found one cluster in the right parietal superior region with positive correlation between MR and GWC (CWP = 0.026, cluster size of 848.6 mm^2), no significant results of interest were observed within this identified region.

Statistical Analysis

Sample Characteristics Analyses

Our primary outcomes are symptom severity (ADOS CSS) at 18 and 36 months of age and discrete diagnosis at age 3 (HR-TD, HR-ASD, HR-non-TD). Mean age at scan acquisitions was 16.5 months (see **Table 1**). Accordingly we considered the evaluations performed at 18 months as the clinical correlate that was the closest in time to the neuroimaging data and thus refer to this as the

[2]http://freesurfer.net/fswiki/

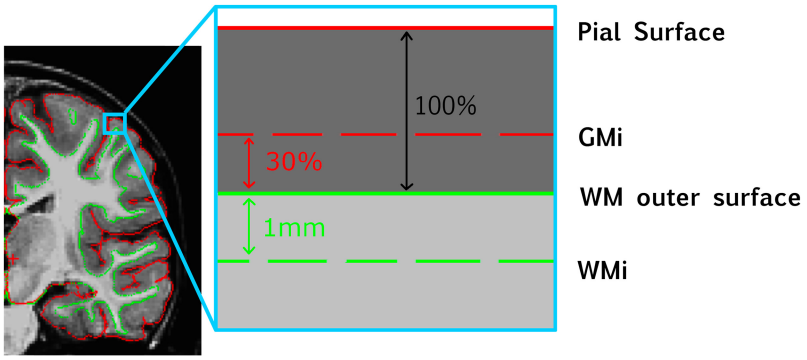

FIGURE 1 | Schematic representation of how GWC is computed at each vertex. GMi, gray matter intensity; WM, white matter; WMi, WM intensity.

phenotype at the time of scan acquisitions. Data derived from assessments at 36 months of age were used to test for associations between early neuroimaging parameters and later clinical outcomes.

We further subdivided the HR-ASD into late and early symptom onset (LOA and EOA). Associations between clinical outcome (HR-TD, HR-ASD and HR-non_TD) and individual characteristics that could represent potential confounding factors in GWC analysis were explored. These parameters included gender, age at scanning (which was calculated as mean age between two scans) and time interval between scans. According to the nature of the tested variables (i.e., discrete or continuous), we used either Pearson correlation, one-way ANOVA, Student's *t*-test (or Mann-Whitney *U*-test when non-parametric distribution of variables was found), or chi-square test.

For descriptive purposes, we tested for differences in behavioral scores (DQ, ADOS CSS at 18 and 24 months) between diagnosis groups (HR-TD, HR-ASD, and HR-non-TD) using one-way ANOVA. We also tested for potential differences in behavioral scores between the two HR-ASD subgroups (EOA and LOA) using either Student's *t*-test or Mann-Whitney *U*-test. Statistics described in this section were performed with Prism v.8.3.0 software with significance threshold set at alpha = 0.05. Behavioral and demographic characteristics of the sample are displayed in **Table 1**.

Surface-Based Analyses

We used the general linear model (GLM) command implemented in FreeSurfer to perform vertex-wise whole-brain surface-based analysis of GWC.

First, to determine the effect of time on GWC in typical development between the age of 12 and 24 months, we performed vertex-wise parametric comparison of ΔGWC values vs. zero in our HR-TD group. We then extracted vertex-wise ΔGWC values from all significant clusters and computed an average ΔGWC value for each hemisphere. This mean hemispheric ΔGWC value was compared between right and left to test for any asymmetry in the effect of age on GWC.

Then, we fit a GLM to test whether GWC and ΔGWC at age 12–24 months are associated with discrete diagnostic outcome at age 3 (HR-ASD or HR-TD). HR-non-TD were not included in this analysis in order to limit the number of group comparisons. Also, HR-non-TD does not represent a clearly distinct group with pure developmental delay but also includes children with autistic traits without a confirmed diagnosis. As such, HR-non-TD can be considered as an intermediate group in the symptom continuum between TD and ASD:

$$\begin{aligned} \text{GWC} &\sim \textit{Diagnostic outcome} + \textit{age}1 + \textit{age}2 \\ \Delta\text{GWC} &\sim \textit{Diagnostic outcome} + \textit{age}1 + \textit{age}2 \end{aligned} \tag{4}$$

We further tested if symptom severity at 18 and at 36 months of age were associated with GWC and ΔGWC. All participants were included in this analysis. The following GLM was conducted:

$$\begin{aligned} \text{GWC} &\sim \textit{ADOS CSS} + \textit{age}1 + \textit{age}2 \\ \Delta\text{GWC} &\sim \textit{ADOS CSS} + \textit{age}1 + \textit{age}2 \end{aligned} \tag{5}$$

Given that GWC before the age of 24 months is influenced by age (Travis et al., 2014), age at scanning was regressed out in all GLM analyses. The *p*-value for each voxel was calculated using two-tailed testing with significance threshold set at alpha = 0.05. Cluster-wise analyses were corrected for multiple comparisons using Monte-Carlo simulation (MCS) with a significance threshold for cluster-wise *p*-value (CWP) of alpha = 0.05. We used cluster-wise and MCS analysis pipelines implemented in FreeSurfer (Hagler et al., 2006).

The same GLM methods were utilized for analyses of CT and ΔCT values.

We then wanted to determine if potential alterations of GWC and ΔGWC found with GLM analysis were associated with the age at which the first ASD-related symptoms emerged. As a further *post hoc* exploratory analysis, for each cluster exhibiting significant GWC or ΔGWC alterations, we computed the average of all vertex-wise GWC or ΔGWC values, respectively, across all vertices in the cluster for each participant. These individual cluster-averaged GWC values were then compared between HR-TD, EOA and LOA using an ANCOVA with age at first scan and age at second scan as regressors. Significance threshold was set at alpha = 0.05. Whenever an ANCOVA reached significance,

post hoc comparisons with multiple *t*-tests applying Bonferroni correction was performed.

RESULTS

Sample Characteristics

Clinical characteristics of the sample are described in **Table 1** and illustrated in **Supplementary Figure 1**. As expected, the HR-ASD group exhibited the most severe symptom severity and the lowest DQ at age 3. There was no significant difference between groups in either DQ or symptom severity at 18 months of age. This may be due to the fact that HR-ASD participants who already received an ASD diagnosis at this age (EOA participants) were too few ($n = 4$) to drive a statistically significant difference. A significant association between age at scanning and discrete diagnostic outcome groups was observed with HR-TD being elder than HR-non-TD and HR-ASD.

HR With Typical Development Show Increased GWC Rate of Change Between Age 12 and 24 Months

In HR children who demonstrated typical development at age 3 (HR-TD), GWC was significantly correlated with time in almost all regions (**Figure 2** and **Supplementary Figure 2**). The highest rates of change were found bilaterally in prefrontal areas, temporal poles and temporo-parietal junctions. Areas with the smallest rates of change were found in lateral and medial occipital lobes bilaterally, right paracentral gyrus, right insula, left subgenual region and left inferior frontal gyrus. No regions exhibited a significant decreasing GWC rate of change. There was no global difference between right and left hemisphere (**Supplementary Figure 2B**).

Increased GWC Between 12 and 24 Months of Age Is Associated With ASD at 36 Months

A significant increase in GWC values in HR-ASD compared to HR-TD was found in the following regions: right supramarginal, right precentral, right precuneus, right inferior parietal, right rostral middle frontal, left middle temporal, left pars orbitalis and left pars opercularis (**Figure 3**). No regions displayed a significant decrease in GWC in HR-ASD compared to HR-TD (**Table 2**). There were no differences in terms of local ΔGWC between HR-ASD and HR-TD.

Increased GWC Between 12 and 24 Months of Age Is Associated With ASD Symptom Severity at 18 and 36 Months

GWC was positively correlated with autism symptom severity (ADOS CSS) at 18-months in the following cortices: right inferior parietal, left middle temporal and left pars opercularis (**Figure 4A**). GWC was also positively correlated with autism symptom severity at age 3 (36-months ADOS CSS) in the right precentral, right precuneus, right inferior parietal, right lateral occipital, left paracentral and left lateral occipital regions (**Figure 4B** and **Table 2**). All clusters are illustrated in **Supplementary Figure 3**.

Slower GWC Rate of Change Between Age 12 and 24 Months Is Exclusively Associated With Symptom Severity at 18 Months

A negative correlation between symptom severity (ADOS CSS) at 18-mo and ΔGWC values was observed in the right pars opercularis, right superior frontal, right inferior parietal, left posterior cingulate, left superior parietal, left supramarginal, left superior frontal, and left middle temporal areas (**Figure 5A**). That is, higher symptom severity at 18 months was associated with slower ΔGWC between 12 and 24 months in these regions. Later symptom severity (36-mo ADOS CSS) as well as diagnosis group comparison (HR-TD vs. HR-ASD) were not associated with any significant differences in ΔGWC between age 1 and 2. See **Table 2** and **Supplementary Figure 4** for detailed results and illustrations.

Alterations in GWC Values Are Influenced by the Age of First Reliable ASD Diagnosis

We performed a further exploratory *post hoc* analyses on HR-ASD subgroups based on whether ASD diagnosis was established at 18 months (EOA) or later (LOA). We found that the clusters with altered GWC values in HR-ASD were mostly driven by participants with an early diagnosis onset. In clusters with higher GWC values in HR-ASD compared to HR-TD the EOA subgroup exhibited increased GWC values compared to HR-TD in right supramarginal, right inferior parietal, left middle temporal and left pars opercularis. The LOA subgroup didn't exhibit any clusters of significantly increased GWC values compared to HR-TD (**Figure 3**).

Among brain regions indicated as having significant correlations between GWC and symptom severity at 18-months, there were significant differences in GWC between EOA and HR-TD in the right inferior parietal cortex and between EOA and LOA (EOA having greater values) in the left middle temporal gyrus. There were no differences in GWC between LOA and HR-TD (**Figure 4A** and **Supplementary Figure 3**). For clusters with significant correlations between GWC and 36 months symptom severity (36-mo ADOS CSS), we found significant differences in GWC between EOA and HR-TD in the right inferior parietal region (**Figure 4B** and **Supplementary Figure 3**).

For clusters with significant correlation between symptom severity at time of scan and ΔGWC significant differences were found in ΔGWC between EOA and LOA in all clusters except those incorporating right inferior parietal and left supramarginal regions. Moreover, EOA exhibited slower ΔGWC compared to HR-TD in the left posterior cingulate and left superior parietal regions. There were no differences between HR-TD and LOA in any of the clusters (**Figure 5** and **Supplementary Figure 4**).

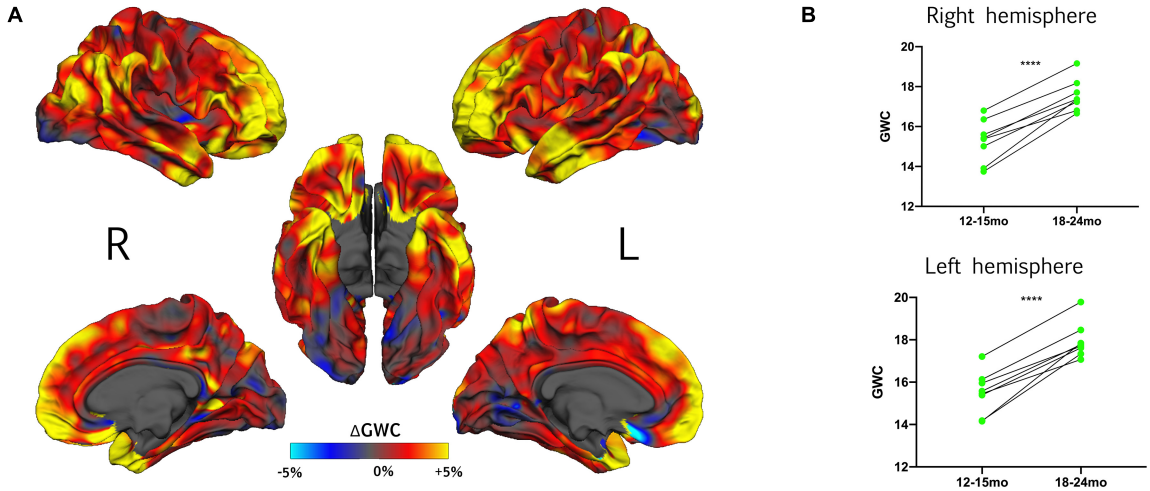

FIGURE 2 | (A) Effect of time on GWC within HR toddlers with typical development outcome at 3 years (HR-TD) represented with vertex-wise ΔGWC values mapped on the common FreeSurfer template. **(B)** For each hemisphere, individual trajectories of gray-white matter contrast (GWC) from 12–15 to 18–24 months within the HR-TD group. Individual GWC displayed are the average of all vertex-wise values extracted from clusters with a significant effect of time on GWC (one per hemisphere, see **Supplementary Figure 2**). Right hemisphere: 12–15-mo GWC = 15.3% ± 1.1; 18–24 GWC = 17.6% ± 0.8. Left hemisphere: 12–15- months GWC = 15.5% ± 1.0; 18–24- months GWC = 17.9% ± 0.8. ****$p < 0.0001$ (Student's *t*-test).

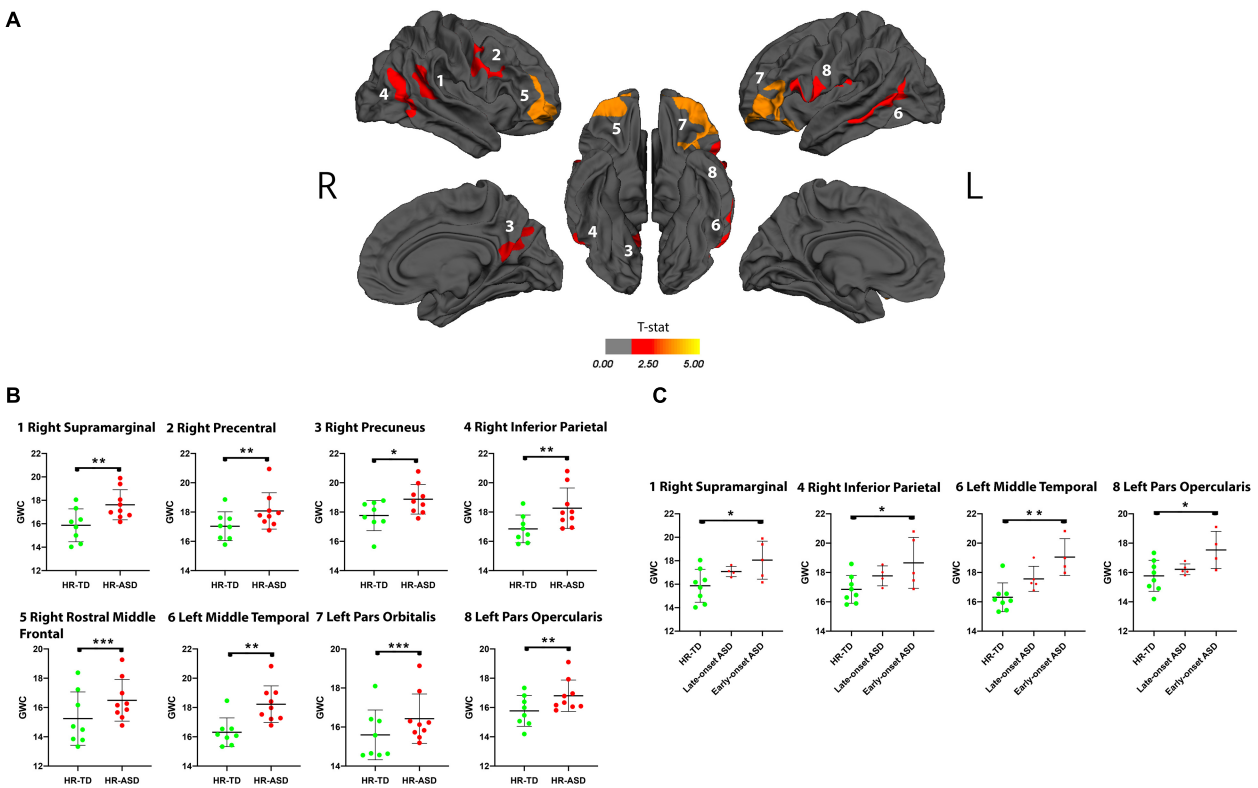

FIGURE 3 | (A) Clusters in which gray-white matter contrast (GWC) at age 12–24 months (computed as mean GWC between the two scan acquisitions) has a significant association with diagnostic outcome at age 3 (HR-TD or HR-ASD). Only clusters with CWP < 0.05 after regression for age are and correction for multiple comparisons are displayed. Within each hemisphere, clusters are numbered in order of decreasing effect size (Cohen's D, see **Table 2**). Color code corresponds to *p*-value of the vertex with the greatest *p*-value within the cluster. *P*-values are represented as T-stat = -log(*p*-value). **(B)** Individual mean GWC values for each significant cluster are displayed. **(C)** Comparison between HR-TD and HR-ASD subgroups (LOA and EOA). Only clusters with a significant ANCOVA ($p < 0.05$) are displayed. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

TABLE 2 | Clusters of significant association between GWC and clinical outcomes.

mGWC	Cluster number	Anatomical label	Size (mm^2)	CWP	Pvm	Effect size
Diagnostic outcome (HR-TD vs. HR-ASD)	1	Right supramarginal	886.4	0.009	3.432	0.748
	2	Right precentral	976.7	0.004	3.429	0.730
	3	Right precuneus	850.4	0.012	4.396	0.705
	4	Right inferior parietal	994.9	0.004	3.684	0.638
	5	Right rostral middle frontal	2013.0	<0.001	3.089	0.559
	6	Left middle temporal	1050.3	0.003	3.682	0.757
	7	Left pars orbitalis	2733.2	<0.001	4.133	0.659
	8	Left pars opercularis	985.8	0.002	4.646	0.651
18 months ADOS CSS	1	Right inferior parietal	713.7	0.038	4.127	0.835
	2	Left middle temporal	803.6	0.015	3.510	0.758
	3	Left pars opercularis	982.1	0.003	4.016	0.603
36 months ADOS CSS	1	Right precentral	785.6	0.022	2.595	0.787
	2	Right precuneus	899.8	0.007	4.648	0.775
	3	Right inferior parietal	728.0	0.034	3.669	0.722
	4	Right lateral occipical	1342.2	<0.001	3.283	0.562
	5	Left paracentral	727.2	0.028	3.215	0.861
	6	Left lateral occipital	1346.8	<0.001	3.303	0.712
ΔGWC						
18 months ADOS CSS	1	Right pars opercularis	1662.9	<0.001	3.334	0.835
	2	Right superior frontal	806.9	0.018	2.865	0.766
	3	Right inferior parietal	1232.7	<0.001	2.506	0.759
	4	Left posterior cingulate	1240.9	<0.001	3.631	0.899
	5	Left superior parietal	840.9	0.011	5.019	0.840
	6	Left supramarginal	1536.1	<0.001	2.915	0.814
	7	Left superior frontal	1280.9	<0.001	3.640	0.790
	8	Left middle temporal	905.0	0.007	3.097	0.746

*Detailed results of surface-based statistical analyses. Cluster numbers refer to numbers displayed in **Figures 3–5** and are listed in order of decreasing effect size within each analysis. Anatomical labels refer to location of vertex with maximal p-value (Pvm) according to FreeSurfer Desikan parcellation atlas. Pvm are reported in T-stat values which is equal to -log(p-value). Effect sizes are expressed with either Cohen's d or R squared (r^2) values. CWP, Cluster-wise p-value.*

Cortical Thickness at 12–24 Months Is Decreased in Medial Superior Frontal Gyrus in the HR-ASD Group

We found decreased CT values in HR-ASD compared to HR-TD in a single cluster located in the left superior frontal region (cluster size of 679.7 mm^2, cluster-wise p-value of 0.042, see **Supplementary Figure 5**). No differences in ΔCT between HR-ASD and HR-TD were observed. There was also no effect of symptom severity (either 18-months or 36-months ADOS CSS) on either CT or ΔCT.

LR-TD Comparison With HR-TD and HR-ASD

As a supplemental analysis, we performed vertex-wise comparisons of GWC and ΔGWC between LR-TD and HR-TD, as well as between LR-TD and HR-ASD. We found that HR-ASD have higher GWC in the left supramarginal area compared to LR-TD (CWP = 0.018, cluster size of 778.6 mm^2). We also found that HR-TD have lower GWC in the right lateral orbitofrontal cortex compared to LR-TD (CWP = 0.045, cluster size of 685.2 mm^2). There was no difference in terms of ΔGWC. Results are displayed in **Supplementary Figure 6**. ΔGWC values within the LR-TD are displayed in **Supplementary Figure 7**.

DISCUSSION

Our aim was to use structural MRI to conduct an exploratory surface-based analysis to determine if alterations in tissue contrast across the gray-white matter boundary in toddlers at high-risk for ASD could represent an early biomarker of clinical outcome (i.e., autism diagnosis) at age 3 and whether alterations in GWC are associated with autism symptom severity. To our knowledge, this is the first study to explore GWC in children aged less than 24 months old who are at a high risk to develop ASD. Firstly, HR children with typical development were found to exhibit widespread increases of GWC values with time from ages 12 to 24 months. This result provides a first normative reference for typical GWC values at this age in HR toddlers. Secondly, ASD outcome at 3 years of age was associated with widespread (though well localized) increased GWC values during the second year of life compared to HR infants with a TD outcome. These results suggest that brain microstructural alterations in ASD are already present at the end of infancy and are associated with clinical outcomes later in development. Lastly, individuals who experienced more severe symptoms of ASD at 18 months of age showed a distinct neurobiological signature characterized by a slower rate of change in GWC between 12 and 24 months of age.

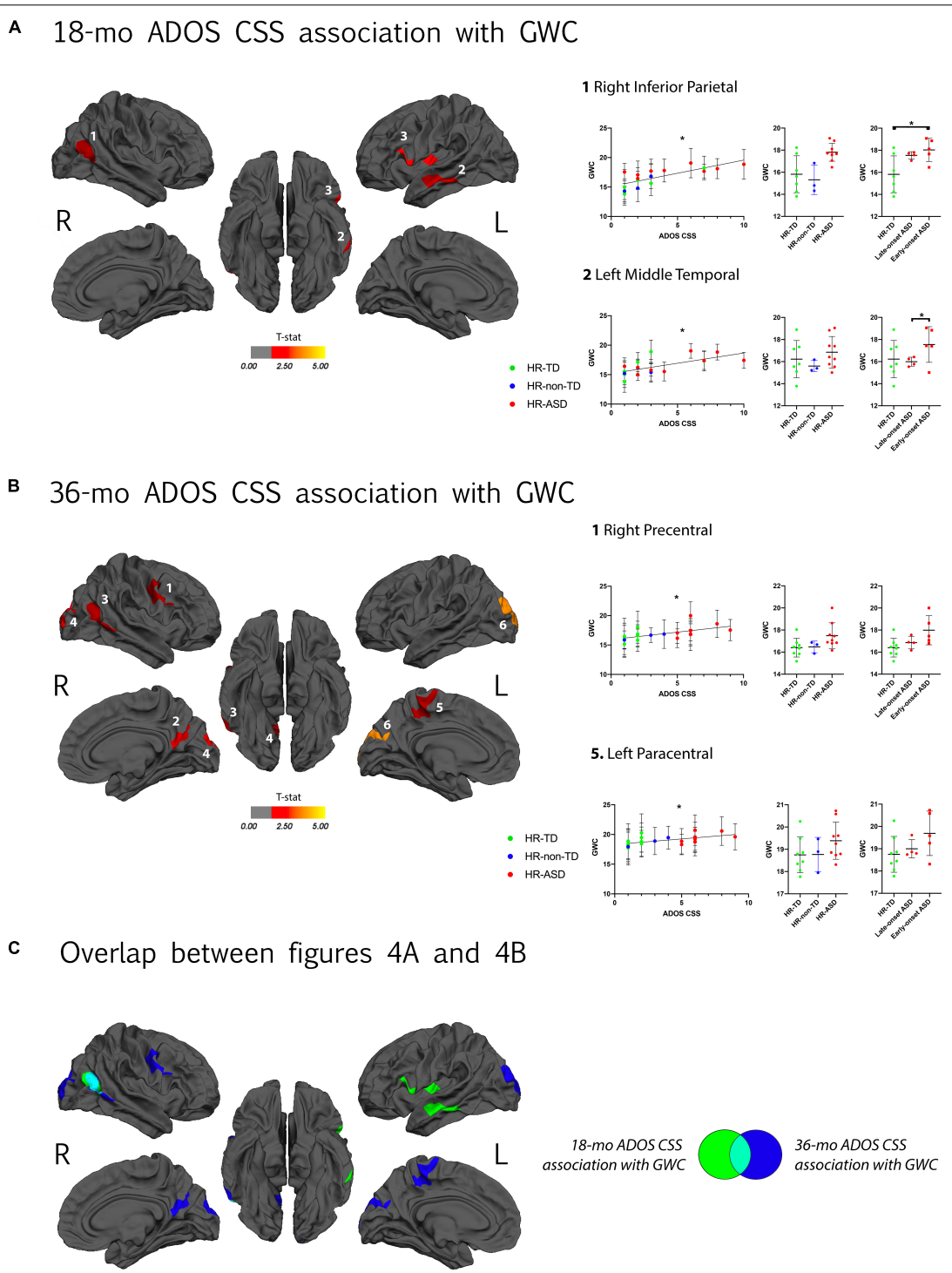

FIGURE 4 | (A) Clusters with a significant association between gray-white matter contrast (GWC) at age 12–24 months (computed as mean GWC between two scan acquisitions) and ADOS calibrated severity score (CSS) at 18 months of age. Only clusters with CWP < 0.05 after regression for age and correction for multiple comparisons are displayed. Color code corresponds to *P*-value of the vertex with maximal *p*-value (Pvm) of each cluster and is represented as T-stat values (see **Table 2**). Within each hemisphere, clusters are listed in order of decreasing effect size (Pearson's R, see **Table 2**). On the right, individual mean GWC values are displayed in function of ADOS CSS for clusters with the greatest effect size. Same individual values are further plotted in function of diagnostic outcome group (HR-TD, HR-non-TD, and HR-ASD) in the middle graph. In the right graph, comparison between HR-TD and HR-ASD subgroups (LOA and EOA) are displayed. Similar graphs for all significant clusters are available in **Supplementary Figure 3A**. **(B)** Association between GWC at age 12–24 months of age and symptom severity at 36 months of age. Detailed results for all clusters are displayed in **Supplementary Figure 3B**. **(C)** Clusters of **(A,B)** displayed on a common template with color code corresponding to the age of clinical assessment (green for 18 and blue for 36 months of age). $^*p < 0.05$.

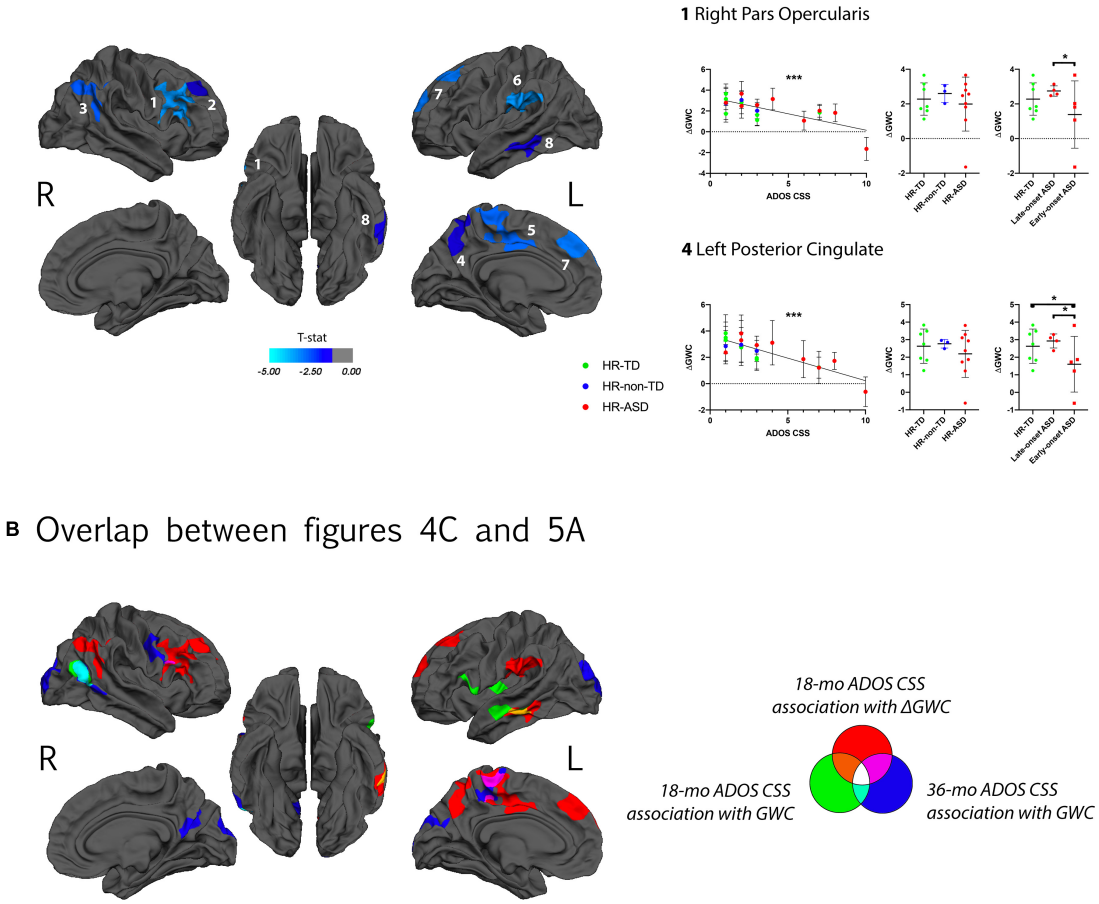

FIGURE 5 | (A) Association between GWC rate of change (ΔGWC) between 12 and 24 months of age and ADOS calibrated severity score (CSS) at 18 months of age. Only clusters with CWP < 0.05 after regression for age and correction for multiple comparisons are displayed. Color code corresponds to *P*-value of the vertex with maximal *P*-value (Pvm) of each cluster (see **Table 2**). In each hemisphere, clusters are listed in order of decreasing effect size (Pearson's R, see **Table 2**). Individual ΔGWC are displayed in function of ADOS CSS on the graph on the left. Only values for clusters with the greatest effect sizes are shown. Same individual values are further plotted in function of diagnostic outcome group (HR-TD, HR-non-TD and HR-ASD) in the middle graph. On the right, comparison between HR-TD and HR-ASD subgroups (LOA and EOA) are displayed. Similar graphs for all significant clusters are displayed in **Supplementary Figure 4**. **(B)** Clusters displayed on **(A)** and clusters with a significant association between GWC at age 12–24 months and ADOS CSS at 18 months and 36 months of age (**Figure 4C**) displayed on a common template. Color code reflects which longitudinal GWC value (either mean GWC or ΔGWC) and which ADOS CSS (either at 18 months or 36 months of age) were used in GLM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Typical Development in a HR Population Is Characterized by Increasing GWC Values Between 12 and 24 Months of Age

The only previous study exploring GWC changes in TD across the same age range as the current study also identified brain regions where GWC values increased between age 12 and 19 months (Travis et al., 2014). Clusters found by Travis et al. (2014) correspond to regions observed in the current study with the highest ΔGWC values in the HR-TD group (left dorso-lateral prefrontal cortex and left anterior temporal lobe). However, Travis et al. (2014) found more focal regions with significant increases in ΔGWC in comparison to our results and exclusively localized in the left hemisphere. Several possible explanations may explain these differences. One is the exploration of a shorter age interval by Travis et al. (2014) (from 12 to 19 months instead of 12 to 24 months in our study) which may result in reduced effects of time on GWC. Another explanation is the more conservative method to correct for multiple corrections used by Travis et al. (2014) (false discovery rate instead of MCS). Nonetheless, collectively these results converge in supporting the idea that GWC tends to increase with time in various regions during the second year of life in TD.

Relations Between Our Results and GWC Alterations Previously Found at Older Ages

Considering existing literature exploring GWC in ASD, GWC differences in regions reported as altered in older populations

with ASD were also observed in the current study. For instance, bilateral middle temporal gyri (MTG) exhibited decreased GWC in Andrews et al. (2017). Furthermore, right precuneus, right occipital gyri and left inferior frontal gyrus (IFG) exhibited decreased GWC values in association with ASD from late childhood to early adulthood in MRC Aims Consortium et al. (2018). Although brain regions indicated by the current study are consistent with these two previous studies of adults, we found that ASD was associated with increased GWC. Although inconsistent, these observations are not necessarily incompatible. One potential explanation is the possibility that between the age explored in our study (i.e., before 2 years of age) and later ages, the GWC rates of change are slower amongst children with ASD compared to TD. This hypothesis is supported by our finding that the second year of life is characterized by slower GWC rates of change in individuals who experienced more severe symptoms of ASD during the period of scan acquisitions. Further studies exploring GWC trajectories in later childhood are needed to confirm that ASD is characterized by slower GWC rates of change at some point as we did not find any group difference in ΔGWC between HR-ASD and HR-TD in our sample.

Some of our results were consistent in location and direction with previous literature, e.g., increased GWC in bilateral occipital and left paracentral gyri was previously described by Fouquet et al. in adults with ASD (Fouquet et al., 2019). This suggests that increased GWC in primary cortices (visual cortex and primary motor areas) could remain higher across lifespan in individuals with ASD compared to TD. Also, regions found to have slower GWC changes with time (ΔGWC) were consistent with areas described to have later decreased GWC in Mann et al. (for bilateral prefrontal and parietal posterior cortices) and Andrews et al. (2017) (for left MTG, left precuneus, and left medial part of superior frontal gyrus).

Increased GWC at 12–24 Months of Age Relates to ASD Outcome at 36 Months of Age

Areas in which increased GWC were associated with ASD diagnosis at age 3 (**Figure 3**) have all been previously implicated in functions altered in ASD, including language and social processing (Amaral et al., 2008). Left middle temporal gyrus (MTG) and left inferior frontal gyrus (IFG), for instance, are both implicated in semantic processing which is one of the most commonly found altered domains of language in ASD (Tager-Flusberg et al., 2006; Huang et al., 2012; Wei et al., 2012). Moreover, left IFG is known to be functionally altered during semantic processing tasks in adults with ASD (Harris et al., 2006). Left MTG and left IFG both exhibit morphometric alterations in adults with ASD (Libero et al., 2014). Right MTG has shown metabolic activation related to multimodal integration of communication cues (i.e., gaze, speech and gesture) (Holler et al., 2015) in TD, processes known to be challenging for individuals with ASD (Stevenson et al., 2014). The right precuneus is a key component of the default mode network (DMN), which is implicated in mentalizing (i.e., building inferences about others' mental states). Such "theory of mind" deficits have been highlighted as a feature of ASD for decades (Baron-Cohen et al., 1985) and there is growing evidence supporting the presence of alterations in the DMN and more specifically precuneus in ASD (Padmanabhan et al., 2017).

Regarding regions in which high GWC values at age 12–24 months were associated with symptom severity at age 3 (**Figure 4B**), some overlap with clusters associated with later diagnosis outcomes are present, such as right precuneus, and right MTG. Nonetheless, some clusters were exclusively associated with symptom severity at age 3 and not later diagnosis outcome. One possible explanation of this apparent discrepancy is the fact that HR-non-TD were excluded from GWC comparison between diagnosis outcomes. HR-non-TD comprises children with borderline ADOS scores reflecting children with some autistic traits but not expressing the full phenotype. It is thus possible that the adjunction of HR-non-TD in the ADOS correlation analyses provided enough statistical power to drive a significant correlation between ADOS and GWC in these clusters that was otherwise missed in the binary outcome comparison.

Another explanation is that some HR-TD present mild autistic traits (i.e., ADOS calibrated severity scores of 2). This heterogeneity within HR-TD is not taken into account in discrete group comparisons but is included within the ADOS correlation analyses. Increased GWC values in primary cortices such as the occipital gyri (visual) and precentral gyrus (motor) in association with symptom severity is consistent with the results reported by Fouquet et al. (2019) on GWC in adults with ASD. It is also consistent with previous reports of disruption of primary motor area organization in children with ASD (Nebel et al., 2014) as well as functional and structural alterations in occipital regions in the same population (Jung et al., 2019). Our results suggest that a common microstructural mechanism during the first years of life could be at play across various cortical areas that all have been independently reported as functionally and/or structurally altered in ASD. Finally, regions exhibiting increased GWC values in relation to symptom severity at 18-months (**Figure 4A**) are mostly overlapping with regions that are associated with later ASD diagnoses (IFG, left MTG) or later symptom severity (right MTG).

Slower GWC Rate of Change Between 12 and 24 Months of Age as a Neural Signature of the ASD Symptom Severity at the Age of 18 Months

A widespread decrease in the rate of GWC change between 12 and 24 months of age was associated with ASD symptom severity at 18 months. Most clusters with slower ΔGWC did not overlap with regions that had increased GWC associated with diagnostic outcome at age 3 (**Figure 5B**). ΔGWC alterations were largely localized within the central executive network (CEN), including bilateral dorso-lateral prefrontal and bilateral posterior parietal cortex (Fox et al., 2006). Functional alterations of CEN have previously been reported in ASD (Perez Velazquez et al., 2009). Decreased GWC rate of change was also observed in left temporo-parietal junction (TPJ), left precuneus and left middle

temporal gyrus (MTG). The TPJ and precuneus are both within the DMN which has been found altered in ASD (Padmanabhan et al., 2017). Furthermore, the left MTG exhibits both increased GWC and decreased ΔGWC between age 12 and 24 months. This alteration of left MTG microstructure further supports its possible role as a key region in the early development of ASD phenotypes. Overall, these results suggest that participants who manifest early symptoms of ASD are characterized by a specific pattern of dynamic GWC changes during the second year of life, largely affecting regions implicated in executive functions.

Neurobiological Interpretations of Altered GWC Values

To understand the neurobiological correlates to our findings, an important step would be to explore if GWC differences result from alterations in cortical gray matter (GM), superficial white matter (WM), or a combination of both structures. Since GWC measures depend on two parameters (WM intensity and GM intensity), changes in GWC values can be caused either by changes in one or both of these variables. The GWC measure is proportional to WM intensity and inversely proportional to GM intensity. In other words, a darker gray matter intensity and a brighter superficial white matter intensity would both result in increased GWC values. The opposite reasoning holds for explaining decreased GWC values. Unfortunately, in qualitative MRI techniques such as T1-weighted scans, the absolute intensity values can be influenced by many factors (type of setup, detectors used, etc.) resulting in great intra- and inter-participants variability (Lutti et al., 2014). It is thus of limited utility to perform statistical analyses on GM and/or WM intensities *per se,* an issue that was already highlighted in early studies using GWC (Westlye et al., 2009).

Despite this intrinsic limitation, we can speculate here on the likelihood of various neurobiological correlates which could explain enhanced GWC values during the second year of life. First, increased GWC could result from decreased GM intensity (i.e., a darker cortical gray matter on T1w images). Many studies have highlighted alterations of cortical cytoarchitecture in ASD which could lead to alterations in GM intensity. For instance, greater density of dendritic spines of pyramidal cells as well as an increased neural density have been reported in various cortical regions and in the amygdala amongst children and adults with ASD (Avino et al., 2018). Evidence suggests that an increased number of minicolumns (which constitute the basic structural units of cortical architecture) could be a potential cause of neural excess in ASD (Casanova et al., 2006). If our results are explained by increased neural density at the end of infancy they would thus bring further support to the hypothesis that ASD is characterized by altered neural proliferation, migration and lamination processes (Packer, 2016). Another explanation for decreased GM intensity would be a delay in intracortical myelination (Fouquet et al., 2019). Nevertheless, deficits in intracortical myelin are not well documented in ASD and limited to animal model studies (van Tilborg et al., 2017).

Increased WM intensity represents an alternative (although not exclusive) explanation to increased GWC. If this were the case, increased myelination would be a likely contributor, since myelin is the most determinant contribution to WM intensity (Koenig, 1991). Early increased myelination in ASD has been suggested by several studies exploring white matter tracts in infancy using diffusion weighted imaging (Wolff et al., 2012, 2015; Solso et al., 2016; The IBIS Network et al., 2017b; Andrews et al., 2019). If true, these results would support the idea that ASD is characterized by an increased myelination processes during the first months of life.

Potential mechanisms underlying decreased GWC rates of change include faster increases in GM intensity (i.e., cortical gray matter becoming rapidly brighter) and/or slower increases in WM intensity (i.e., superficial white matter becoming slowly brighter). Slower WM intensity changes could be explained by a delay in myelination of superficial WM. This hypothesis would be consistent with previous reports of decreased myelin in superficial WM in adolescent and adults with ASD (Hong et al., 2019). Furthermore, this hypothesis would converge with previous studies who showed an early developmental pattern that could be indicative of increased myelin content in various WM tracts during infancy followed by a delayed myelination process after the age of 12 months in ASD compared to TD (Wolff et al., 2012; Solso et al., 2016; Andrews et al., 2021). Thus, decreased myelin in superficial WM could be part of a more generalized alteration of the myelin as Ameis and Catani (2015) concluded that adults with ASD present a globally decreased connectivity after a review of diffusion imaging literature.

To overcome limitations in the biological interpretation of GWC measures, future studies would benefit from implementing MRI techniques that offer a quantitative measure of the local intensity to decipher respective contributions of cortical gray matter and superficial white matter to GWC alterations. One solution could be the use of imaging methods that precisely "map" the physical T1 or T2 properties of the tissue to allow local quantification and inter-participants comparison of microstructure content (Marques et al., 2010; Hilbert et al., 2018).

GWC Alterations as a Specific Neurobiological Signature of ASD Diagnosis and Symptom Severity at 18 Months of Age

The current findings indicate that differences in GWC at 12–24 months are related to age at which first ASD symptoms occur. First, symptom severity at 18 months (i.e., ADOS CSS at 18 months) was associated with a pattern of GWC alterations that was distinct from alterations associated with later clinical outcome (ADOS CSS and diagnosis outcome at 36 months). E.g., slower GWC rates of change were specifically observed in relation to symptom severity at time of scan and were not linked to any later clinical outcome. Second, our *post hoc* exploratory analyses found that the subset of HR-ASD with established diagnosis at 18 months (i.e., early onset autism) exhibited the greatest magnitude in GWC alterations within all clusters across all analyses compared to HR-ASD with a later onset of ASD after 18 months (**Supplementary Figures 3**, **4**). Since the early and late onset subgroups did

not exhibit any difference in either symptom severity or global development (DQ) at age 3 (see **Table 1**), these differences can solely be explained by age of first reliable diagnosis onset and not by later symptom severity or cognitive level. Together, these results suggest that children with ASD experiencing more severe symptoms and a reliable diagnosis at 18 months are characterized by a specific pattern of early GWC alterations at the age of 1–2 years which consists of widespread slower GWC rate of change and a trend for all GWC alterations to be greater in magnitude in comparison to the rest of individuals with ASD.

Minor Differences Between LR-TD and HR-ASD

Compared to our analysis performed within the HR population, differences between LR-TD and HR groups (HR-TD and HR-ASD) were scarce. HR-ASD exhibit one cluster with increased GWC compared to LR-TD in the left supramarginal region. This suggests that higher GWC is a specific signature of HR-ASD compared to both LR-TD and HR-TD. The reason why we found so few differences in GWC between HR-ASD and LR-TD are not clear. One could hypothesize that HR-ASD and HR-TD would be less different than HR-ASD and LR-TD since HR children share some common genetic risk together and have some similarities in their development (Messinger et al., 2013). Our results highlight that HR-TD have a different neurodevelopmental trajectory compared to HR-ASD in the early years. This could be caused by neural compensatory mechanisms that seek to counter neural predisposition to ASD. Our results also highlight the fact that HR-TD and LR-TD represent distinct population in terms of neurodevelopment and should be analyzed separately.

LIMITATIONS AND FURTHER PERSPECTIVES

Some limitations to our study need to be highlighted. First is the small size of our sample that limits our study to an exploratory purpose. Indeed, small samples have been shown to be associated with a lower degree of replicability in functional MRI (Turner et al., 2018; Grady et al., 2021) as well as in structural MRI studies (Katuwal et al., 2015; Schaer et al., 2015). This limitation especially holds for our *post hoc* analyses on ASD subgroups. We nevertheless considered that these subgroup analyses offered an interesting deciphering of our main results and provide interesting hypotheses on which future research can build upon. Replication with larger samples will be necessary, especially to better delineate different phenotypic subgroups according to their distinct GWC alterations.

Second, motion artifacts (Fouquet et al., 2019) as well as alterations of cortical thickness could represent confounding factors to our results (Westlye et al., 2009). Nevertheless, vertex-wise analyses only revealed a single focal cluster with decreased mean cortical thickness in the HR-ASD group. The vast majority of regions found to have altered GWC or ΔGWC values in relation to ASD showed no significant differences in cortical thickness. Additionally, motion in our sample was only correlated with GWC in the right superior parietal region and did not overlap with any cluster from our main results. We can thus reasonably rule out alterations of cortical thickness as well as motion as confounding factors.

Finally, one could wonder how observed alterations are specific to ASD and not related to broader developmental delay. In our sample, HR-ASD exhibited a significant global delay (DQ = 77.4 in average, see **Table 1**) and it is impossible to exclude that this delay was in part responsible for the observed differences. Further studies including either a group of children with developmental delay without ASD or children with ASD without developmental delay are needed to address this limitation.

CONCLUSION

In conclusion, our results support the hypothesis that ASD is associated with widespread microstructural alterations at the gray-white matter boundary during the first 2 years of life. These alterations were linked to symptom severity at 18 months of age, and also with later diagnosis outcomes and symptom severity at 3 years of age. GWC alterations in ASD consisted of increased contrast across many brain regions relevant for social processing, language acquisition as well as in primary visual and motor cortical regions. In parallel, children who experienced more severe symptoms of autism at 18 months of age exhibited slower GWC rates of change during the second year of life in many regions that are important for attentional and executive processing. Finally, all the GWC alterations that we reported were globally stronger in toddlers who already received a reliable ASD diagnosis at 18 months compared to those who developed ASD later. A potential neurobiological explanation of these findings might involve delayed myelination of superficial white matter, a hypothesis which will need to be assessed by further quantitative neuroimaging studies. Together, our results suggest that early enhancement of GWC in many regions is associated with later autism diagnosis and symptom severity, and that autism symptom severity at the age of 18 months is associated with a specific corresponding early developmental brain signature.

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 University of California at Davis Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SO, DGA, and CWN conceived of and designed the study and acquired all clinical and neuroimaging data. DGA, JL, and GY provided technical assistance. MG prepared and analyzed the data under the supervision of MS and CWN. MG wrote the manuscript with the input from all other authors. All authors participated in interpretation of results, read and approved the final manuscript.

FUNDING

This research was supported by the Swiss National Foundation Synapsy (Grant No. 51NF40–185897) and the Swiss National Foundation for Scientific Research (Grant Nos. 323630-191227 to MG and 163859 and 190084 to MS). This work was also supported by the National Institutes of Health (Grant Nos. R01MH068398 to SO, R01MH104438 to CWN R01MH103284 to MS, and R01MH103371 to DGA) and the University of California (UC) Davis MIND Institute. The MIND Institute Intellectual and Developmental Disabilities Research Center (Grant No. U54HD079125) and the Autism Center of Excellence (Grant No. P50 HD093079) awarded by the National Institute of Child Health and Development (NICHD) also supported this project. DSA was supported by the MIND Institute Autism Research Training Program (Grant No. T32MH073124).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.669194/full#supplementary-material

Supplementary Figure 1 | Graphic representation of individual ADOS CSS at 18 and 24 months of age in our sample (n = 20 HR participants). Color code represents individual diagnosis outcome at age 3. HR-TD: high risk for ASD with typical development; HR-non-TD: high risk for ASD with atypical development; HR-ASD: high risk for ASD with ASD.

Supplementary Figure 2 | (A) Clusters with a significant effect of time on gray-white matter contrast (GWC) in the HR-TD group. Clusters with cluster-wise P-value (CWP) < 0.05 only are displayed. Color code corresponds to P-value of the vertex with the maximal P-value (Pvm) of each cluster. **(B)** On the right are plotted for each hemisphere the individual GWC rates of change (ΔGWC) within each significant cluster (one per hemisphere). We found no significant difference between both clusters (paired t-test, $p = 0.15$).

Supplementary Figure 3 | Results displayed on **Figure 4** (Association between GWC at age 12–24 months and symptom severity at 18 months of age) with individual GWC values displayed for each significant cluster in function of ADOS calibrated severity score (CSS) (upper-side graphs), diagnostic outcome group (left down-side graphs) and HR-ASD subgroups (right down-side graphs). **(A)** Results for clusters with significant correlation between GWC and 18-mo ADOS CSS. **(B)** Clusters with significant correlation between GWC and 36-mo ADOS CSS. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Supplementary Figure 4 | Results displayed on **Figure 5** (Association between symptom severity at 18 months of age and GWC rate of change (ΔGWC) between 12 and 24 months of age) with individual ΔGWC displayed for each cluster in function ADOS calibrated severity score (CSS) (upper-side graph), diagnostic outcome group (left down-side graphs) and HR-ASD subgroups (right down-side graphs). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Supplementary Figure 5 | Association between cortical thickness at age 12–24 months and diagnostic outcome at 36 months of age (HR-ASD or HR-TD). The single cluster with significantly smaller CT in HR-ASD compared to HR-TD (CWP < 0.05) is displayed. We found no significant cluster in the right hemisphere. Color code corresponds to P-value of the vertex with maximal P-value (Pvm) of the displayed cluster. On the right, individual CT-values are displayed in function of diagnosis outcome for the displayed cluster. $^{*}p < 0.05$. CWP, cluster-wise P-value.

Supplementary Figure 6 | Results of supplementary analysis comparing LR-TD with HR-ASD **(A)** and LR-TD with HR-TD **(B)**. Color code corresponds to P-value of the vertex with maximal P-value (Pvm) of the displayed clusters. On the graphs, individual GWC values are displayed in function of diagnosis outcome for the displayed clusters.

Supplementary Figure 7 | (A) Effect of time on GWC within LR toddlers with typical development outcomes at 3 years (LR-TD) represented with vertex-wise ΔGWC values mapped on the common FreeSurfer template. **(B)** Clusters with a significant effect of time on gray-white matter contrast (GWC) in the LR-TD group. Clusters with cluster-wise P-value (CWP) < 0.05 only are displayed. Color code corresponds to P-value of the vertex with the maximal P-value (Pvm) of each cluster.

Supplementary Table 1 | Demographic and behavioral information on HR-ASD subgroups (LOA and EOA). P-value of statistical comparison between both subgroups.

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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.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 29 September 2021
doi: 10.3389/fnins.2021.705890

DRD1 and *DRD2* Receptor Polymorphisms: Genetic Neuromodulation of the Dopaminergic System as a Risk Factor for ASD, ADHD and ASD/ADHD Overlap

*Maria Addolorata Mariggiò[1], Roberto Palumbi[1], Angela Vinella[1], Riccardo Laterza[1], Maria Giuseppina Petruzzelli[1], Antonia Peschechera[1], Alessandra Gabellone[1], Ottavio Gentile[2], Alessandra Vincenti[1] and Lucia Margari[1]**

[1] Department of Biomedical Sciences and Human Oncology, University of Bari Aldo Moro, Bari, Italy, [2] Pediatric Surgery Unit, Giovanni XXIII Hospital, Bari, Italy

OPEN ACCESS

Edited by:
Eugenia Conti,
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Francesca Felicia Operto,
University of Salerno, Italy

***Correspondence:**
Lucia Margari
lucia.margari@uniba.it

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: 06 May 2021
Accepted: 07 September 2021
Published: 29 September 2021

Citation:
Mariggiò MA, Palumbi R, Vinella A, Laterza R, Petruzzelli MG, Peschechera A, Gabellone A, Gentile O, Vincenti A and Margari L (2021) DRD1 and DRD2 Receptor Polymorphisms: Genetic Neuromodulation of the Dopaminergic System as a Risk Factor for ASD, ADHD and ASD/ADHD Overlap. Front. Neurosci. 15:705890. doi: 10.3389/fnins.2021.705890

The dopaminergic system (DS) is one of the most important neuromodulator systems involved in complex functions that are compromised in both autism spectrum disorder (ASD) and attention deficit/hyperactivity disorder (ADHD), conditions that frequently occur in overlap. This evidence suggests that both disorders might have common neurobiological pathways involving the DS. Therefore, the aim of this study was to examine the *DRD1* and *DRD2* dopamine receptor single nucleotide polymorphisms (SNPs) as potential risk factors for ASD, ADHD, and ASD/ADHD overlap. Genetic data were obtained from four groups: 75 ASD patients, 75 ADHD patients, 30 patients with ASD/ADHD overlap, and 75 healthy controls. All participants were between 2 and 17 years old. We compared the genotypic and allelic frequency of 18 SNPs among all of the study groups. Moreover, in the case of statistically significant differences, odds ratios (OR) were obtained to evaluate if the presence of SNPs might be a risk factor of developing a specific clinical phenotype. This study found that *DRD1* and *DRD2* receptors SNPs might be considered as potential risk factors for ASD and ADHD. However, only *DRD2-12* (*rs7131465*) was significantly associated with a higher risk for the ASD/ADHD overlap. These data support the hypothesis of the genetic neuromodulation of the DS in the neurobiology of these conditions.

Keywords: autism spectrum disorder, ADHD, ASD/ADHD overlap, dopaminergic system, dopamine receptors, polymorphisms, neuromodulation, neurobiology

INTRODUCTION

Autism spectrum disorder (ASD) and attention deficit/hyperactivity disorder (ADHD), as well as bipolar disorder or schizophrenia, are neuropsychiatric disorders characterized by strong genetic bases (Sullivan et al., 2012; Woodbury-Smith and Scherer, 2018; Rylaarsdam and Guemez-Gamboa, 2019; Grimm et al., 2020). The dopaminergic system (DS) is involved in the regulation and the neuromodulation of some central nervous system (CNS) functions, such as social skills, the perception and the reward mechanisms for social activities, and attention and motor functions (Pavăl, 2017; Klein et al., 2019; Madadi Asl et al., 2019). Moreover, over the last two decades, several studies underlined that alterations in DS contribute to both ASD and ADHD (Iversen and Iversen, 2007; Cousins et al., 2009; Del Campo et al., 2011; Dichter et al., 2012; Owen et al., 2017).

These alterations may be related to different consequences: a selective deficit of dopamine (DA), and genetic mutations to the genes involved in synaptic homeostasis, as DA receptors, membrane transporters, or the enzymes designated to DA degradation or reuptake.

Genome-wide association studies (GWAS) significantly contributed to the identification of several genome variants known as single nucleotide polymorphisms (SNPs) associated with neuropsychiatric disorders (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013, 2019; Cross-Disorder Group of the Psychiatric Genomics Consortium, Lee et al., 2013). These genomic variations may remain silent, without functional implications. In other cases, SNPs can give rise to missense or non-sense mutations, gene expression, or splicing alterations. When a DS receptor region is involved, SNPs can cause increase or reduction, until the absence, of receptor protein. Alternatively, binding potential or binding affinity of receptor proteins for the ligand can also be modified (Sullivan et al., 2012; Cross-Disorder Group of the Psychiatric Genomics Consortium, Lee et al., 2013).

Given the multitude of gene variants and possible mechanisms, several studies investigated the correlation between the SNPs involving the DS and ASD or ADHD.

Attention deficit/hyperactivity disorder is a neurodevelopmental disorder (NDD) characterized by a persistent pattern ofattention deficit, hyperactivity, and impulsivity; it is one of the most common NDDs with a complex etiology and a strong genetic component (Nigg, 2013; Matthews et al., 2014; Demontis et al., 2019; Grimm et al., 2020). The clinical symptomatology of ADHD is linked to a series of alterations of functions regulated by the DS in the CNS. Furthermore, functional neuroimaging evidence has offered results about dopaminergic dysfunction in patients with ADHD, supporting the possible role of catecholaminergic dysregulation in the neurobiology of the disorder (Nigg, 2013).

As in ADHD, the DS is also involved in the ethology of ASD (Pavăl, 2017; Madadi Asl et al., 2019). ASD is a disorder characterized by two main core symptoms: a social communication and interaction deficit and the presence of repetitive and restricted interests and behaviors. Most of the functions disrupted in ASD are regulated by the DS. For example, the prefrontal cortex and the mesocorticolimbic circuit are both involved in executive functions and social cognition, while a nigro-striatal pathway alteration might explain the motor symptoms of ASD (Pavăl, 2017).

Recent studies have already identified hundreds of ASD-related gene variant encoding for synaptic proteins, transcription factors, epigenetic modulators and molecules involved in intracellular signaling (Castellanos and Tannock, 2002; Wise, 2004; Yin and Knowlton, 2006; Balleine et al., 2007; Hettinger et al., 2012). The DS plays a role in motor functions, reward and motivation which are altered in ASD. Patients with ASD display inappropriate social behavior (Mayes et al., 2011; Neale et al., 2012; Lamanna et al., 2017). Furthermore, some genetic studies have identified several SNPs or gene mutations related to the DS in patients with ASD (Craig et al., 2015, 2016).

Autism spectrum disorder and ADHD share common clinical features related to the impairment of several functions, such as attention skills, executive functions, and motor and social skills (American Psychiatric Association, 2013; Craig et al., 2015, 2016; Antshel and Russo, 2019; Gudmundsson et al., 2019). The overlap between ASD and ADHD is the clinical condition in which the two disorders are comorbid and the respective symptoms occur in the same patient. Since the publication of the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), ADHD is no longer an exclusion criteria for an ASD diagnosis and vice versa (American Psychiatric Association, 2013).

According to a recent review, the prevalence of ASD/ADHD overlap has increased over the years, and these disorders seem to share genetic heritability and some clinical features (Antshel and Russo, 2019). Other studies aimed to identify possible risk factors for these condition (Craig et al., 2015, 2016; Lamanna et al., 2017; Gudmundsson et al., 2019), but its neurobiology is still unclear.

Therefore, the purpose of this study was to provide new results that might confirm and support the involvement of DS in the pathogenesis of ASD, ADHD, and their overlap, focusing on dopaminergic receptor SNPs as possible genetic risk factors for these conditions.

MATERIALS AND METHODS

Participants

For the study, patients diagnosed with ASD, ADHD, and ASD/ADHD overlap were recruited at the Childhood and Adolescence Neuropsychiatry Unit, University of Bari Aldo Moro, from 2015 to 2019.

The inclusion criteria were patients diagnosed with ASD, ADHD, and ASD/ADHD overlap, and aged between 2 and 17 years. The diagnoses were made according to the diagnostic criteria of the DSM-5 (American Psychiatric Association, 2013). We decided to consider ASD/ADHD overlap as an individual group in order to identify dopamine receptor SNPs as possible genetic risk factors of this distinct clinical disorder. The clinical diagnostic procedures included a full medical history interview, a neurological examination, and the administration of standardized protocols. We recruited 75 patients with ASD, 75

patients with ADHD and 30 patients with ASD/ADHD overlap. All patients included in the study were Caucasian.

The exclusion criteria were patients suffering from ASD and ADHD attributable to known genetic syndromes or other medical conditions (e.g., ASD-like symptoms might occur in fragile X syndrome; ADHD-like symptoms might be caused by drug intoxication or fetal alcohol syndrome).

For comparison and risk assessment of genotypes, 75 subjects aged between 2 and 17 years that had surgery and without any neurodevelopmental disorders were recruited at the Pediatric Surgery Unit, Giovanni XXIII Hospital, Bari, as controls.

The study was approved by the Local Ethical Committee (protocol number 592/12) and for all participants, informed consent was collected from their parents.

Genotyping

The choice of polymorphisms was influenced by several factors. *DRD1* and some *DRD2* SNPs involved in this study were already known in the literature. Furthermore, using http://www.ncbi.nlm.nih.gov/nuccore/209977039?report=genbank&to=72685, we searched for all polymorphisms of the *DRD2* gene that are currently identified.

Since the methylation profiles of regions containing CpG islands could influence the levels of gene expression, using the CpGplot program of the EMBOSS package (available at https://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/) we identified two regions within the introns of the *DRD2* gene that are unusually enriched with CpG dinucleotides; the first extends from nucleotide 4,634 up to nucleotide 5,660 (therefore longer than 1 kb), and the second extends from nucleotide 5,740 up to 5,953 (214 base pair long).

In these regions, we selected SNPs having an allelic frequency not less than 10% (0.1) in principle and, among these, only those that could be discriminated using the restriction fragment length polymorphism (RFLP) technique were considered.

This technique involves the use of restriction enzymes that recognize and cut specific DNA sequences. The enzymatic cutting is usually carried out in correspondence with the polymorphic sequence, allowing the recognition of the nucleotide variation.

The search for restriction enzymes to be used was conducted using the programs available on the New England Biolabs website[1]. The first program used was NEBcutter®[2], which allows the identification of restriction enzymes able to discriminate the polymorphic sequence. We then moved on to the Primer3 program (see 0.4.0) (available at http://bioinfo.ut.ee/primer3-0.4.0/primer3/) to design amplification primers for the restriction sites and, finally, the REBsites program[3] was used to predict the length of the fragments obtained after the restriction enzyme cutting. The genotyping of the recruited subjects was carried out using venous blood samples from patients and controls. To isolate the leukocytes of the study subjects, a sample was taken in tubes containing sodium citrate. A total of 10 ml of peripheral blood was mixed in a 1:1 ratio with Emagel (Piramal Healthcare, Northumberland, United Kingdom) heparinized (5 U.I. of heparin per ml of Emagel). The obtained solution was placed on a rotor for 10 min at the end of which the red cells were left to settle. The supernatant thus obtained was centrifuged at 1,600 rpm for 10 min. The pellet was re-suspended in 5 ml of 1X PBS and centrifuged at 1,600 rpm for 10 min. To remove the present cells, an osmotic shock was applied: the pellet was then re-suspended in 1 ml of 0.2% NaCl and vortexed for 1 min. Subsequently, 1 ml of 1.6% NaCl was added and the suspension was then centrifuged at 1,200 rpm for 10 min. Where necessary, the osmotic shock was repeated. The pellet was finally re-suspended in 1 ml of physiological solution and the leukocytes were counted in the Burker chamber. After cell counting, 10×10^6 cell aliquots were used to extract DNA using DNAzol® Reagent (Life Technologies, Carlsbad, CA, United States).

The DNA concentration was measured by spectrophotometer and the solution was diluted with H2O RNasi and DNasi free (SIGMA) to obtain a final value of 100 ng/μl. Each polymorphic region was amplified using 100 ng DNA, 5 μl 10X PCR buffer, 3 μl 25 mM MgCl2, 2 μl 10 mM dNTPs mix, 0.5 μl AmpliTaq Gold 5 U/μl (Life Technologies, Carlsbad, CA, United States) and 1 μl of specific primer (IDT Inc., Coralville, IA, United States). The thermal protocol used was the same for all reactions, with an annealing temperature of 57°C and several cycles equal to 40. **Table 1** shows the 18 polymorphisms selected for the study, their related gene and expected PCR amplicon size. Individual amplicons electrophoretic runs are displayed in **Figure 1**.

Restriction Fragment Length Polymorphism

All of the endonucleases used were purchased from Thermo Scientific (Carlo Erba reagents, Cornaredo, Italy) except for the enzyme *Cac*8I, which was purchased from New England Biolabs (Ipswich, MA, United States). Then, 10 μl of amplified obtained from the PCR reaction was used for enzymatic cutting. The digestion mix was prepared using 2 μl of specific digestion buffer and 1 U of the enzyme in a total volume of 20 μl. The reaction was carried out for 1 h in a thermostatic bath by varying the temperature depending on the enzyme used, as specified in **Supplementary Table 1**.

The information about each polymorphism is obtainable from the NCBI database; db SNPs with the relative expected digestion fragments predicted by the REBsite software are described in detail in the **Supplementary Material**.

An example of genotyping, regarding *DRD1-B* (*rs4532*) polymorphism, is shown in **Figure 2**.

Statistical Analyses

To determine the relationship between *DRD* SNPs under study and the risk of childhood ADHD, ASD and ASD/ADHD overlap phenotypes, both genotypic and allelic frequencies related to each SNP were compared among the groups reported above and the group of subjects unaffected by any neuropsychiatric pathology (control group) by the Chi-squared test or the Fisher's Exact test, where appropriate, (empirical *P*-value).

[1] https://www.neb.com/

[2] http://tools.neb.com/NEBcutter2/

[3] http://tools.neb.com/REBsites/index.php

TABLE 1 | List of analyzed polymorphisms of *DRD1* and *DRD2* genes and of the primer sequences with the expected amplicon size.

Gene (rsID)	SNP primer sequences	Expected amplicon size (bp)
DRD 1-A (rs686)	*FOR: 5′-GTGTGTTGGAAAGCAGCAGA-3′* *REV: 5′-CCATCACACAAAACGGTCAG-3′*	*166*
DRD 1-B (rs4532)	*FOR: 5′-GGCAGAGGTGTTCAGAGTCC-3′* *REV: 5′-CGGTCCTCTCATGGAATGTT-3′*	*187*
DRD 1-C (rs265973)	*FOR: 5′-GCATGCCAATTTGCTCTTG-3′* *REV: 5′-GGATTAAAGAGGATCCAGTCCA-3′*	*100*
DRD 1-D (rs265975)	*FOR: 5′-CCTCTCATGTCCCTCTCCAA-3′* *REV: 5′-GAGCAAGGACAACAGGAAGC-3′*	*232*
DRD 2-A (rs1076560)	*FOR:5′-GACAAGTTCCCAGGCATCAG-3′* *REV:5′-GGCAGAACAGAAGTGGGGTA-3′*	213
DRD 2-B (rs1800497)	*FOR:5′- AAATTTCCATCTCGGCTCCT-3′* *REV:5′-GAGGAGCACCTTCCTGAGTG-3′*	293
DRD 2-C (rs1079597)	*FOR: 5′-TTTCCCTTCTGTGGGATGAG-3′* *REV: 5′-GGAGGTTGCAATAGGCAAGA-3′*	274
DRD 2-E (rs7118900)	*FOR: 5′- CGCAGTAGGAGAGGGCATAG-3′* *REV: 5′-ATGGGAGCTTCAAAGGGAAG-3′*	*348*
DRD 2-1 (rs144851051)	*FOR: 5′- CTCAGCCTCCCAAGTAGCTG-3′* *REV: 5′- GCTGTCCACATGCTGAAGAA-3′*	*346*
DRD 2-2 (rs11608185)	*FOR: 5′-GTGTGCATGGCTGTGTCC-3′* *REV: 5′- GCTGCTGTGAGGGTTATATAGGA-3′*	396
DRD 2-7 (rs35352421)	*FOR: 5′-CCTGCACCCCAGATTCAG-3′* *REV: 5′- CTGTTTCCTCTCTGCCAACC-3′*	375
DRD 2-8 (rs2245805)	*FOR: 5′-CTCCTAGGCATCCAACCAAA-3′* *REV: 5′- GTGGCTCCCAAGTACTGGTC-3′*	373
DRD 2-10 (rs67800399 merged into rs2734832)	*FOR: 5′- TCAGGTCATTTTGGAAGTTGC-3′* *REV: 5′-AGGGAAGGGGTTGTTGAAAG-3′*	249
DRD 2-11 (rs1962262)	*FOR: 5′-CCTCAGCCTCCCAAGTATCT-3′* *REV: 5′-TCTTGGTAACCCTGGGAGTC-3′*	240
DRD 2-12 (rs7131465)	*FOR: 5′-GCCTGTAATCCCAGCACTCT-3′* *REV: 5′-AAGGGAAAACATGGCAAATG-3′*	366
DRD 2-15 (rs61902807)	*FOR: 5′-CCTCTAAGCACCAGACAGAGC-3′* *REV: 5′-ACCTCAAGAGCCACCGAAA-3′*	250
DRD 2-16 (rs10789943)	*FOR: 5′-TAGCCTCCTCGCCACTTAGA-3′* *REV: 5′-CGAAAGTTCAGGACCAAGGA-3′*	362
DRD 2-17 (rs10789944)	*FOR: 5′-TAGCCTCCTCGCCACTTAGA-3′* *REV: 5′-CTCTCCCCCATCCTTAGCTT-3′*	300

DRD, Dopamine Receptor; FOR, forward primer; REV, reverse primer; bp, base-pair.

Further the genotypic association analysis under the dominant and recessive models of inheritance were performed.

The differences were considered statistically significant if the *P*-value was < 0.05.

For the latter, the odds ratio (OR) and the 95% confidence interval (95% CI) were then calculated to assess the risk of expressing or not expressing the pathological phenotype for the group under examination compared to the reference group, based on the presence of the minor allele.

A multiple testing correction (false discovery rate) was performed to guard against the potential for false positive associations (corrected *P*-value). Data were analyzed with R version 4.0.2.

RESULTS

We recruited 75 patients with ASD, 75 patients with ADHD and 30 patients with ASD/ADHD overlap. Demographic features are summarized in **Table 2**, while allele frequencies distribution, regarding 18 analyzed SNPs, is shown in **Table 3**.

Among *D1* and *D2* receptor genes, Chi-squared test identified six and seven SNPs, respectively, in genotypic and allelic distribution, characterized by a statistically significant difference both in the case–control comparison and between the pathological groups, with empirical *P*-values < 0.05 (**Tables 4**, **5**).

About *D1* receptor polymorphisms, the SNP *rs4532* appeared to be associated with a greater risk for ASD (OR = 1.8; 95%IC = 1.115–2.912; empirical *P*-value = 0.02).

The most relevant results came from the analysis of D2 receptor polymorphisms. Indeed, *rs2245805* and *rs7131465* appeared to be associated with the increased risk of developing ASD/ADHD overlap compared to the other clinical phenotypes.

The presence of the minor allele in *rs144851051* and *rs2734832* seems to promote the development of a singular clinical disease, that is ADHD vs. controls for *rs144851051* (OR = 2.6; 95%IC = 1.0054–7.073; empirical *P*-value = 0.04) and ADHD

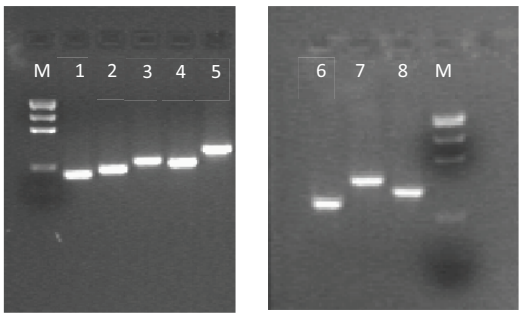

FIGURE 1 | Example of gel electrophoresis pattern of PCR amplicons about some analyzed *DRD* SNPs with their size. M, Precision Molecular Mass Ruler, Bio-Rad Laboratories, Inc., (size of DNA fragments: 1000, 700, 500, 200, 100 bp). PCR amplicons: 1, *DRD 1-A* (166 bp); 2, *DRD 1-B* (187 bp); 3, *DRD 1-D* (232 bp); 4, *DRD 2-A* (213 bp); 5, *DRD 2-B* (293 bp); 6, *DRD 2-15* (250 bp); 7, *DRD 2-16* (362 bp); 8, *DRD 2-17* (300 bp).

or ASD vs. overlap for *rs2734832* (OR = 1.9; 95%IC = 1.0564–3.5671; empirical *P*-value = 0.03 and OR = 2.1; 95%IC = 1.1196–3.7949; empirical *P*-value = 0.02, respectively).

By contrast, *rs11608185* and *rs61902807* could be protective factors for the development of the overlap condition.

However, the false discovery rate method dramatically reduced the number of significantly different SNPs and only *rs7131465* (*DRD2-12*), both in genotypic and allelic distribution, remained after the correction.

The presence of the minor allele in SNP *rs7131465*, located in the 5′-terminal untranslated region (5′ UTR) of the *DRD2* gene, seems to be a strong risk factor of developing ASD/ADHD overlap vs. a singular clinical disease, that is ASD or ADHD (OR = 2.7; 95%CI = 1.4701–5.024; corrected *P*-value = 0.003 and OR = 2.8; 95%CI = 1.5581–5.3447; corrected *P*-value = 0.003, respectively).

Actual results are impacted by the reduced sample size of overlap group and low statistical power of the comparison groups. To solve a similar situation, Ma et al. (2021) merged their data to form a single aggregated clinical group to be compared against a single aggregated control one. The advantage of this method is enlarging the comparison group size and thus increasing statistical power.

On our side, an enlarged ADHD or ASD group, including ADHD/ASD overlap, would lead to the loss of distinctive feature and prediction of specific risk for overlap patients.

To support our findings about rs7131465 SNP, we took a different approach that is dominant and recessive models of inheritance (**Table 6**; Liu et al., 2021; Ma et al., 2021).

The group with the C*/C* homozygous minor allele or the A/C* heterozygous genotypes of *rs7131465* showed an increased risk of overlap comparing to healthy controls (OR = 3.25, 95% CI = 1.11–11.91, empirical *P*-value = 0.04), ASD (OR = 4.84, 95% CI = 1.68–17.61, empirical *P*-value = 0.007) or ADHD (OR = 5.69, 95% CI = 1.198–20.69, empirical *P*-value = 0.003) in a dominant model, but not a recessive model. The last two of them survived the multiple testing correction and remained statistically significant, i.e., overlap vs. ASD (corrected *P*-value = 0.021), overlap vs. ADHD (corrected *P*-value = 0.017).

No significant difference in the genotype distribution of *rs7131465* in children with ASD or ADHD and healthy controls was observed, both in recessive or dominant model.

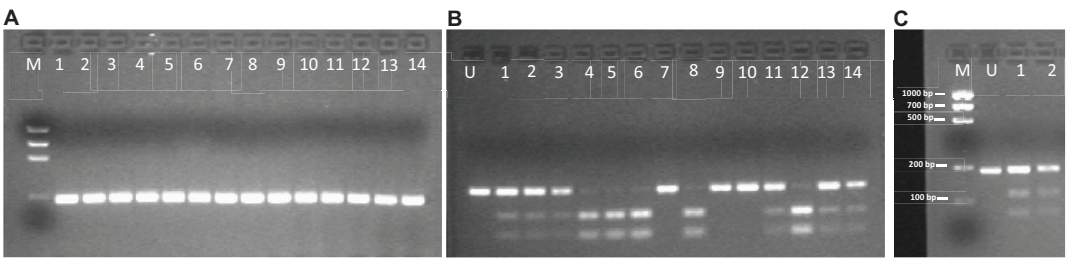

FIGURE 2 | Gel Electrophoresis pattern about *DRD 1-B* SNP (*rs4532*). **(A)** Electrophoretic gel of 14 patients *DRD 1-B* amplicons (187 bp). **(B)** *Bpu*10I enzyme digestion pattern on the same 14 patients PCR amplicons. Restriction site is on T allele. Single 187-bp electrophoresis band denotes a C/C homozygous genotype; 116-bp and 71-bp banding pattern is for a T/T homozygous genotype; the presence of three bands of 187-bp, 116-bp and 71-bp is for C/T heterozygous genotype. **(C)** Molecular length (bp) of restriction fragments derived from *rs4532 digestion by Bpu*10I. M, DNA Molecular Weight Marker (Precision Molecular Mass Ruler, Bio-Rad Laboratories, Inc.); U, undigested PCR amplicon (187 bp).

TABLE 2 | Demographic features of the study groups.

Participants		ADHD	ASD	ADHD/ASD overlap	Controls
Number		75	75	30	75
Average age, (years)		10.36	10.57	11.57	12.23
Gender	Male (%)	83	85	76.6	75
	Female (%)	17	15	23.4	25

ADHD, attention deficit/hyperactivity disorder; ASD, autism spectrum disorder.

TABLE 3 | Allele frequencies distribution of SNPs in study groups.

Gene	db SNP (rsID)	Minor/major allele	Minor allele frequency			
			ADHD ($n = 75$)	ASD ($n = 75$)	Overlap ($n = 30$)	Controls ($n = 75$)
DRD 1	*DRD 1-A (rs686)*	*G/A*	0.346	0.386	0.383	0.293
	DRD 1-B (rs4532)	*C/T*	0.346	0.420	0.383	0.286
	DRD 1-C (rs265973)	*T/C*	0.427	0.467	0.433	0.460
	DRD 1-D (rs265975)	*T/C*	0.327	0.346	0.317	0.427
	DRD 2-A (rs1076560)	*A/C*	0.14	0.14	0.150	0.16
	DRD 2-B (rs1800497)	*T/C*	0.17	0.16	0.150	0.21
	DRD 2-C (rs1079597)	*A/G*	0.11	0.12	0.150	0.16
	DRD 2-E (rs7118900)	*A/G*	0.14	0.17	0.167	0.21
	DRD 2-1 (rs144851051)	*T/C*	*0.1*	*0.05*	*0.050*	*0.04*
	DRD 2-2 (rs11608185)	*C/T*	*0.66*	*0.67*	*0.500*	*0.61*
DRD 2	*DRD 2-7 (rs35352421)*	*T/G*	*0.93*	*0.93*	*0.950*	*0.96*
	DRD 2-8 (rs2245805)	*A/C*	*0.23*	*0.21*	*0.350*	*0.23*
	DRD 2-10 (rs67800399 merged into rs2734832)	*C/A*	*0.34*	*0.33*	*0.500*	*0.39*
	DRD 2-11 (rs1962262)	*T/C*	0.11	0.12	0.176	0.16
	DRD 2-12 (rs7131465)	*C/A*	0.33	0.34	0.567	0.43
	DRD 2-15 (rs61902807)	*C/T*	0.40	0.45	0.333	0.47
	DRD 2-16 (rs10789943)	*A/G*	0.15	0.19	0.133	0.13
	DRD 2-17 (rs10789944)	*A/C*	0.16	0.19	0.133	0.14

SNP, single nucleotide polymorphism; ADHD: attention deficit/hyperactivity disorder; ASD, autism spectrum disorder.

DISCUSSION

In this study, we aimed to investigate if specific *DRD1* and *DRD2* receptor polymorphisms might be considered as potential genetic risk factors for ASD, ADHD, and ASD/ADHD overlap.

Our study found that two specific polymorphisms of the *D2* receptor, *rs2245805* and *rs7131465,* respectively, *DRD2-8* and *DRD2-12*, might be associated with ASD/ADHD overlap when compared with ASD, ADHD, and control groups. However, only the SNP *rs7131465* (*DRD-12*) showed a statistically significant higher risk for the ASD/ADHD overlap.

TABLE 4 | Results of the comparative analysis of genotype distribution of the SNPs among the study groups.

Polymorphism (rsID)	Compared groups	Empirical *P*-value*	Corrected *P*-value for false discovery rate
DRD 2-8 (rs2245805)	Overlap vs. ASD	0.05	0.20
DRD 2-10 (rs2734832)	Overlap vs. ASD	0.04	0.15
	Overlap vs. ADHD	0.05	0.15
DRD 2-12 (rs7131465)	Overlap vs. ASD	0.005	0.015
	Overlap vs. ADHD	0.003	0.015
DRD 1-B (rs4532)	ASD vs. CTR	0.04	0.12
DRD 1-D (rs265975)	ADHD vs. CTR	0.04	0.12
DRD 2-2 (rs11608185)	Overlap vs. ADHD	0.05	0.15

**Empirical P-value: result of Chi-squared test.*
SNPs, single nucleotide polymorphisms; ADHD, attention deficit/hyperactivity disorder; ASD, autism spectrum disorder.

DRD2-12 is within the intronic region between exon 1 and exon 2 of 5′ UTR. This intronic region is large 50,391 base pair and *rs7131465* is located near the beginning of exon 2. Currently, no study has been conducted that examined the effect of this polymorphism. Since UTRs are the regulatory elements of genes, acting as controllers of translation and RNA decay, as well as targets for RNA interference (RNAi) and playing a central role in post-transcriptional regulation, it should be no surprise that polymorphisms in 5′ UTRs have been linked to many human, mainly oncological and neurological, diseases (Halvorsen et al., 2010). These SNPs can promote tumorigenesis by increasing c-Myc expression (Chappell et al., 2000), translation inhibition (Cazzola and Skoda, 2000), and transcription activity (Fan et al., 2013). 5′ UTR alterations was also involved in neurological disease such as spinocerebellar ataxia type 1 (Rachna et al., 2020), Parkinson's disease (Rubino et al., 2020), bipolar disorder type I (Alizadeh et al., 2019) and Alzheimer's disease (Lahiri et al., 2003).

To the best of our knowledge, no previous studies were carried out to investigate genetic polymorphisms of ASD/ADHD overlap. This is probably related to the fact that the nosographical recognition of the comorbidity between the two disorders occurred only after the publication of the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders in 2013.

However, from the results of the present study, it is hypothesized that dopaminergic neuromodulation may also be involved in the pathogenesis of the overlap, probably with different genetic risk aspects compared to those of ASD and ADHD. Even if these two disorders share a common clinical

TABLE 5 | Results of the comparative analysis of allelic distribution of the SNPs among the study groups with the corresponding OR values.

Polymorphism (rsID)	Compared groups	Empirical *P*-value*	Corrected *P*-value for false discovery rate	OR (95% CI)
DRD 2-1 (rs144851051)	ADHD vs. CTR	0.04	0.21	2.6 (1.0054–7.073)
DRD 2-8 (rs2245805)	Overlap vs. CTR	0.04	0.08	1.9 (1.0317–3.7817)
	Overlap vs. ASD	0.02	0.08	2.1 (1.1095–4.1077)
	Overlap vs. ADHD	0.04	0.08	1.9 (1.0317–3.7817)
DRD 2-10 (rs2734832)	ASD vs. Overlap	0.02	0.09	2.1 (1.1196–3.7949)
	ADHD vs. Overlap	0.03	0.09	1.9 (1.0564–3.5671)
DRD 2-12 (rs7131465)	Overlap vs. CTR	0.04	0.32	1.8 (1.0255–3.451)
	Overlap vs. ASD	0.001	0.003	2.7 (1.4701–5.024)
	Overlap vs. ADHD	0.001	0.003	2.8 (1.5581–5.3447)
DRD 1-B (rs4532)	ASD vs. CTR	0.02	0.12	1.8 (1.115–2.912)
DRD 2-2 (rs11608185)	Overlap vs. ASD	0.02	0.09	0.5 (0.2718–0.9197)
	Overlap vs. ADHD	0.03	0.09	0.5 (0.2803–0.9467)
DRD 2-15 (rs61902807)	Overlap vs. CTR	0.04	0.24	0.5 (0.2742–0.9695)

**Empirical P-value: result of Chi-squared test or Fisher's Exact test.*
SNPs, single nucleotide polymorphisms; OR, odds ratio; ADHD, attention deficit/hyperactivity disorder; ASD, autism spectrum disorder.

TABLE 6 | The genotype distribution of SNP *DRD 2-12* between overlap and the other clinical groups and risk prediction for overlap disorder, under the most significant genetic model of inheritance.

db *SNP* (rsID)	Compared groups	Most significant model	Genotype	Group1/Group2 (n, %)	#OR (95% CI)	Empirical *P*-value	Corrected *P*-value
DRD 2-12 (rs7131465)	Overlap vs. Controls	Dominant	C*/C* + A/C*	26 (87)/50 (67)	3.25 (1.11–11.91)	0.04	0.08
			A/A	4 (13)/25 (33)			
	Overlap vs. ASD	Dominant	C*/C* + A/C*	26 (87)/43 (57)	4.84 (1.68–17.61)	0.007	**0.021**
			A/A	4 (13)/32 (43)			
	Overlap vs. ADHD	Dominant	C*/C* + A/C*	26 (87)/40 (53)	5.69 (1.98 –20.69)	0.003	**0.017**
			A/A	4 (13)/35 (47)			

OR, odds ratio; SNP, single nucleotide polymorphism; CI, confidence interval.
**Minor allele.*
Dominant model: homozygous minor allele plus heterozygous vs. homozygous major allele.
#OR value associated with the minor allele genotype.
Significant SNPs after multiple testing correction bolded.

ground, including the impairment in cognitive functions (e.g., attention skills), in social abilities, and in the executive functions, recent studies underlined that both ASD and ADHD retain qualitative and quantitative clinical differences in their phenotype (Craig et al., 2015; Antshel and Russo, 2019). SNP *rs7131465* in 5′ UTR might be involved in alternative splicing resulting in mRNA instability and producing different isoforms of *DRD2* transcript.

Other *D1* and *D2* receptors have been previously identified in patients with ASD (Hettinger et al., 2008, 2012). A study on murine models showed that excessive striatal dopaminergic activation, deriving from specific mutations of the *D1* receptor, might promote autistic symptoms in mice, such as social deficits and repetitive behaviors. This interpretation was supported by the evidence that murine behavioral changes induced by excessive dopaminergic activity were inhibited by specific *D1* receptor antagonists (Lee et al., 2018).

Interestingly, Liu et al. (2020) demonstrated that certain SNPs of dopaminergic system genes might have a modulator effect on facial/emotion recognition in patients with ASD (Liu et al., 2020). Nevertheless, a recent Chinese study showed that some serotonin HTR2A receptor SNP might also be associated with a higher risk for ASD (Liu et al., 2021). Moreover, previous meta-analyses showed a significant association between some *D2* receptor polymorphisms and ADHD (Sullivan et al., 2012; Wu et al., 2012; Pan et al., 2015). As for ASD, some studies investigated the possible effects of gene polymorphisms of the DS on the functional activity of the dopaminergic circuits involved in ADHD. Different models have been proposed to explain the symptomatology of the disorder; among these, the executive functions model is the most described and studied (Arnsten and Li, 2005; Willcutt et al., 2005; Craig et al., 2016).

Lastly, more recent neuroimaging studies showed that the presence of some *DRD2* and *DRD4* might, respectively, modulate the gyrification and the functional activity of cortical areas involved in cognitive processes that are impaired in ADHD and other psychiatric disorders (Palaniyappan et al., 2019; Overs et al., 2021).

CONCLUSION

In conclusion, we found that carrying specific *DRD1/DRD2* SNPs could increase the risk for ASD, ADHD, even if only one SNP showed a statistically significant association with a higher risk

for and ASD/ADHD overlap. These findings might support the hypothesis of the involvement of the dopaminergic system in the neurobiology of these conditions. However, this study has some limitations that need to be mentioned. The study protocol approved by the Local Committee did not include also genetic examination of the parents' patient; therefore, we were not able to verify if a SNP was inherited or it is a *de novo* mutation. Moreover, this was a genetic preliminary study, so we did not proceed with a functional validation of the analyzed SNPs and with a correlation phenotype/genotype analysis; however, all these analyses would be considered for future investigations.

In addition, further studies on larger groups might explore more in-depth how the dopaminergic system SNPs could represent biomarkers for a clinical phenotype and eventually how they could modulate the efficacy of the pharmacological or rehabilitation therapy in these disorders.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Local Ethical Committee—Policlinico of Bari (protocol number 592/12). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MM, RP, and LM: conceptualization, writing—review and editing, supervision, and project administration. MM, RP, AnV, AlV, and RL: methodology and data curation. AnV, AlV, and RL: formal analysis. MM, RP, MP, AP, AG, OG, and RL: investigation. AnV, MP, AP, AG, and OG: resources. MM and RP: writing—original draft preparation. 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/fnins.2021.705890/full#supplementary-material

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frontiers in Neuroscience

ORIGINAL RESEARCH
published: 21 October 2021
doi: 10.3389/fnins.2021.733775

Alexithymia and Autistic Traits: Associations With Social and Emotional Challenges Among College Students

Potheini Vaiouli† and Georgia Panayiotou*‡

Department of Psychology, Center for Applied Neuroscience, University of Cyprus, Nicosia, Cyprus

OPEN ACCESS

Edited by:
Marco Turi, Fondazione Stella Maris Mediterraneo Onlus, Italy

Reviewed by:
Chenjie Shen, Massachusetts Institute of Technology, United States
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Natasha Baxter, University of York, United Kingdom, in collaboration with reviewer HH

***Correspondence:**
Potheini Vaiouli
vaiouli.potheini@ucy.ac.cy

†*This author share first authorship*

‡*This author share senior authorship*

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: *30 June 2021*
Accepted: *30 September 2021*
Published: *21 October 2021*

Citation:
Vaiouli P and Panayiotou G (2021) Alexithymia and Autistic Traits: Associations With Social and Emotional Challenges Among College Students. Front. Neurosci. 15:733775. doi: 10.3389/fnins.2021.733775

Background: Alexithymia is a multifaceted personality construct defined by marked difficulties in identifying and describing feelings and in externally oriented thinking. Given its intrinsic role in social-emotional processing, alexithymia is now recognized as a trans-diagnostic trait in a range of neurodevelopmental disorders, including autism. Research has pinpointed to the co-occurrence of autism with characteristics typical of alexithymic normative samples, such as social-communication difficulties and decreased emotion regulation abilities. Nonetheless, the role of individual facets of alexithymia in predicting challenges in social communication functioning is still understudied.

Methods: In total, 275 young adults completed the Toronto Alexithymia Scale, the Autism Spectrum Quotient (short form), the Interpersonal Competence Questionnaire, and the Difficulties in Emotion Regulation Scale self-reported questionnaires for assessing alexithymic and autistic traits, social-communication abilities, and emotion regulation difficulties. We used regression models to establish cross-sectional associations between autism, alexithymia, and social-emotional difficulties. Also, we ran a parallel mediation analysis to determine whether the relationship between autistic traits and emotion regulations challenges are mediated by Alexithymia facets.

Results: Analysis showed a significant positive association between autistic traits and alexithymic traits and between autistic traits and emotion regulation difficulties while, as expected, autistic traits were negatively correlated with social skills. A significant relationship was found among the participants' levels of alexithymia and their interpersonal skills with two of three alexithymic subscales significantly contributing to the model. Similarly, a significant relationship was found among alexithymia subscales and emotion regulation difficulties with all three alexithymia subscales being statistically significant. Finally, analysis on two mediator models indicated a significant effect of autistic traits on social skills mediated by alexithymic traits as well as a significant indirect effect of autistic traits on emotion regulation difficulties mediated by alexithymic traits.

Conclusion: The results of this study provide evidence of the influence of different alexithymic facets on the relationship between autistic traits and social-emotional challenges in young adults. Longitudinal studies may explore further alexithymia and its associations with social-emotional difficulties in autism as well as the potential implications of these findings in intervention and treatment programs.

Keywords: alexithymia, autism, TAS-20, emotion regulation, social skills

INTRODUCTION

Alexithymia is a multifaceted personality construct with a dimensional nature that negatively impacts affective processing, dimensions of emotional regulation and the interpretation and recognition of emotional stimuli (both verbal and non-verbal). First described by Sifneos (1973), the alexithymia construct is broken down into several facets, the most widely recognized of which are difficulties in identifying feelings, difficulties in describing feelings, and poor external oriented thinking (Bagby et al., 1994). Elevated levels of alexithymia are implicated in social-emotional and mental health outcomes, including a range of neurodevelopmental conditions that affect social and emotional understanding (Berthoz et al., 2011; Bird and Cook, 2013). That is, individuals with high alexithymic traits are reported to experience significantly more interpersonal difficulties, difficulties describing their emotions, poor affect regulation, and an impaired ability to recognize bodily sensations compared to those individuals with low alexithymic traits (i.e., Spitzer et al., 2005; Vanheule et al., 2007; Bird and Cook, 2013).

Given its intrinsic role in social-emotional processing, alexithymia is now recognized as a trans-diagnostic risk factor in a range of mental health including autism. Autism spectrum disorders (ASD) are among the most common neurodevelopmental disorders with prevalence rates ∼1.9%, (Maenner et al., 2020) characterized by increased difficulties in the emotional and social domain [American Psychiatric Association (APA), 2013]. In addition, the term broader autism phenotype (BAP) is used to describe individuals who display clinical or personality characteristics similar to those typical of ASD, although it is not a formal diagnosis (Parr and Le Couteur, 2013). Individuals with BAP or autistic traits frequently present challenges in social communication and social relating, and have increased emotional difficulties, such as reduced emotion regulation abilities (Samson et al., 2012), along a spectrum of severity.

Elevated levels of alexithymia are a robust finding in adult populations with autism, with prevalence rates of alexithymia in the autism population being between 65 and 85% (Bird and Cook, 2013; Brewer et al., 2015; Kinnaird et al., 2019); higher prevalence among relatives of individuals with autism (Szatmari et al., 2008) compared to the general population. Several studies that examined autistic traits as a continuum rather than focusing on autism diagnosis, also found a strong association between alexithymic and autistic traits (see Shah et al., 2016; Nicholson et al., 2018). There are several pathways through which this association could be explained, for example, that alexithymia is the etiology of the socio-emotional difficulties associated with autistic traits, or vice versa, or that the link is indirect, and explained by other shared characteristics between autism and alexithymia. For example, alexithymia itself may cause anxiety and related sleep issues (i.e., Tani et al., 2004), and the difficulty to externalize and process emotions may lead to a variety of psychosomatic manifestations (Poquérusse et al., 2018). Further, emotion processing difficulties, which is a central alexithymic trait, are correlated with depression, which may ultimately be the common link that associates alexithymia and autism (Hill et al., 2004).

Suggesting a more direct path between the two traits, Bird and Cook (2013) have proposed that emotion processing deficits in autism stem from co-occurring alexithymia rather than ASD, known in the literature as the "alexithymia hypothesis." Individuals with high alexithymic traits experience significantly more social-emotional difficulties than those with lower alexithymic traits (i.e., Spitzer et al., 2005), similar to individuals with high levels of autistic traits. Research findings indicate that alexithymic individuals may have a limited repertoire of emotion regulation skills (Darrow and Follette, 2014) involving inflexibility and disruptions in emotion processing (Panayiotou and Constantinou, 2017; Panayiotou et al., 2020) and poor emotion awareness (da Silva et al., 2017). Finally, studies that explored language in alexithymia have identified consistent challenges in language expression such as reduced complexity, openness and emotional content (Luminet et al., 2021), which are linked to deficits of empathy (Grynberg et al., 2018), core challenges found in autism as well.

Evidence in support of the alexithymia hypothesis, has begun to accumulate. Alexithymia total scores and scores on alexithymic facets appear to explain a number of difficulties in the processing, regulation and social expression of emotion, in individuals either meeting the autism diagnosis or characterized by autistic traits. Alexithymic traits have been shown to relate to difficulties in the production of emotion expressions (Trevisan et al., 2016) and empathy toward others (Bird et al., 2010) in individuals with autism. These skills are considered prerequisites for successful relationships and social interactions during daily routines (Trevisan et al., 2016) and their absence is known to disrupt everyday social functioning. Also, associations have been found between parent-reported alexithymic traits and the Autism Diagnostic Observation Schedule (ADOS) assessment scores (Hobson et al., 2020).

Looking at the role of alexithymia facets, the difficulty in identifying and describing feelings facet has been associated with difficulties in emotional reactivity and emotional regulation

(Samson et al., 2012), difficulties in recognizing verbal and non-verbal emotional expressions (Heaton et al., 2012; Cook et al., 2013; Oakley et al., 2016), and challenges in the ability to experience and understand emotions (Herbert et al., 2011) in autistic individuals. However, more research is required to evaluate this hypothesis, especially with regards to the specific role of alexithymic facets, on particular difficulties associated with autistic traits, and with regards to individuals with different levels of autistic traits, when looking at them as a continuum of characteristics, found in the general population.

The Current Study

In spite of the emergence of work on the association between facets of alexithymia and specific difficulties in autism (i.e., Liss et al., 2008; Oakley et al., 2020), more work is required to fully understand how sub-factors of these two constructs are related, as well as which are unrelated and distinct. In this study we examine alexithymic and autistic traits as a continuum of characteristics within the general population of young adults in Cyprus (college students), in order to provide data that will further clarify the nomological network of the link between the difficulties encompassed by the two constructs. Establishing that autism and alexithymia are indeed related but distinct allows us to make valid arguments in cases in which one construct can be used to explain difficulties related to the other. As both alexithymia and autism are conceptualized as being in a continuum, conducting such studies in the general population can help establish hypotheses on the contribution of alexithymia to the difficulties of autistic individuals that can then be tested in clinical samples.

Specifically, we aimed to assess cross-sectional associations between alexithymia (including its individual facets) and socioemotional and communication difficulties frequently observed in the BAP, including emotion regulation difficulties and interpersonal social skills, in a cohort of neurotypical young adults with varied levels of autistic and alexithymic characteristics. Extending previous literature on the co-occurrence of autistic and alexithymic traits, we focused on the followings: (1) Characterize the degree of association between alexithymic and autistic traits in the population of young, neurotypical colleges students of both genders. (2) Examine which facets of alexithymia are most strongly related to autistic traits and to difficulties typically related to autism. (3) Examine which facets of alexithymia may help explain (i.e., mediate) the association between autistic traits and emotional/interpersonal difficulties, specifically emotion regulation and social skills.

MATERIALS AND METHODS

Participants

A total of 275 Greek-Cypriot undergraduate university students (73.8% females; *M*age = 21.01, SD = 2.49 years) were recruited from introductory undergraduate courses of psychology and received extra course credit or were included in a prize-draw for their participation. Students pass competitive exams to enter the particular university, while those who enter based on disability criteria meet a specific performance cutoff relative to their cohort; therefore, although the intellectual ability of participants was not assessed, all students can be assumed not to have significant diversions from at least average intellectual ability. The majority of them were women (see **Table 1**), which is consistent with the composition of the student body at the particular university. From the total pool, 23 cases (9.1%) were excluded from analyses due to incomplete questionnaires on the autism measure. In total, 252 participants with available data on all measures were included in this study. No further exclusion criterial were involved and both autistic traits and alexithymia were assessed *via* self-report, conceptualized as continuous traits. Therefore, no clinical diagnostic procedures were followed. The study is part of a larger project on personality and mental health that received approval from the Cyprus National Bioethics Committee. **Table 1** shows the sample's characteristics.

Measures

Alexithymia

The 20-item self-report Toronto Alexithymia Scale (TAS-20; Bagby et al., 1994) was administered to measure alexithymia. The TAS-20, the most frequently used measure of alexithymia in adults and in prior research pertaining to autism (Vaiouli and Panagiotou, 2021) results in a total score and three subscales: difficulties in identifying feelings, difficulties in describing feelings, and externally oriented thinking. Higher scores indicate higher alexithymia, with scores $\geq$ 61 indicating "severe" (i.e., clinically relevant) alexithymia (Parker et al., 2003).

Autism Traits

The short form of the Autism Spectrum Quotient questionnaire (Baron-Cohen et al., 2001) for adults (AQ-10) was used for measuring the severity of autistic traits. The self-report questionnaire assesses the potential neurodevelopmental impairment with 10 items rated on a 4-point-Likert scale ranging from 1 (I strongly disagree) to 4 (I strongly agree). Total scores range from 0 to 5, such that lower scores on AQ indicate fewer autistic traits; total scores above 6 indicate more autistic traits. For this study, the total score was used as a continuous measure of symptom severity. The scale had very good internal consistency with a Cronbach's $\alpha = 0.90$ for the current sample. The AQ-10 was used instead of the original Autism Spectrum Quotient (Baron-Cohen et al., 2001) as the AQ-10 was more economic with only 10 items and there is little performance difference between the AQ-10 and the full AQ-50 (Booth et al., 2013).

Emotion Regulation Difficulties

The Difficulties in Emotion Regulation Scale (DERS; Gratz and Roemer, 2004) is a 36-item, self-report measure. It was utilized to assess participants' emotion regulation deficits. The DERS provides an overall emotion dysregulation score, and scores for six factors that reflect the multifaceted nature of emotion regulation: non-acceptance of emotional responses (Non-acceptance); difficulties engaging in goal-directed behavior whilst distressed (Goals); impulse control difficulties whilst distressed (Impulse); lack of emotional awareness (Awareness); limited access to emotion regulation strategies (Strategies); and

TABLE 1 | Correlations among all variables included in the study.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	Autism																		
2	Alexithymia	0.206**																	
3	TASDIF	0.202**	0.846**																
4	TASDDF	0.193**	0.847**	0.617**															
5	TASEOT	0.045	0.522**	0.123*	0.250**														
6	DESRS total	0.185**	0.669**	0.698**	0.523**	0.205**													
7	DERS non-acceptance	0.179**	0.430**	0.465**	0.353**	0.085	0.768**												
8	DERS goals	0.091	0.303**	0.350**	0.307**	−0.045	0.698**	0.448**											
9	DERS impulse	0.159*	0.539**	0.616**	0.363**	0.153*	0.856**	0.579**	0.525**										
10	DERS awareness	−0.008	0.445**	0.321**	0.303**	0.410**	0.406**	0.079	0.086	0.252**									
11	DERS strategies	0.200**	0.529**	0.576**	0.419**	0.116	0.888**	0.686**	0.627**	0.748**	0.152*								
12	DERS clarity	0.160*	0.768**	0.778**	0.608**	0.261**	0.772**	0.497**	0.344**	0.613**	0.423**	0.600**							
13	ICQtotal	−0.204**	−0.383**	−0.223**	−0.354**	−0.324**	−0.340**	−0.238**	−0.117	−0.219**	−0.365**	−0.286**	−0.324**						
14	ICQ initiation	−0195**	−0.320**	−0.215**	−0.322**	−0.195**	−0.248**	−0.144*	−0.090	−0.129*	−0.269**	−0.215**	−0.294**	0.854**					
15	ICQ.NegAssert, Interpersonal Competence Questionnaire, Negative Assertion.	−0.183**	−0.315**	−0.151*	−0.211**	−0.415**	−0.289**	−0.245**	−0.026	−0.205**	−0.336**	−0.231**	−0.277**	0.821**	0.573**				
16	ICQ disclosure	−0.177**	−0.282**	−0.184**	−0.228**	−0.249**	−0.315**	−0.240**	−0.135*	−0.196**	−0.329**	−0.268**	−0.257**	0.867**	0.739**	0.664**			
17	ICQ emotional support	−0.109	−0.304**	−0.090	−0.399**	−0.249**	−0.137*	−0.112	−0.017	−0.018	−0.231**	−0.118	−0.152*	0.746**	0.585**	0.466**	0.519**		
18	ICQ conflict management	−0.168**	−0.342**	−0.284**	−0.272**	−0.214**	−0.422**	−0.244**	−0.227**	−0.376**	−0.331**	−0.357**	−0.356**	0.793**	0.568**	0.660**	0.627**	0.427**	

***Correlation is significant at the 0.01 level (two-tailed); *Correlation is significant at the 0.05 level (two-tailed).*

lack of emotional clarity (Clarity). For our sample, reliability was excellent 0.93.

Social Skills

This 40-items Interpersonal Competence Questionnaire (ICQ) scale assessed participants' social skills (Buhrmester et al., 1988), through a 5-point scale (1 = I am poor at this, 5 = I am extremely good at this). ICQ involves five subscales; initiating relationships, providing emotional support, asserting influence, self-disclosure, and conflict resolution (eight items per subscale). In the standardization study, internal consistency for the five domains of the ICQ was good, ranging from 0.77 to 0.87 (Buhrmester et al., 1988). For our sample, reliabilities for the five subscales were good to excellent ranging between 0.89 and 0.94.

Procedure

The study was completed in one phase of questionnaire completion over a period of 3 months. Participants were invited to complete the aforementioned self-report questionnaires, as part of a larger study. All questionnaires were administered to the participants in the same order through an online platform (LimeSurvey) and the duration for the full package was approximately 2 h. Informed consent was obtained by all participants.

Plan of Analyses

Data processing and analysis was performed using SPSS v25 and RStudio (statistical programming language R). First, we performed Pearson's *r* bivariate correlations among study variables to examine associations among the study constructs. Then, we addressed the respective associations between autistic and alexithymic traits and socioemotional difficulties, using multiple linear regression models. Dependent variables were in two separate models, respectively, social skills and emotion regulation difficulties, i.e., the negative socio-emotional outcomes often associated with autism spectrum. Independent variables were age, gender, alexithymia, and autism trait scores.

Finally, to determine whether the relationship between autistic traits and social-emotional difficulties were mediated by alexithymic traits (TAS-20 DIF, DDF, and EOT), two structural equation modeling (SEM) models were conducted with R studio. In each of the two models, the three TAS-20 subscales were entered as mediators between AQ and ICQ (first model) or DERS (second model) (**Figure 1**). Given the cross-sectional nature of the study, our ordering of the variables in the model was based on both theoretical and developmental considerations. As per the "alexithymia hypothesis" alexithymia is believed to explain (i.e., is the mechanism) the link between autism and negative socio-emotional outcomes, therefore indicating alexithymia as the mediator. Furthermore, autistic traits are considered largely hereditary (Colvert et al., 2015), and its effects on development can be seen early in life. Although less is known about the developmental origin of alexithymia, several models suggest that it may be a result of early traumatic experiences, or an acquired coping approach involving pervasive avoidance of emotions (Badura, 2003; Krystal, 2015; Panayiotou et al., 2020), therefore it is assumed to appear somewhat later in life than autism characteristics.

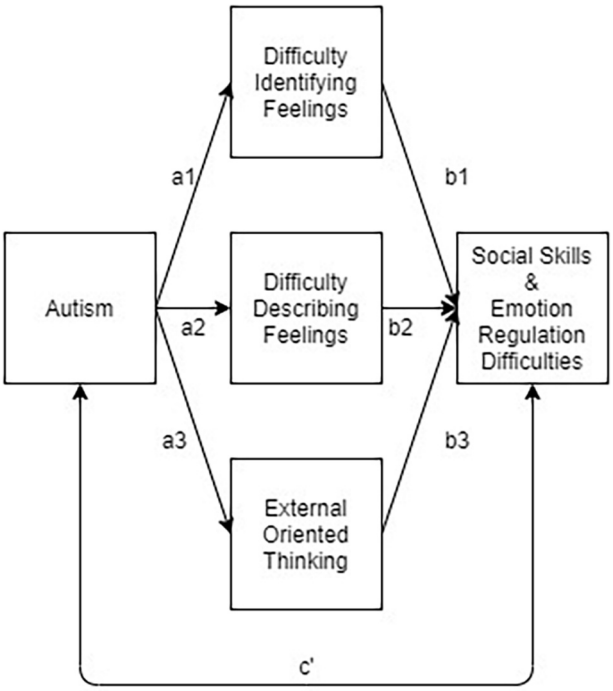

FIGURE 1 | Proposed mediation model of the relationship between ASD traits (AQ), alexithymic traits (TAS-20) and social skills (ICQ), and emotion regulation abilities (DERS). Separate analyses were conducted for the ICQ and DERS, respectively.

RESULTS

Associations on Social-Emotional Challenges, Alexithymic, and Autistic Traits

Pearson's *r* correlation examined associations among all variables in the study and showed a significant positive association between autistic traits and alexithymic traits ($r = 0.21$, $p < 0.01$), which however is not high enough to suggest multicollinearity; this finding addressed question 1, but showing that the two constructs are related but independent. There were also significant associations between autistic traits and emotion regulation difficulties (DERS) ($r = 0.18$, $p < 0.05$) while, as expected, autistic traits were negatively correlated with social skills (ICQ) ($r = -0.20$, $p < 0.01$).

Specifically, there were significant negative associations between autistic traits and all facets of social skills, including the ability to initiate and sustain social interactions and manage conflicts (as measured by subscales of the ICQ). Also, autistic traits positively and significantly correlated with two of the three facets of alexithymia (i.e., Difficulty Identifying Feelings and Difficulty Describing Feelings), showing once more the

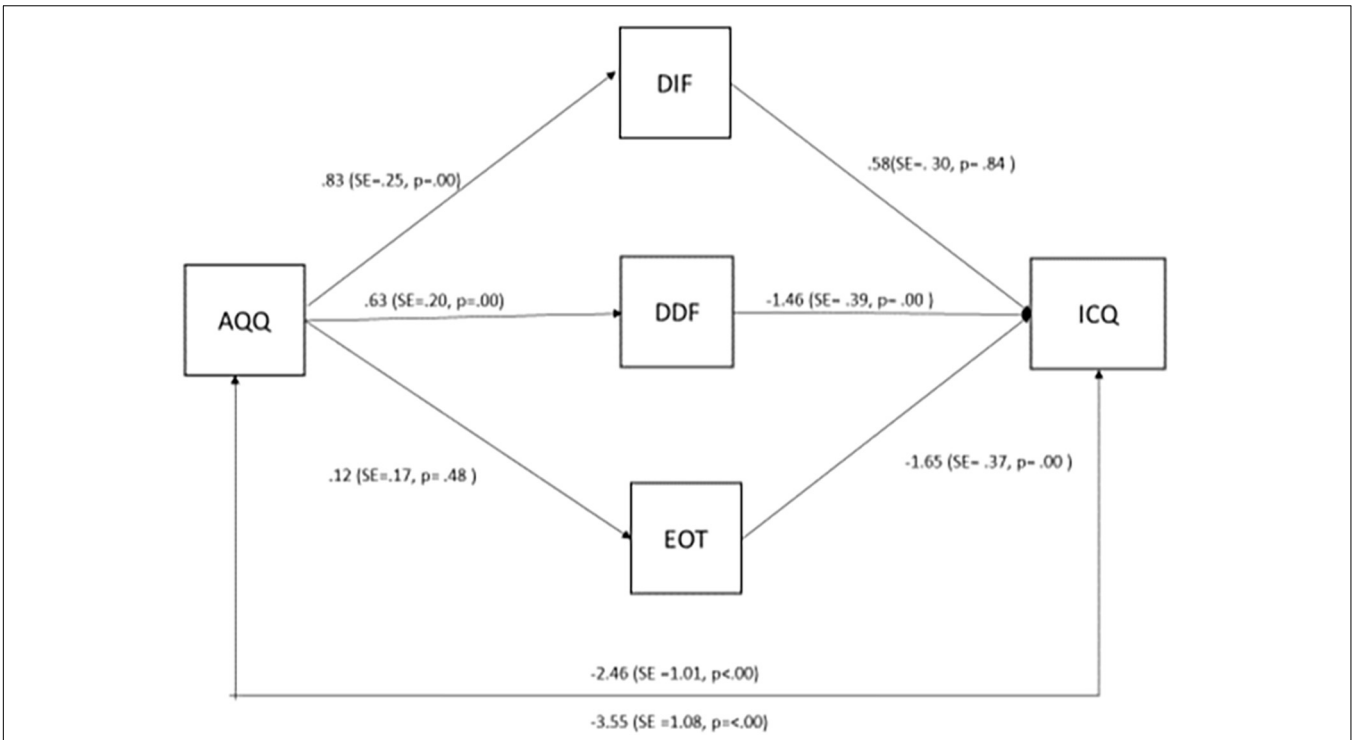

FIGURE 2 | Structural equation model 1.

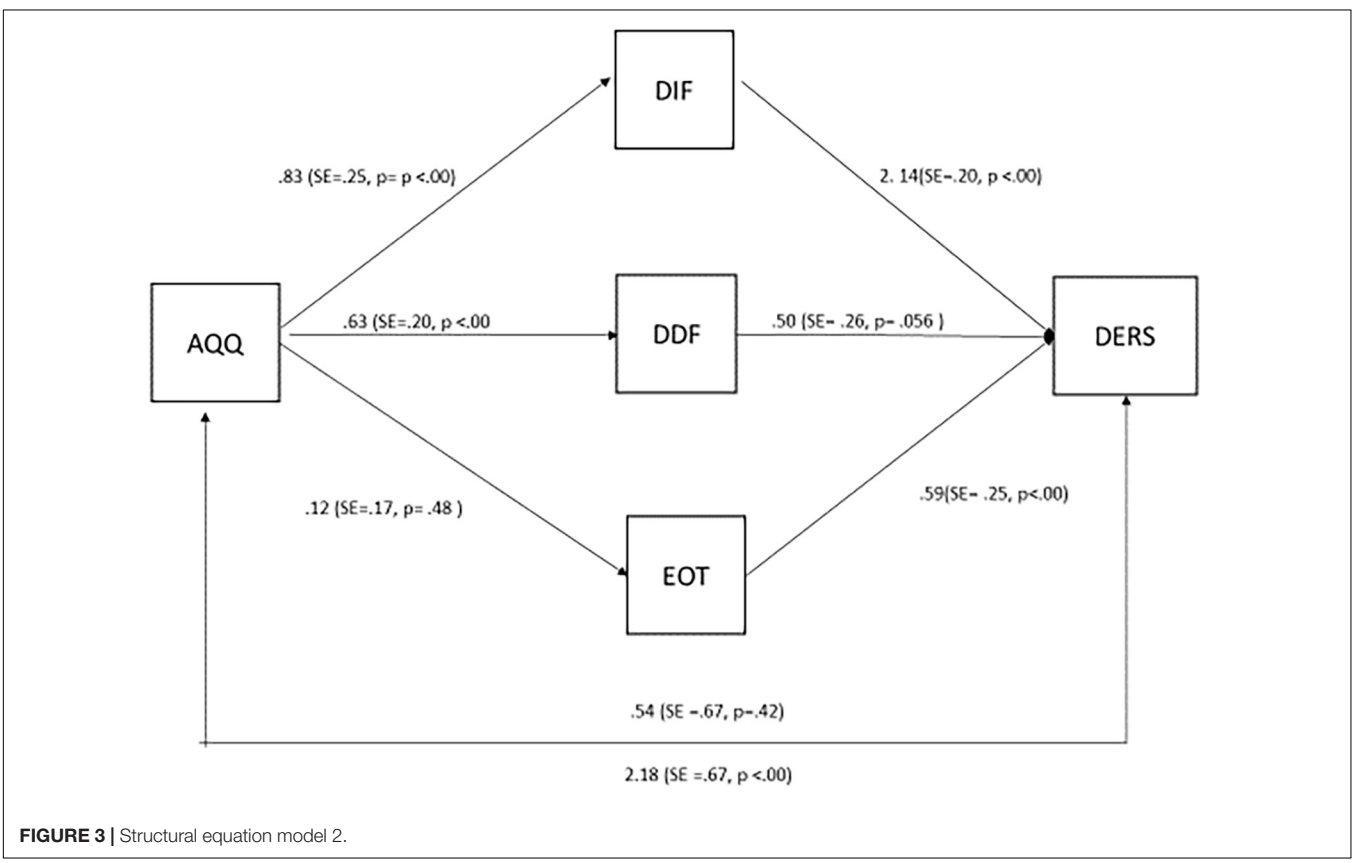

FIGURE 3 | Structural equation model 2.

relatedness between the two traits; in response to question 2, and in accord with the few previous studies that addressed this issue, results concur that among neurotypical young adults, the strongest association is between autistic traits and the two "emotional" facets of alexithymia. Furthermore, autistic traits were significantly related to four aspects of emotion regulation difficulties (but not with the difficulty in awareness and goals; see **Table 1**).

Prediction of Social Skills/Emotional Difficulties by Alexithymic and Autistic Traits

To examine the role of alexithymia and its facets in the socioemotional difficulties associated with autism (question 3), we first conducted regression analyses to affirm that both autistic and alexithymic traits predict these forms of difficulties. Multiple-linear regressions were calculated to predict interpersonal social skills and emotion regulation challenges respectively, based on the self-reported levels of alexithymia and autism of the participants. Gender and age were entered as covariables but they were not statistically significant in any of the models and they were completely removed from the models. Also, exploratory analyses (i.e., repetition of the regression analyses described here) were run separately for each gender, and similar associations were found to hold for both genders. Therefore, gender is not further discussed here, and our unequal gender samples do not appear to have affected outcomes of main analyses.

In model 1, alexithymic subscales were entered as the predictors and interpersonal social skills were the outcome. A significant relationship was found among the participants' levels of alexithymia and their interpersonal skills [$F(3,251) = 19.17$, $p < 0.01$], $r^2 = 0.17$ with two of three alexithymic subscales significantly contributing to the model (DDF: $B = -1.56$, $p < 0.01$; EOT: $B = -1.65$, $p < 0.01$) while Difficulty in Identifying Feelings did not ($B = -0.008$, $p = 0.98$).

In model 2, we ran the same model but entered all alexithymic subscales as predictors of the emotion regulation challenges (as measured by DERS). Similarly, a significant relationship was found among alexithymia subscales and emotion regulation difficulties [$F(3,270) = 92.08$, $p < 0.01$], $r^2 = 0.51$. All three subscales of alexithymia were statistically significant predictors (DIF: $B = 2.23$, $p < 0.01$; DDF: $B = 0.55$, $p < 0.05$, and EOT: $B = 0.59$, $p < 0.05$).

Next, we ran two additional models in which autistic traits (total scores) were entered as the predictors with interpersonal social skills and emotion regulation challenges respectively as the outcomes. A significant relationship was found among the participants' levels of autism and their interpersonal skills [Model 3: $F(1,251) = 10.81$, $p < 0.01$], $r^2 = 0.04$ as well as the participants emotion regulation challenges [Model 4: $F(1,251) = 8.81$, $p < 0.01$], $r^2 = 0.03$. **Table 2** presents all information on the regression analysis.

Structural Equation Modeling

Following the verification of the presence of significant associations between both the predictor (autistic traits) and the proposed mediator (alexithymia) with the outcome variables by the regression analyses above, SEM was used to determine whether the relationship between autistic traits (as measured by the AQ) and social skills (ICQ) and emotion regulations challenges (DERS), respectively are significantly mediated by Alexithymia factors (TAS-20, DIE, DDE, and EOT), and which ones. We ran two different SEM models, using a bootstrap resampling value of 10,000, and 95% bias-corrected confidence intervals (CIs).

In the first mediator model (**Figure 2**), autistic traits were the predictors and social skills were the outcome, mediated by the three alexithymia subscales. The analysis verified that autistic traits were a significant independent predictor on social skills ($B = -2.46$, SE = 1.01, $p < 0.005$). As predicted, there was also a significant indirect effect of autistic traits on social skills mediated by alexithymic traits ($B = -3.55$, SE = 1.08; $p < 0.005$) with Difficulty Describing Feelings being a significant [CI (−1.92, −0.21)] mediator, while Difficulty Identifying Feelings [CI (−0.49, 0.63)] and Externally Oriented Thinking [CI (−0.76, 0.33)] not being significant.

In the second mediator model (**Figure 3**) autistic traits were entered as the predictors of emotion regulation difficulties, mediated by alexithymic traits. Autistic traits were not a significant direct predictor on Emotion Regulation Difficulties ($B = 0.54$, SE = 0.67, $p = 0.42$). There was a significant indirect effect of Autistic traits on Emotion Regulation Difficulties mediated by Alexithymic traits ($B = 2.18$, SE = 0.67; $p \leq 0.05$) with DIF being a significant mediator [CI (0.72, 2.99)], while DDF [CI (−0.43, 0.83)] and EOT [CI (−0.12, 0.32)] were not. The latter model indicates that the association between autistic traits and emotion regulation difficulties is not direct but fully mediated by the presence of specific alexithymic characteristics.

DISCUSSION

Alexithymia and Social-Emotional Difficulties in Autism

This study extends existing work that examines the role of alexithymia in the relationships between autism and socio-emotional difficulties, specifically, emotion regulation and social skills. In this study, we conceptualized autistic traits on a continuum of severity, found in neurotypical young adults, and therefore aimed to show that similar association among alexithymia and autistic difficulties can be found not only among diagnosed individuals with autism, but at all levels of severity of the autism spectrum. The results provide specific evidence of the influence of different alexithymic facets on the relationship between autistic traits and social-emotional challenges in the young adults in our sample. To our best knowledge this is one of the few studies to date to assess cross-sectional associations between social-emotional difficulties and all alexithymic facets, beyond the total score in alexithymia scales (see also Oakley et al., 2020). While other studies have provided similar results (i.e., Liss et al., 2008; Kinnaird et al., 2019), suggesting that alexithymia is an important trait with implications for individuals on the autism spectrum, we identify the importance of specific facets of alexithymia in relation to specific social and emotional difficulties.

TABLE 2 | Regression coefficient for autistic and alexithymic traits, as predictors of social and emotional skills.

		B	SE	Beta	*p* <
Model 1	TAS DIF	−0.008	0.31	−0.002	0.98
	TAS DDF	−1.56	0.39	−0.29	0.00
	TAS EOT	−1.65	0.38	−0.25	0.00
	r^2 = 0.177				
Model 2	TAS DIF	2.23	0.20	0.61	0.00
	TAS DDF	0.55	0.25	0.12	0.03
	TAS EOT	53	0.24	0.09	0.03
	r^2 = 0.503				
Model 3	Autism	−3.55	1.08	−0.20	0.00
	r^2 = 0.038				
Model 4	Autism	2.73	0.92	0.18	0.00
	r^2 = 0.030				

First, we found that, as expected based on prior research, autistic traits and alexithymia aspects are indeed significantly associated, but not multicollinear (associations <0.8, typically considered as the criterion for multicollinearity), suggesting related but independent constructs. Next, we found that autistic traits and the participants' self-reported social and emotional difficulties were not only associated with the total alexithymia scores but most strongly with two of the three facets of alexithymia (that is difficulties in identifying/describing feelings). Research that looked specifically into facets of alexithymia in autistic individuals have provided similar results, indicating that the "emotional" aspects of alexithymia, difficulties with describing and identifying feelings, are also the facets most strongly associated with social and emotional challenges (i.e., Kloosterman et al., 2009; Milosavljevic et al., 2016; Schaller and Rauh, 2017).

These findings provide evidence on the intersection between alexithymic traits and common characteristics of autism, especially in the social and emotional domain. They indicate that alexithymia is closely related to the emotional/social challenges involved in autism while lack of significant evidence on the externally oriented thinking facet weakens the associations between alexithymia and cognitive challenges of individuals with autistic characteristics. For example, autistic individuals experience challenges in recognizing specific emotions, such as fear and disgust (Humphreys et al., 2007; Wallace et al., 2008), anger (Ashwin et al., 2006), or sadness (Corden et al., 2008), in interpreting facial expressions, and in describing the emotional content of verbal, auditory, and visual stimuli (Allen et al., 2013; Cook et al., 2013). Notably those are pre-requisites for successful emotional and social understanding. As such, one possible explanation for the observed associations between alexithymia and social-emotional difficulties is that hindered communication, challenges in social relationships, and disruption of everyday social interactions (Trevisan et al., 2016) which have been associated with autistic traits, may actually be interpreted by co-occurring emotional facets of alexithymia.

Of interest to this study, different facets of alexithymia were related with different aspects of social and emotional challenges in our findings. Specifically, two alexithymic subscales (difficulty describing feelings and externally oriented thinking) were found to predict participants' interpersonal social skills and all alexithymic subscales were found to predict participants' emotional regulation challenges. Social interactions are complex phenomena, requiring both emotional and cognitive skills (e.g., ability to empathize, and ability to pay attention to conversation). It appears that both the emotional and cognitive facets of alexithymia, i.e., the difficulty in verbally communicating emotion, and the difficulty in being attentive to ones' internal experiences (in order to know them and communicate them), disrupt the ability to socially interact. In the case of skills to regulate one's own emotions, all aspects of alexithymia had negative effects, as emotion regulation implicates a wide range of strategies (as measured here by the DERS) that may rely on different sorts of abilities (e.g., difficulty describing feelings may hinder emotional sharing, communication and support seeking, difficulty in identifying feelings may hinder emotional awareness and acceptance, and externally oriented thinking, may be associated with avoidance of internal experiences, leading to their poor regulation).

However, only the difficulty in describing feelings facet significantly mediated the relationship between autistic traits and social skills and only the difficulty in identifying feelings facet significantly mediated the relationship between autistic traits and emotion regulation difficulties in our sample. These effects stress the role of the ability to communicate emotions, in the interpersonal difficulties of individuals with autistic traits, and the ability to "know" one's emotions, for effective emotion regulation (Barrett, 2004). In both cases we observed significant mediation by alexithymia facets, in support of the alexithymia hypothesis, which argues that it is alexithymia that may be responsible for the socio-emotional challenges in autism. Importantly, in model one, both direct and indirect associations were found between autistic traits and social skills– suggesting that the alexithymia hypothesis is supported partially: that is, autistic traits themselves

contribute directly to these difficulties over and above the indirect association *via* alexithymia characteristics. In the case of emotion regulation difficulties, however, the direct path between autistic traits and these difficulties was not significant, showing that these difficulties may be fully described by alexithymia.

The co-occurrence of autism with characteristics typical of alexithymia, such as difficulty identifying emotions (Silani et al., 2008; Kloosterman et al., 2009), difficulties with emotion regulation (Weiss et al., 2014; Costa et al., 2017), and decreased levels of empathy (Lartseva et al., 2015) is well-documented in the literature. Collectively, these findings align with the conceptualization of alexithymia as pertaining to emotional difficulties and a growing body of research that explores the alexithymia hypothesis (Bird and Cook, 2013), according to which the emotion processing deficits in autism stem from co-occurring alexithymia rather than autism.

Findings like our own, also suggest possible directions for potential interventions, for individuals with various degrees of autistic traits. As suggested by our mediation models, training in the ability to recognize and describe emotions, perhaps through psycho-educational activities, and in staying attuned to one's internal experiences, in order to know, process, and ultimately accept them (e.g., through meditation), may be of paramount importance in addressing the interpersonal and emotional challenges encountered in individuals with autistic traits. It remains to be seen, if our models can replicate at higher levels of clinical autistic characteristics, which would suggest similar interventions for autism spectrum.

CONCLUSION

Over the last decades, special attention has been given to the complex emotional abilities of autistic individuals, providing evidence for deficits in the basic processing of emotions, abnormalities in emotional reactivity and emotional regulation (i.e., Samson et al., 2012). However, the complexity and intensity of deficits do not appear to be universal within the autistic population. In line with the alexithymia hypothesis (Bird and Cook, 2013), the strongest explanation for this inconsistency is alexithymia. It may be necessary for future studies on this and related areas to control for alexithymia and particularly its three facets in their design and analysis. That will allow to explore the extent to which the manipulation of specific aspects of emotional processing could improve emotional responding with a specific focus on clinical applications. This includes recognizing alexithymic traits in the clinical and therapeutic care of individuals with autistic traits, as alexithymia may act as a modifier, negatively affecting social and emotional difficulties in autism (Costa et al., 2017; Oakley et al., 2020).

Limitations and Future Steps

The current study had several limitations. Participants were not explicitly asked to report whether they had diagnosis of ASD. While high scores on AQ may indicate more autistic traits, autistic individuals may report low on AQ scores, or have received social skills interventions and therefore report exhibiting fewer traits. Further, this study included a non-clinical sample and it is not clear whether the relations reported in this largely non-autistic sample would hold for a clinical sample. In addition, this was a relatively homogeneous sample (consisting of a larger proportion of women), and it is unclear whether the relationships observed would be found in a more diverse group. Also, this study was cross-sectional; therefore, no inferences about the causal relationships between the variables investigated can be made.

Regarding measurement, emotion identification is considered a counter-intuitive approach for measuring constructs associated with problems on emotion identification (Griffin et al., 2016). This may introduce further bias than what is inherent in self-report measures, as participants may have challenges to assess their own emotional abilities, either due to alexithymic and autistic traits or other variables (e.g., self-esteem and over or under-reporting of their perceived qualities). Further, work for adolescents and children with autism has indicated that parent and child reports of alexithymia do not correlate (Griffin et al., 2016; Hobson et al., 2020; Hobson and Bedem, 2021). This highlights further the issue of self-reporting these variables, especially in clinical samples and pinpoint to the need for multiple data sources and the further development of behavioral measures to assess the alexithymia construct in younger ages.

Overall, the results are preliminary and need to be verified in longitudinal research. Investigating how alexithymic traits interact with dimensions of affective processing, physiology, and emotion regulation through a multi-method approach provides important directions in research and insights on social-emotional challenges of autistic individuals with a specific focus on clinical applications. Future research may focus on the associations between the two traits using both a longitudinal and rigorous experimental approach with the inclusion of clinical autism. This would help understand both autism and alexithymia and why they overlap, clarify how much of deficits alexithymia may explain in autism, and explore social and emotional challenges in autistic individuals with the potential to provide novel directions in clinical interventions and 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 Cyprus National Bioethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

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

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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.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 28 October 2021
doi: 10.3389/fnins.2021.732611

B and T Immunoregulation: A New Insight of B Regulatory Lymphocytes in Autism Spectrum Disorder

*Andrea De Giacomo†, Concetta Domenica Gargano, Marta Simone†, Maria Giuseppina Petruzzelli, Chiara Pedaci, Donatella Giambersio, Lucia Margari and Maddalena Ruggieri**

Department of Basic Medical Sciences, Neuroscience, and Sense Organs, University of Bari "Aldo Moro", Bari, Italy

OPEN ACCESS

Edited by:
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***Correspondence:**
Maddalena Ruggieri
maddalena.ruggieri@uniba.it

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: *29 June 2021*
Accepted: *27 September 2021*
Published: *28 October 2021*

Citation:
De Giacomo A, Gargano CD, Simone M, Petruzzelli MG, Pedaci C, Giambersio D, Margari L and Ruggieri M (2021) B and T Immunoregulation: A New Insight of B Regulatory Lymphocytes in Autism Spectrum Disorder. Front. Neurosci. 15:732611. doi: 10.3389/fnins.2021.732611

Introduction: Autism Spectrum Disorder (ASD) is a heterogeneous neurodevelopmental disorder characterized by a complex pathogenesis, by impairment social communication and interaction, and may also manifest repetitive patterns of behavior. Many studies have recognized an alteration of the immune response as a major etiological component in ASDs. Despite this, it is still unclear the variation of the function of the immune response.

Aim: Our aim is to investigate the levels of immunological markers in peripheral blood of children with ASD such as: regulatory B and T cells, memory B and natural killer (NK) cells.

Materials and Methods: We assessed various subsets of immune cells in peripheral blood (regulatory B and T cells, B-cell memory and natural killer cells) by multi-parametric flow cytometric analysis in 26 ASD children compared to 16 healthy controls (HCs) who matched age and gender.

Results: No significant difference was observed between B-cell memory and NK cells in ASDs and HCs. Instead, regulatory B cells and T cells were decreased ($p < 0.05$) in ASD subjects when compared to HCs.

Discussion: Regulatory B and T cells have a strategic role in maintaining the immune homeostasis. Their functions have been associated with the development of multiple pathologies especially in autoimmune diseases. According to our study, the immunological imbalance of regulatory B and T cells may play a pivotal role in the evolution of the disease, as immune deficiencies could be related to the severity of the ongoing disorder.

Keywords: ASD, autism, immune system, immune tolerance, regulatory T lymphocytes, regulatory B lymphocytes

INTRODUCTION

Autism spectrum disorder (ASD) is a complex heterogeneous neurodevelopmental disorder characterized by impairments in social relationship and communication, manifesting repetitive and stereotyped behavior, and deficits in verbal and non-verbal interaction (APA, 2013).

ASD has an extensive clinical heterogeneity and it is correlated with numerous comorbidities including: social anxiety disorder, attention deficit disorder, immune system abnormalities, gastrointestinal disorders, mitochondrial dysfunction, sleep disturbances, and epilepsy (Mannion and Leader, 2013, 2016).

ASD is prominent for its complex disorder and uncertain pathogenesis. However, genetic, epigenetic, and environmental factors contribute to its development (Abrahams and Geschwind, 2008; Rossignol et al., 2014). Although the specific etiologies of ASD remain unknown, there are many hypotheses about the potential involvement of the immune system in the etiopathogenesis of the disease. Many studies have recognized an alteration of the immune response in individuals diagnosed with ASD. Evidence of alterations in the functioning of the central and peripheral immune systems reveal that there is a subset of individuals with ASD who have some form of immune dysregulation (Goines and Van de Water, 2010; Gottfried et al., 2015).

Besides, a high prevalence of other immune-related comorbidities including: autoimmune diseases, allergies, and psoriasis have been found in children with ASD compared to healthy controls (Zerbo et al., 2015). Alterations of the immune system include: improper stimulation of immune cells, generation of autoantibodies, cytokine/chemokine imbalance, and increased permeability of the blood-brain barrier (Gładysz et al., 2018). Individuals diagnosed with ASD often have an immunomodulation of the lymphocytes (T lymphocytes, B lymphocytes, monocytes, Natural Killer (NK) cells and dendritic cells).

Immune system aberrations, including altered cytokine profiles, are thought to associate with ASDs (Ashwood et al., 2006; Enstrom et al., 2009b; Goines and Van de Water, 2010; Jyonouchi, 2013; Gottfried et al., 2015; Masi et al., 2015; Mead and Ashwood, 2015; Gładysz et al., 2018; Hughes et al., 2018). Increased frequency of monocytes, myeloid dendritic cells (associated with bigger amygdala size and more aberrant behaviors), NK cells have been reported in children with ASD (Enstrom et al., 2009a; Ashwood et al., 2011; Breece et al., 2013).

Regulatory T and B lymphocytes play an important role in self-tolerance as they limit autoimmune responses by suppressing proinflammatory modes of action (Dasguptaa et al., 2020). It has been demonstrated that Tregs were decreased in some autoimmune disorders such as Multiple Sclerosis (MS), rheumatoid arthritis (RA) (Toubi et al., 2005) and clinically active systemic lupus erythematosus (LES) (Miyara et al., 2005). Alterations of Bregs instead have been found in the same diseases, in tumors and infectious diseases (Rincon-Arévalo et al., 2015). Variation in frequencies of T cells and in both mature and activated B cells have also been reported in ASDs. In particular, an altered function or a decrease in regulatory T lymphocytes (Tregs) was highlighted (Mostafa et al., 2010; Ashwood et al., 2011; Hughes et al., 2018). Tregs are essential for maintaining homeostasis of the immune system, limiting the extent of effector responses by allowing the creation of immunological tolerance.

Moreover, two main types of Tregs have been identified: natural Tregs and inducible ones. Natural Tregs are developed in the thymus compared to Inducible regulatory T (iTregs) which are "$CD4^+$ lymphocytes" that express the transcription factor forkhead box P3 (Foxp3) and are developed outside the thymus under different conditions (Bilate and Lafaille, 2012). Recently, regulatory B lymphocytes (Bregs) have contributed to the maintenance of peripheral tolerance by limiting excessive inflammatory responses that occur during either autoimmune diseases or uncontrolled infections. Interleukin 10 (IL-10) is essential for Bregs function as it inhibits proinflammatory cytokines and supports the differentiation of Tregs (Mauri and Bosma, 2012).

It has been spotted that there is an increase in both T-helper 17 (Th17) lymphocytes (Th17) and cytokine Interleukin 17 (IL-17) in ASDs. A downregulation of Tregs and a decrease in Transforming Growth Factor beta (TGF-β) and Interleukin 10 (IL-10) production have also been detected. Therefore, a high "Th17" or "Treg ratio" has been attributed to severe ASD (Basheer et al., 2018; Moaaz et al., 2019). Reduced levels of the regulatory cytokine TGF-b1 are associated with reduced adaptive behavior and worsening behavioral symptoms (Ashwood et al., 2008; El Gohary et al., 2015).

To further underpin immunodeficiency disorder, Heuer et al. (2008) demonstrated that a reduction of IgM and IgG levels correlate with high behavioral severity in ASD patients. Therefore this review is designed to explore the alterations in various peripheral immune cell subpopulations and their roles in ASDs compare to healthy controls. The alteration of the immune regulation and inflammatory response are considered possible risk factors in the etiopathogenesis of ASD.

The main objective of our study is to focus on ASDs with Bregs and T regs alterations, and lack of immune tolerance. We hypothesized that a reduction of regulatory B and T cells could be related to the severity of the disease. Therefore, a better approach of the immune dysregulation and inflammation in ASDs might be essential in the diagnosis of this neurodevelopmental disorder.

MATERIALS AND METHODS

Patients

Twenty-six patients (PTs) (mean age 8.3 ± 3.6 years) affected by ASD and 16 Healthy Controls (HCs) (mean age 9.9 ± 5.7 years) were enrolled for a follow—up at the Department of Child Neuropsychiatry, University of Bari, Italy (**Table 1**). The study-protocol was approved by the Local Ethics Committee and all

TABLE 1 | Demographic and clinical data.

Variable	ASD	HCs	*p*-value
Age; year (mean ± SD)	8.3 ± 3.6	9.9 ± 5.7	NS
Disorder duration; month (mean ± SD)	56.5 ± 35.9		
Gender, n (%)			
Male	21 (80.8)	12 (75.0)	NS
Female	5 (19.2)	4 (25.0)	NS

SD, Standard deviation; n, sample size; %, percentage; NS, no statistically difference.

patients agreed to the written informed consents. Hereafter, they were then diagnosed through clinical interviews with parents following DSM-5 criteria (APA, 2013) and through. Autism Diagnostic Observation Schedule (Lord et al., 2012). The ADOS provides an algorithm with cut-offs for autism and ASDs and four different modules were used, based on the language ability of the child.

Exclusion criteria in PTs are: the presence of genetic syndrome in particular Fragile-X syndrome, Rett's syndrome and Tuberous Sclerosis, history of allergies, autoimmune disease, disabling neurological disease, pharmacological therapy, febrile seizure, and PTs receiving a special diet and with recent febrile illness.

Exclusion criteria in HCs are: the presence of neurodevelopmental disease, psychiatric or neurological disorders, pharmacological therapy, autoimmune diseases or allergic disorders.

Demographic and clinical data were collected from both ASD PTs and HCs: age, gender and disorder duration (period from the date of first diagnosis) (**Table 1**) and PTs were assessed for neurological disability which includes ASD level, IQ and language disability (**Table 2**). We opted for the *Wechsler Intelligence Scale for Children, fourth edition (WISC-IV)* to assess total/verbal IQ in scholar children (Wechsler, 2012); *Leiter International Performance Scale Revised-Visualization and Reasoning Battery (Leiter-R)* to assess not-verbal IQ in pre-scholar children (Roid and Miller, 2002); *Test di Valutazione del Linguaggio (TVL)* to evaluate language disability (Cianchetti, 2010).

Hence, patients with greater impairments were examined according to four parameters: severe ASD level (severe: level 3; mild to moderate: level 1 and 2 according to DSM-5), intelligence quotient (IQ), language disability and the presence of all these parameters to identify patients with full impairments. The outcome result is as follows: 6 patients with severe ASD level, 14 patients with IQ < 70, 17 patients with language disability and 5 patients with full impairments.

Flow Cytometric Analysis of Peripheral Lymphocytes Subsets

Blood samples were collected in fast blood specimens in ethylene diamine tetra acetic acid (EDTA) (8.55 mg/tube) for immunophenotyping by flow cytometry. Hundred microliter of peripheral blood samples were incubated with specific monoclonal antibodies (Beckman Coulter, United Kingdom) at room temperature (RT) for 15 min in the dark. After incubation, red cells were lysed with VersaLyse lysing solution (REF A09777, Beckman Coulter, United Kingdom) for 15 min at RT in the dark and then analyzed by flow cytometry. Analysis for samples incubated with Anti-FoxP3 was performed using PerFix-nc (no centrifuge assay Kit) (REF B31167, Beckman Coulter, United Kingdom), for intra- and extra-cellular staining preparation.

Lymphocytes subpopulations were identified by the recognition of surface molecules belonging to the family of Cluster of Differentiation (CD) and by intracellular FoxP3 transcription factor. Analyzed events were subjected to gating analysis (total lymphocytes and $CD45^+$ vs. side scatter) (CD45-FITC, REF A07782, Beckman Coulter, United Kingdom) and exclusion of doublets and dead cells. Lymphocytes subpopulations were identified by using the monoclonal antibodies against $CD3^+/CD19^+/CD27^+$ for B memory cells (CD3-PC5, REF A07749; CD19-PC7, REF IM3628; CD27-PE REF IM2578, Beckman Coulter, United Kingdom); $CD19^+/CD38^+/CD24^+$ for B regulatory cells (CD19-PC7, REF IM3628; CD38-PE REF AO7779, CD24-PC5.5 REF B23133, Beckman Coulter, United Kingdom); $CD4^+/CD25^+/CD127^+/\text{Anti-FoxP3}^+$ for T regulatory cells (CD4-FITC, REF A07750; CD25-PE REF A07774, CD127-PC7 REF A64618, Anti-FoxP3-PC7 REF B46032, Beckman Coulter, United Kingdom); $CD3^+/CD16^+/CD56^+$ for Natural Killer cells (CD3-PC5, REF A07749; CD16-PC7 REF 6607118; CD56-PE REF A07788, Beckman Coulter, United Kingdom). Samples were acquired on a Beckman Coulter CytoFLEX flow cytometer and analyzed using CytExpert software (Beckman Coulter, United Kingdom). Data are shown as the mean of percentage of events ± SD.

TABLE 2 | ASD level, IQ, and language disability.

Patients with severe ASD level, n (%)	6 (26%)
Patients with IQ < 70, n (%)	14 (54%)
Patients with language disability, n (%)	17 (65%)
Patients with full impairment, n (%)	5 (19%)

n, sample size; %, percentage of patients with the mentioned characteristic among the whole group of patients.

Statistical Analysis

The lymphocytes' subsets percentage in ASD patients and HCs one are reported as mean ± standard deviation (SD). Lymphocytes subsets values were not normally distributed, and therefore a Spearman non-parametric test was performed to compare these parameters with patients' clinical and demographic data. Student's *t*-test was used for statistical analysis on the percentage means of LS and Pearson Correlation coefficient was used for statistical analysis on participants' gender. *P*-values < 0.05 were considered statistically significant. Thus, further analysis was conducted using GraphPad Prism 8.0.

RESULTS

Lymphocytes Subsets

Lymphocyte subsets (LS) cytometric analysis revealed some differences between patients (PTs) and HCs. Interestingly, statistical differences were shown for regulatory B cells subset (**Figures 1B,E**, Mean ± SD: HCs, 39.56 ± 17.6; PTs, 29.29 ± 14.5, $P = 0.04$) and for regulatory T cells subset (**Figures 1C,F**, Mean ± SD: HCs, 9.88 ± 23.1; PTs, 1.33 ± 1.9, $P = 0.045$) which were involved in immune tolerance mechanisms. Moreover, no statistical difference were found in B memory lymphocytes (**Figure 1A**, Mean% ± SD: HCs, 1.95 ± 2.3; PTs, 2.29 ± 2.9, $P = 0.35$) and NK cells (**Figure 1D**, Mean% ± SD: HCs, 8.76 ± 4.4; PTs, 6.87 ± 4.4, $P = 0.09$).

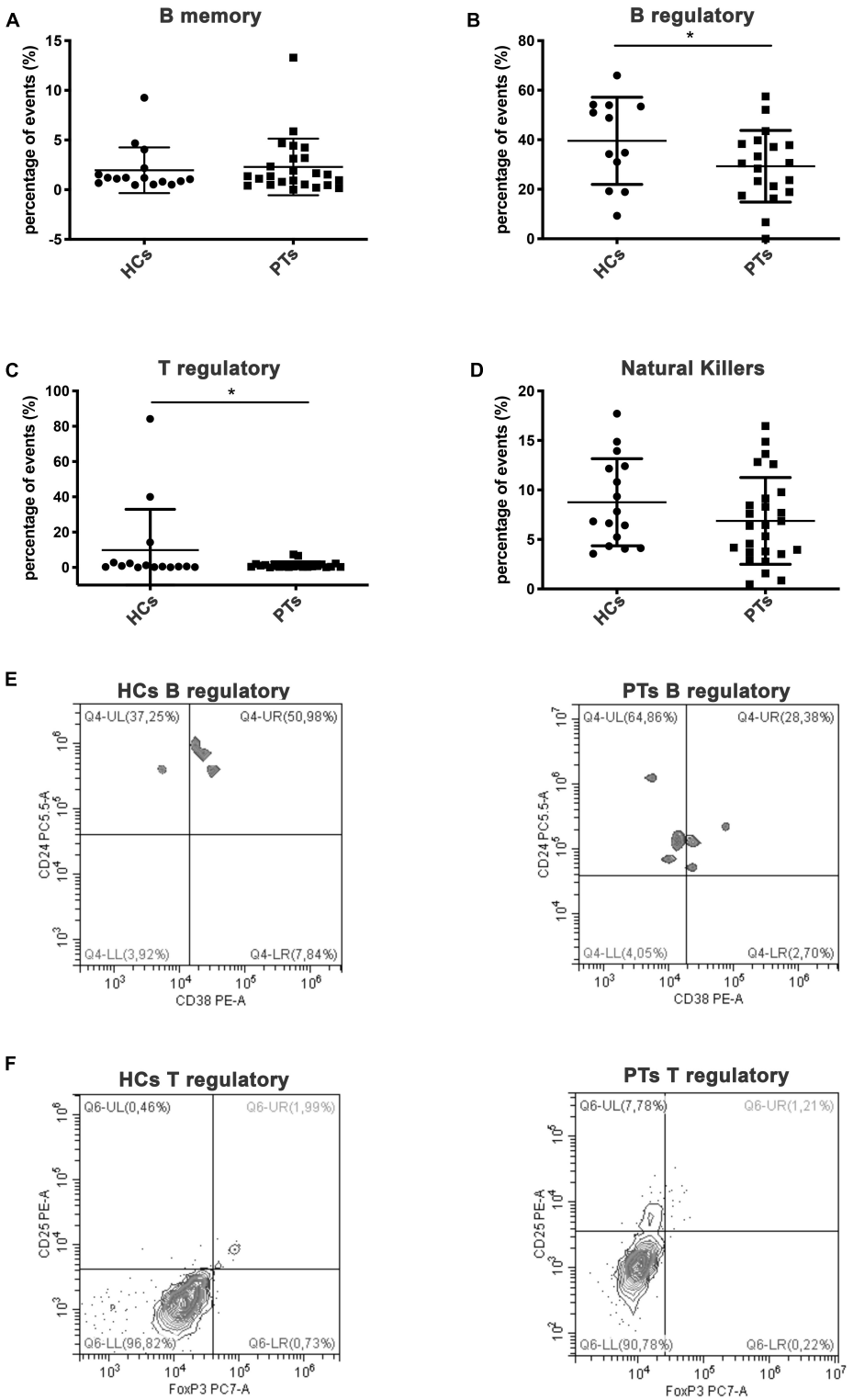

FIGURE 1 | Lymphocytes subpopulations. Scatter plot graphs representing the percentage of events of lymphocyte subpopulations **(A)** memory B cells ($CD45^+/CD3^+/CD19^+/CD27^+$), **(B)** regulatory B cells ($CD45^+/CD19^+/CD38^+/CD24^+$), **(C)** regulatory T cells ($CD4^+/CD25^+/FoxP3^+$), **(D)** natural killer ($CD45^+/CD3^+/CD16^+/CD56^+$); *$p$-value < 0.05 (Student's t-test). Representative dot plot graphs of flow cytometry analysis of lymphocytes subpopulations. **(E)** B regulatory lymphocytes of HCs and PTs are shown in Q4-UR (underlined) representing $CD38^+/CD24$ events previously gated on $CD45^+/CD19^+$. **(F)** T regulatory lymphocytes of HCs and PTs are shown in Q6-UR (underlined) representing $CD25^+$/anti-FoxP3 events previously gated on $CD4^+$.

TABLE 3 | B and T regulatory subsets means: comparison between total PTs and sub- groups of severity.

Variable	Total PTs	Severe ASD	10 < 70	L. disability	Full impairment	*p*-value
B reg (mean%)	29.29	20.11	30.40	30.16	20.11	NS
T reg (mean%)	1.33	0.86	1.31	1.23	0.98	NS

PTs, patients; L, disability: language disability; full impairment: PTs with severe ASD level, 10 < 70 and language disability; B reg, B regulatory subpopulation; T regulatory subpopulation; mean%, mean of lymphocyte subpopulation in percentage; NS, no statistically difference.

For a better survey of regulatory B and T lymphocytes subsets involvement in ASD, we compared the mean percentage of LS of patients with the most severe conditions to the mean percentage of LS of all patients. **Table 3** identifies the mean percentage of regulatory B and T subsets in all PTs and in different subgroups of severity. The percentage of variation between these two lymphocytes population is shown in **Table 4**. Four out of six patients with severe ASD level showed a reduction of 35.3% in the T reg subset compared to the T reg subset of all patients (T reg mean% of level 3 vs. level 1, 2: 0.86 vs. 1.33). A reduction of 31.3% in the B reg subset was observed in patients with severe ASD level, compared to the B reg subset of all patients (B reg mean% of level 3 vs. level 1, 2: 20.11 vs. 29.29). Lymphocytes subsets are quite similar when compared to patients with IQ < 70 and with language disability to all the other patients. However, patients with full impairment showed a reduction of 26.3% in the T reg subset in comparison with children with mild to moderate autism (T reg mean% of level 3 vs. level 1, 2: 0.98 vs. 1.33) and a reduction of 31,3% in the B reg subset compared to all patients (B reg mean% of level 3 vs. level 1, 2: 20.11 vs. 29.29). No statistically differences were found comparing ASD patient groups on the basis of disease severity (P-value > 0.05; Student's t-test).

DISCUSSION

The alteration of regulatory T and B lymphocytes has been reported to be involved in ASD. It is difficult to make direct comparisons between results of our study and previous studies due to differences in analytical technique, age range of subjects, diagnostic criteria and the use of siblings as controls. In our case, children with autism had significantly lower frequency of both Tregs and B regs ($p < 0.05$) compared to HCs. On the contrary, there was no significant difference in the proportion of B memory and NK cells. Four out of six patients diagnosed with severe ASD (level 3, according to DSM-5) showed lower value of Tregs (reduction of 35.3%) compared to children with mild or moderate autism (level 1 and 2, according to DSM-5) (**Tables 3**, **4**). However, the above results did not reach statistical significance.

TABLE 4 | Percentage of variation vs. all patients.

	T reg	B reg	*p*-value
LS of PTs with severe ASD level, (%)	−35.3	−31.3	NS
LS of PTs with IQ < 70, (%)	−1.5	+3.8	NS
LS of PTs with language disability, (%)	−7.5	+2.9	NS
LS of PTs with full impairment, (%)	−26.3	−31.3	NS

LS, Lymphocytes Subpopulation; PTs, patients; full impairment: PTs with severe ASD level, IQ < 70 and language disability; T reg, T regulatory subset; B reg, B regulatory subset; NS, no statistically difference.

According to our results, Mostafa et al. (2010) demonstrated a significantly lower frequency of Tregs in Egyptian autistic children compared to HCs. Children with severe autism had a major decrease in the frequency of Tregs in peripheral blood than children with mild and moderate autism (Mostafa et al., 2010). Another study found a dysregulation of Tregs related to transcription factors, in particular a downregulation of Foxp3 within $CD4^+$ T cells in peripheral blood of autistic children. These results suggest that a reduction in Foxp3 expression is associated to a decrease of Tregs in children with autism (Ahmad et al., 2017). Moreover, Ahmad et al. also highlighted a systemic Treg deficiency, focusing on dysregulation of Th1, Th2, Th17, and Treg-related transcription factors. A deficiency of the $Foxp3^+$ Tregs protein was also found in upregulation of Th1/Th2/Th17 related transcription factors. These findings suggest that transcription factor signaling is altered in ASDs, which results in immunological imbalance, and therefore, the restoration of the transcription factor signaling could have a greater therapeutic potential in the treatment of autistic disorders (Ahmad et al., 2017).

To validate our findings, there is also a study (Moaaz et al., 2019), demonstrating that Th17/Treg imbalance in ASDs was significantly skewed toward a Th17 response compared to their control. This means that there is a significant reduction in Treg percentage in ASDs and this also confirmed the downregulation of the related transcription factor (Foxp3) and cytokines (TGF-β and IL-10) in their peripheral blood. Moaaz et al. (2019) have also discovered a negative correlation between Tregs and disease severity, showing that children with severe autism have lower values of Tregs than children with mild or moderate autism.

Regulatory T cells function in children with autism showed a decrease of TGF-β1 levels (Ashwood et al., 2008) and a IL-10 production (Ashwood and Wakefield, 2006). Changes in social behaviors, for example, are associated with reduced TGFβ1 levels (Ashwood et al., 2008). Tregs reduction have been reported in subjects with allergic disorders such as asthma (Mamessier et al., 2008). The presence of comorbid allergic disorders could be a confounding factor in immunity studies in both individuals with ASD and the control group. However, neither subjects with ASD nor the HCs had such a comorbid allergic diagnosis. Disagreeing with our results, Basheer et al. (2018) found no significant differences between ASDs and the control groups in proportion of Tregs. Future studies could investigate the clinical effects of a therapy based on restoring the number of Tregs in the subgroup of ASD patients with Treg deficiency (June and Blazar, 2006). We found that Bregs decreased ($p < 0.05$) in subjects with ASD

if compared to HCs as well. No other studies have highlighted these findings. A Breg reduction seems to be linked to worsen severity in autism. In fact, in our findings, four out of six patients with severe autism showed a drastic reduction of about 31.3% if compared to the B reg subset of all patients Bregs are also reduced (−31.3%) in PTs with full impairment compared to all PTs (**Tables 3**, **4**).

Heuer et al. (2008) studied B cells functions and observed a decrease in the total levels of both IgG and IgM in peripheral blood of autistic children compared to typically developing controls (Heuer et al., 2008). They subsequently investigate whether the reduced plasma levels of IgG and IgM were the result of defective development, activation or function of B cells. Heuer et al. (2012) showed no differences in the number of B memory cells. This indicates that the decrease of Immunoglobulins (Igs) in autism is not the result of B cell dysfunction but it depends on the involvement of many immune cells (Heuer et al., 2012).

In agreement with our findings, Basheer et al. (2018) found no significant differences in the frequency of NK cells in subjects with ASD. They also found no significant alterations in frequencies of various subsets of B cells in ASDs compared to previous study, in which a higher number of mature, activated B cells was reported (Ashwood et al., 2011; Basheer et al., 2018). Contrarily to our results, previous studies have shown a significantly higher number of B and NK cells in children with autism than in controls (Enstrom et al., 2009a; Ashwood et al., 2011).

Some studies have demonstrated that autistic children undergoing Intravenous Immunoglobulins (IVIG) treatment have improvements in total aberrant behavior, irritability, hyperactivity, and social withdrawal and in some cases, the complete resolution of ASD symptoms. The therapeutic mechanisms of Intravenous Immunoglobulins (IVIG) are complex; they may provide therapeutic benefits for both autoimmune and inflammatory diseases through multiple different processes (Galeotti et al., 2017; Rossignol and Frye, 2021). Other agents instead could decrease antibodies, including MMF (immunosuppressive therapy), methotrexate, rituximab, and bortezomib. These therapies in ASDs are, however, controversial and under debate because of the limitations of the data from *in vitro* or animal-based studies, the high cost of the treatment and the undefined immunopathology of autism (Wong and White, 2016).

Some limitations are present in this study due to the small sample size, the reduced number of HCs compared to ASD subjects and the difference in frequency between males and females. The wide age range of participants too could be considered as another limitation because of the different impact on the immune cells value. Further studies are needed to scrutinize for one or more reliable biomarkers that will help elucidate the mechanism underlying the causes of autism and this could help to identify different subgroups within the autistic population designing effective treatments and redefining ASD on molecular, immunological, and biochemical background. Therefore, it is important to carry on with this research on Bregs which play an important role in immune tolerance and these data could be useful for future biomarkers and therapies in ASD subjects.

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 Comitato Etico Policlinico. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AD and MS: wrote the first draft of the manuscript and design a work. MR and CDG: substantial contributions to the conception or design of the work, the acquisition, and analysis and interpretation of data for the work. All authors contributed to the manuscript revision, read and approved the submitted version.

FUNDING

AD was funded by "Ex Fondo 60%"—Ministero dell'Università Di Ricerca.

ACKNOWLEDGMENTS

We thank to Dr. Mariangela Mastropasqua from the Department of Basic Medical Sciences, Neuroscience, and Sense Organs, University of Bari "Aldo Moro" for her technical support and we thank to Dr. Maria Gloria Campa for data collection.

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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.

The reviewer PI declared a shared affiliation, though no other collaboration, with the authors to the handling editor.

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frontiers in Neuroscience

ORIGINAL RESEARCH
published: 23 November 2021
doi: 10.3389/fnins.2021.734177

Autistic Traits and Empathy in Children With Attention Deficit Hyperactivity Disorder, Autism Spectrum Disorder and Co-occurring Attention Deficit Hyperactivity Disorder/Autism Spectrum Disorder

Stefania Aiello[1†], David Vagni[1†], Antonio Cerasa[1,2,3], Elisa Leonardi[1], Cristina Carrozza[1], Francesca Famà[1], Agrippina Campisi[1], Flavia Marino[1], Rosamaria Siracusano[4], Maria Ausilia Alquino[5], Francesco Mainiero[6], Eva Germano[5], Gennaro Tartarisco[1], Giovanni Pioggia[1‡], Antonella Gagliano[7‡] and Liliana Ruta[1‡]*

[1] Institute for Biomedical Research and Innovation (IRIB), National Research Council of Italy (CNR), Messina, Italy, [2] S. Anna Institute, Crotone, Italy, [3] Pharmacotechnology Documentation and Transfer Unit, Preclinical and Translational Pharmacology, Department of Pharmacy, Health Science and Nutrition, University of Calabria, Arcavacata, Italy, [4] Division of Child Neurology and Psychiatry, Federico II University Hospital Naples, Naples, Italy, [5] Division of Child Neurology and Psychiatry, Department of the Adult and Developmental Age Human Pathology, University of Messina, Messina, Italy, [6] Department of Psychology, Faculty of Medicine and Psychology, Sapienza University of Rome, Rome, Italy, [7] Child and Adolescent Neuropsychiatry Unit, Department of Biomedical Sciences, University of Cagliari, Cagliari, Italy

Attention Deficit Hyperactivity Disorder (ADHD) and Autism Spectrum Disorders (ASD) are two of the most represented neurodevelopmental conditions in childhood. The diagnostic shift introduced by the DSM-5, allowing a combined diagnosis of ADHD and ASD, poses different clinical challenges related to diagnostic overshadowing, accuracy of clinical judgment and potential delay in an ASD diagnosis in children presenting with ADHD. Here we tried to disentangle the clinical phenotype and specificity of the two co-occurring conditions in relation to autism traits and empathy, by comparing children with ASD with and without comorbid ADHD with children presenting ADHD only and children with typical development. The child versions of the Autism Quotient (C-AQ) and Empathy Quotient (C-EQ) were administered to a total sample of 198 male children between 6 and 14 years old with age appropriate language skills and normal intelligence. Univariate analysis demonstrated no significant differences in the C-AQ total and subscale scores as well as the C-EQ between children with ASD and children with ASD + ADHD, while children with ADHD alone presented an intermediate phenotype between ASD and TD. Furthermore, a receiver operating characteristic (ROC) analysis was applied to discriminate among the different phenotypes. We found that the C-AQ and C-EQ were accurate at distinguishing with satisfactory reliability between: (a) ASD vs. non- ASD (N-ASD) groups comprising both ADHD and TD children (Area Under the Curve AUC 88% for C-AQ and 81% for C-EQ); (b) ASD and TD (AUC 92% for C-AQ

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Edited by:
Francesco Craig, University of Calabria, Italy

Reviewed by:
Chenjie Shen, Massachusetts Institute of Technology, United States
Francesca Felicia Operto, University of Salerno, Italy

***Correspondence:**
Liliana Ruta
liliana.ruta@cnr.it

[†] These authors have contributed equally to this work and share first authorship

[‡] These authors have contributed equally to this work and share last authorship

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: *30 June 2021*
Accepted: *04 November 2021*
Published: *23 November 2021*

Citation:
Aiello S, Vagni D, Cerasa A, Leonardi E, Carrozza C, Famà F, Campisi A, Marino F, Siracusano R, Alquino MA, Mainiero F, Germano E, Tartarisco G, Pioggia G, Gagliano A and Ruta L (2021) Autistic Traits and Empathy in Children With Attention Deficit Hyperactivity Disorder, Autism Spectrum Disorder and Co-occurring Attention Deficit Hyperactivity Disorder/Autism Spectrum Disorder. Front. Neurosci. 15:734177. doi: 10.3389/fnins.2021.734177

and 95% for C-EQ); (c) ASD and ADHD (AUC 80% for C-AQ and 68% for C-EQ). Our data confirm the reliability of the C-AQ and C-EQ as behavioral markers to differentiate ASD (regardless of comorbid ADHD) from an ADHD condition and TD. Interestingly, in our sample an ADHD condition does not increase the severity of the clinical phenotype in terms of autism traits distribution and empathy, suggesting that the psychological measures detected by the two quantitative instruments are independent of ADHD traits. This evidence will contribute to the translational efforts in developing better tailored treatments and preventive strategies.

Keywords: autistic traits, empathy, ADHD, ASD, gender

INTRODUCTION

The Diagnostic and Statistical Manual of Mental Disorders fifth edition (DSM-5) (American Psychiatric Association [APA], 2013) introduced a new conceptualization of Neurodevelopmental Disorders, providing a fundamental shift from a categorical to a dimensional system of diagnosis (Hudziak et al., 2007; Jones, 2012). In the DSM-5, remarkable modifications affected one of the currently most represented neurodevelopmental conditions in childhood: Autism Spectrum Disorder (ASD).

The major changes in the ASD diagnostic category were: (i) the rearrangement of the three core domains down to two core domains (social communication and restricted and repetitive behaviors); (ii) the removal of sub-diagnoses (Autistic Disorder, Asperger Syndrome, Pervasive Developmental Disorder Not Otherwise Specified, Disintegrative Disorder) in favor of a dimensional approach of symptoms' severity; and (iii) the possibility of a comorbid diagnosis of ADHD.

Within the autism phenotype, restricted repetitive behaviors have been linked to cognitive rigidity (South et al., 2007; Lemonda et al., 2012; Faja and Nelson Darling, 2019), hyperfocus and difficulties in predictive coding (van de Cruys et al., 2014). Different studies have replicated an impairment in executive functions (EF) such as verbal working memory, inhibition and quick visual scanning in the ASD population (Rommelse et al., 2015; Operto et al., 2021). Furthermore, EF is known to self-regulate emotional and empathic abilities, also referred to as "Hot Executive functions" (Yang et al., 2011), which result impaired in children with autism (Harmsen, 2019), with significantly higher deficits in emotional regulation (ER) than other neurodevelopmental and psychiatric disorders (Samson et al., 2012; Joshi et al., 2018). Additionally, difficulties in controlling emotions often result in internalizing problems such as anxiety and depression (Gadow et al., 2012; Operto et al., 2021).

Impairments in EF and self-regulation represent core neuropsychological features of ADHD. Interestingly, poor quality of social perspective's assumptions, impaired social cognition, lack of pragmatic language and empathy have also been observed in children with ADHD (Marton et al., 2009; Bora and Pantelis, 2016; Lasmono et al., 2021). With respect to empathy subdomains, it has been reported that ADHD is mainly characterized by a deficit in affective empathy (sharing and responding to another individual's emotions) rather than to its cognitive domain (understanding another person's perspective) (Abdel-Hamid et al., 2019; Cristofani et al., 2020; Fantozzi et al., 2021) as it is usually observed in ASD (Rueda et al., 2015). The emotional and behavioral profile in ADHD children results in both internalizing and externalizing problems such as mood and anxiety disorders, aggressive and/or oppositional behaviors, with consequent difficult peer relationships (Nijmeijer et al., 2008; Harkins et al., 2021). Furthermore, many children with ADHD also show hyperfocus on topics that meet their personal interests (Groen et al., 2020) and demonstrate difficulties in processing speed and working memory (Molavi et al., 2020; Operto et al., 2021).

Previous evidence confirmed the symptoms overlap between ADHD and ASD, with 18–50% of children with ADHD presenting with clinical levels of ASD symptoms (Ronald et al., 2008; Kochhar et al., 2011; Van Der Meer et al., 2012; Grzadzinski et al., 2016) and conversely, 40–70% of children with ASD displaying co-occurring ADHD (Salazar et al., 2015; Joshi et al., 2017; Antshel and Russo, 2019).

Diagnostic accuracy may be even more challenging when ASD and ADHD co-occur. In fact, the neuropsychological and behavioral overlapping between the two conditions poses important questions related to diagnostic overshadowing and need to be in depth investigated to explain possible shared etiologies (Antshel and Russo, 2019). Furthermore, studying the co-occurrence of the two conditions at early stages is crucial to be aware of how they evolve and, consequently, how their pathways vary along with child development. This longitudinal perspective is fundamental to enhancing targeted treatment. Previous findings suggest that, in the socio-communicative domain, ADHD lies intermediately between ASD and control groups (Grzadzinski et al., 2011; van de Cruys et al., 2014; Salley et al., 2015; Groen et al., 2018). ADHD and ASD could then belong to the same continuum and represent two different manifestations of a common spectrum disorder. A recent study showed that compared to only ASD, an ADHD comorbidity was associated with reduced cognitive task performance (Mansour et al., 2021). Other studies demonstrated that an ASD + ADHD condition increased deficits in social interaction and was associated with lower adaptive functioning, higher anxiety, lower empathy (Colombi and Ghaziuddin, 2017; Shephard et al., 2019; Dellapiazza et al., 2021) and a poorer quality of life (Gadow et al., 2009; Sikora et al., 2012; Joshi et al., 2017). Furthermore, with regard to their emotional and behavioral profile, a recent study has demonstrated that children with comorbid ASD and ADHD

result with higher externalizing problems than ASD alone, and lower externalizing symptoms than children with ADHD alone (Carta et al., 2020). Similarly, children with a diagnosis of ADHD who displayed higher autistic traits showed lower cognitive and social skills and the presence of autistic traits determined a more severe outcome (Kotte et al., 2013; Green et al., 2016; Joshi et al., 2020; Sesso et al., 2020).

Based on existing literature we aimed to disentangle the clinical phenotype and specificity of the two co-occurring conditions in relation to autistic traits and empathy, by comparing children with mild to moderate ASD, with normal intelligence and functional language, with and without comorbid ADHD, with children presenting with ADHD only and typically developing (TD) children. Specifically, we intend to explore if comorbid ADHD is associated with an increase in the expression of autistic traits and a decrease of empathy as detected by two dimensional measures such as the child versions of the Autism-Spectrum Quotient (C-AQ) (Auyeung et al., 2008; Ruta et al., 2012) and the Empathy Quotient (C-EQ) (Auyeung et al., 2009). In particular, we are interested in investigating whether the C-AQ and C-EQ are able to distinguish between ASD with or without ADHD and ADHD alone, as well as against typical development.

MATERIALS AND METHODS

Subjects

All the children presenting a clinical diagnosis of ADHD and/or ASD were assessed at the clinical facilities of the National Research Council of Italy (IRIB—CNR) and the Polyclinic University Hospital "AOU G. Martino" both located in Messina, Italy, while TD children were recruited in three big mainstream schools in the metropolitan area of Messina. Clinical diagnosis was examined by medical records and was then confirmed by an experienced child neuropsychiatrist (A.G., E.G., and R.S.) and a chartered clinical psychologist of the team (S.A., E.L.) according to the DSM-5 criteria, with the support of the Autism Diagnostic Observation Schedule-2nd edition (ADOS-2, Module 3) and the parent version of the Conners scale-3rd edition, respectively. All the ASD children included in the study, received a diagnosis of ASD without disorder of intellectual development and with no impairment of functional language (6A02.0) according to ICD-11. All the children had an IQ between 70 and 130 (mean = 98.7, *SD* = 14), assessed using the Wechsler Intelligence Scale for Children-4th edition (WISC-IV).

Exclusion criteria included children with (1) intellectual disability; (2) syndromic (secondary) autism; (3) no fluent language; (4) level 3 (severe) at the DSM-5 severity classification, because for those children many questions on the C-AQ and C-EQ were not applicable or a positive answer would capture difficulties collateral to ASD and not nuclear to it (e.g., "when my child talks on the phone, he doesn't know when it's his turn to talk").

The study was reviewed and approved by the Ethics Committees of CNR (ethical clearance, 01.08.2018) and parents of the children included in the study provided their written informed consent.

Behavioral Measures

The child version of the Autism Spectrum Quotient (C-AQ) and Empathy Quotient (C-EQ) were used to assess Autistic Traits (ATs) and empathy in all the children. The C-AQ and C-EQ were originally developed to study dimensional autistic traits and empathy as a continuum. The C-AQ (Auyeung et al., 2008; Ruta et al., 2012) is a 50-items parent-report questionnaire to assess five areas associated with autism: social skills, communication, imagination, attention switching, and attention to details. It is an adapted version of the AQ for adults, a reliable tool able to identify autistic traits in the general population also at a subthreshold level, discriminating high functioning autism from non-autistic individuals. Items specifically map into the clinical features of autism (ex. She/he prefers to do things the same way repeatedly). Answers are measured with a Likert scale, from 1 to 4 (Definitely Disagree, Slightly Disagree, Slightly Agree, Definitely Agree) with higher scores indicating more autistic traits and behaviors.

The C-EQ (Auyeung et al., 2009) is a 27-items parent-report questionnaire to measure the degree of empathy expressed in real life situations, experiences, and interests. It is an adapted version of the EQ for adults, a trustworthy instrument to evaluate individual behaviors into situations where empathizing skills are required (ex. My child often doesn't understand why some things upset other people so much). Items are presented with a Likert format from 1 to 4 (Definitely Disagree, Slightly Disagree, Slightly Agree, Definitely Agree) with higher scores indicating higher empathic skills. The EQ demonstrated a significant sex difference in the general population (with boys usually resulting as less empathizing than girls) and a negative correlation with autistic traits.

Statistical Analyses

All statistical analyses were conducted using the SPSS Statistics Release 26.0 (IBM SPSS, New York, NY). A two-way ANOVA was performed separately for the C-AQ and the C-EQ. We performed a univariate analysis of variance (ANOVA) to verify between-group differences in the total scores of the C-AQ and C-EQ; the four diagnostic groups were used as dependent variables, the C-AQ and C-EQ were used as independent variables. Levene's Test was used in ANOVA and Box's Test in MANOVA to test the equality of error variances. Given that the groups size was uneven, in case of unequal variances a robust Bias-corrected accelerated (BCa) Bootstrap with 1,000 samples, stratified by group, will be used (Krishnamoorthy et al., 2007). Age and IQ were not matched across all the groups, hence all the analyses were controlled for these measures.

Furthermore, we executed a MANOVA, with the same factors and covariates, to analyze between-group differences in AQ subscales. Multiple comparisons were performed by applying Sidak correction. Pillai's Trace was used to test the null hypothesis.

A receiver operating characteristic (ROC) curve of the C-AQ and C-EQ total scores was calculated to plot sensitivity and 1-specificity in the whole ASD group (ASD+ and ASD−) vs. N-ASD group (ADHD and TD together). The area under the

curve (AUC) is a measure of the overall predictive validity, where an AUC = 0.50 indicates a random prediction of the independent variable, AUC > 0.70 indicates fair validity and AUC > 0.90 indicates excellent validity. Potential cut-off scores on the AQ and EQ for differentiating between children with and without ASD were also evaluated using ROC analysis to determine the cut-point corresponding to the best combination of sensitivity and specificity.

RESULTS

Group Differences on the Child Versions of the Autism Quotient and Empathy Quotient Total Scores

A total sample of 198 male children aged between 6 and 14 years old (mean = 8.8, *SD* = 2.2) has been recruited and tested in the study. *N* = 77 children presented a diagnosis of ASD only (ASD−); *n* = 24 children received a diagnosis of ASD and ADHD (ASD+); *n* = 33 children had ADHD only and *n* = 64 children had typical development (TD) (see **Table 1**).

Equality of variance conditions were not met for C-AQ. Therefore, we decided to use a stratified BCa for both outcome measures to have more robust error ranges. We detected a main effect of group for both the C-AQ [$F_{(3,192)} = 60.0$, $p < 0.001$ $\eta_p^2 = 0.448$] and C-EQ [$F_{(3,\ 149)} = 32.1$, $p < 0.001$, $\eta_p^2 = 0.393$]. We found neither the age effect [$F_{(1,\ 192)} = 0.60$, $p = 0.438$] nor the IQ effect [$F_{(1,\ 192)} = 1.21$, $p = 0.272$] on C-AQ and C-EQ [$F_{(1,\ 149)} = 0.253$, $p = 0.616$ and $F_{(1,\ 149)} = 3.10$, $p = 0.081$, respectively].

On both measures, pairwise comparisons showed that the ASD+ and ASD− groups had comparable scores, with, a mean difference of 5.18 [−2.93, 14.27], $p = 0.237$, for the C-AQ and −0.52 [−5.25, 4.22], $p = 0.787$ for the C-EQ (**Figure 1**).

Furthermore, ASD− scores were significantly different from both the ADHD (−19.54 [−25.95, 13.25], $p = 0.001$ for the C-AQ; 5.16 [8.20, 2.07], $p = 0.001$ for the C-EQ) and the TD group (−34.17 [−40.01, −27.96], $p = 0.001$ for the C-AQ and 14.94 [11.94, 18.13], $p = 0.001$ for the C-EQ), respectively. Children with ADHD, in turn, displayed, on both the C-AQ and C-EQ, intermediate mean scores, but significantly different than TD children (14.63 [8.36, 20.61], $p = 0.001$ for the C-AQ and −9.78 [−14.84, −4.71], $p = 0.001$ for the C-EQ).

Group Differences in Child Versions of the Autism Quotient Subscales Scores

Equality of variance conditions were not met for the social and the attention to details subscales. As before, we performed a stratified BCa. The main effect of the group, reported on the C-AQ total score, was confirmed by the multivariate analysis [$F_{(15,570)} = 8.49$, $p < 0.001$, $\eta_p^2 = 0.183$]. We found no effect of age [$F_{(5,188)} = 0.719$, $p = 0.610$], while IQ had a significant effect [$F_{(5,188)} = 4.20$, $p = 0.001$, $\eta_p^2 = 0.100$]. Univariate analysis led to a main effect of group for communication [$F_{(3,\ 192)} = 44.5$, $p < 0.001$, $\eta_p^2 = 0.410$], social [$F_{(3,192)} = 57.5$, $p < 0.001$, $\eta_p^2 = 0.437$], attention switching [$F_{(3,192)} = 24.5$ $p < 0.001$, $\eta_p^2 = 0.277$], and imagination [$F_{(3,192)} = 29.4$ $p < 0.001$,

TABLE 1 | Demographic data and explored variable comparison for the sample.

Variable	ASD− (*N* = 77)	ASD+ (*N* = 24)	ADHD (*N* = 33)	TD (*N* = 64)	Statistics	*Post hoc*
Age (95% C.I.)	9.11 (8.64, 9.60)	8.46 (7.98, 8.89)	8.51 (7.95, 9.09)	9.83 (9.41, 10.23)	$F_{(3,197)} = 5.631, p = 0.001$	ASD− = TD; TD > ASD+ = ADHD
IQ (95% C.I.)	97.6 (94.5, 100.8)	106.6 (100.7, 111.9)	101.0 (96.4, 105.7)	96.6 (93.3, 99.8)	$F_{(3,197)} = 3.676, p = 0.013$	ASD + > TD; ASD− = ADHD = TD
AQ tot (95% C.I.)	88.2 (83.1, 93.1)	94.0 (87.7, 100.0)	68.7 (64.3, 72.6)	54.4 (50.6, 57.9)	$F_{(3,\ 192)} = 52.0, p < 0.001, \eta_p^2 = 0.448$	ASD + = ASD− > > ADHD > > TD
AQ soc (95% C.I.)	17.7 (16.6, 18.8)	18.6 (16.6, 20.6)	11.2 (9.5, 12.9)	7.79 (6.55, 9.02)	$F_{(3,\ 192)} = 57.5, p < 0.001, \eta_p^2 = 0.473$	ASD + = ASD− > > ADHD > TD
AQ att (95% C.I.)	18.3 (17.2, 19.4)	18.9 (16.9, 21.0)	15.0 (13.3, 16.7)	11.6 (10.4, 12.9)	$F_{(3,\ 192)} = 24.5, p < 0.001, \eta_p^2 = 0.277$	ASD + = ASD− > ADHD > TD
AQ det (95% C.I.)	16.4 (15.1, 17.6)	17.1 (14.9, 19.4)	15.3 (13.4, 17.2)	15.9 (14.5, 17.3)	$F_{(3,\ 192)} = 0.573, p = 0.634, \eta_p^2 = 0.009$	ASD + = ASD− = TD = ADHD
AQ com (95% C.I.)	19.1 (17.8, 20.3)	21.4 (19.1, 23.8)	14.6 (12.7, 16.6)	9.14 (7.72, 10.55)	$F_{(3,\ 192)} = 44.5, p < 0.001, \eta_p^2 = 0.410$	ASD + = ASD− > > ADHD > > TD
AQ imm (95% C.I.)	16.8 (15.6, 17.9)	17.5 (15.6, 19.5)	12.7 (11.0, 14.3)	9.81 (8.60, 11.02)	$F_{(3,\ 192)} = 29.4, p < 0.001, \eta_p^2 = 0.315$	ASD− = ASD+ > > ADHD > TD
EQ tot (95% C.I.)*	20.6 (18.7, 22.4)	20.0 (17.3, 22.5)	25.7 (23.6, 27.9)	35.5 (33.3, 40.0)	$F_{(149)} = 32.1, p < 0.001, \eta_p^2 = 0.393$	ASD− = ASD+ < ADHD < < TD

*Covariates and estimates appearing in the model are evaluated at the following values: Age = 9.17, IQ = 98.9. Bootstrap results are based on 1,000 bootstrap samples stratified by group and computed using Bias-correction. *EQ participants: ASD+ = 24, ASD− = 70, ADHD = 32, TD = 29.*

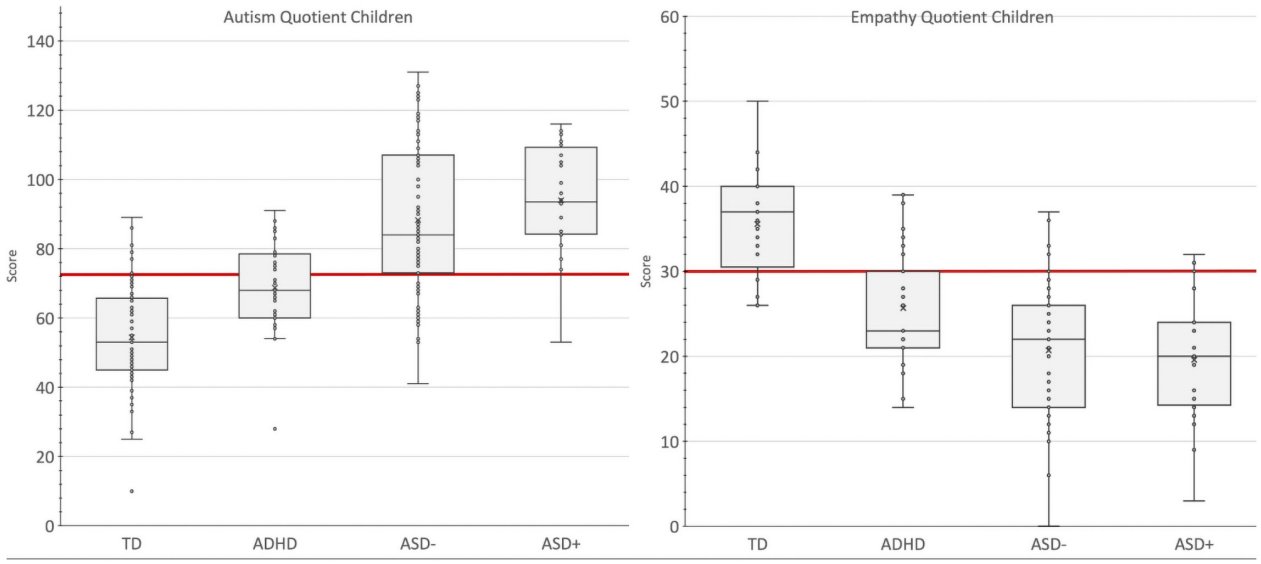

FIGURE 1 | Group comparison on the C-AQ and C-EQ total scores. Box and Whisker Plot. A box is drawn from the first quartile to the third quartile, while a line is drawn at the median and the cross is the mean value. The whiskers extend from each quartile to the minimum or maximum. The Red Line is the Cut-off Value with the Highest Accuracy in Discriminating Autism Spectrum Disorder. TD, Typical Development Group; ADHD, Attention Deficit and Hyperactivity Group; ASD–, Autism Spectrum Disorder Group; ASD+, ASD with comorbid ADHD group.

$\eta_p^2 = 0.315$] subscales. There was no group effect for the attention to details subscale [$F_{(3,192)} = 0.57$ $p = 0.634$, $\eta_p^2 = 0.009$)]. The effect of IQ was significant for the social skills and attention to details subscales [$F_{(1,192)} = 5.81$ $p = 0.017$, $\eta_p^2 = 0.029$ and $F_{(1,192)} = 4.02$ $p = 0.046$, $\eta_p^2 = 0.021$, respectively].

Pairwise comparisons showed that, on the social, communication, imagination and attention switching subscales, ASD+ and ASD− groups had comparable scores (all p-values > 0.386), both groups scored higher than the ADHD only and TD group (all p-values < 0.021), and in turn, ADHD scores were higher than the control group (all p-values < 0.045). There were no significant differences among the four groups on the attention to detail score (all p-values > 0.787). **Figure 2** displays the group differences on the C-AQ subscales scores.

Accuracy of the Child Versions of the Autism Quotient and Empathy Quotient in Predicting Group Differences

Using ROC curve analysis for a positive ASD diagnosis (ASD vs. N-ASD), independent of the ADHD status, the AUC was 0.877 [0.830, 0.923] for C-AQ, and 0.806 [0.737, 0.874] for C-EQ (**Figure 3**).

We also determined the best threshold able to discriminate between ASD and N-ASD with maximum accuracy. The cut-off was 73 (equal or higher) for the C-AQ and 26 (equal or less) for the C-EQ. Using those values, for C-AQ the sensitivity was 82% and the specificity was 79% leading to an overall accuracy of 81%, while for C-EQ the sensitivity was 75% and the specificity was 72%, with an accuracy of 73% (**Table 2**).

A comparison between ASD and TD, led to an AUC of 0.918 [0.877, 0.959] with a cut-off of 73 for AQ-C scores, corresponding to a sensitivity of 82% and a specificity of 89% with an accuracy of 86%. On the C-EQ, AUC was 0.945 [0.907, 0.983] with a cut-off of 26, a sensitivity of 75%, a specificity of 100% and an accuracy of 87%.

Finally, the direct comparison of the whole ASD group (ASD+ and ASD−) vs. the ADHD group, led, for the C-AQ, to an AUC of 0.795 [0.719, 0.872] with a cut-off of 77 corresponding to a sensitivity of 72% and a specificity of 73% with an accuracy of 73%. For the C-EQ, AUC was 0.680 [0.581, 0.779] using a cut-off of 18, sensitivity was 36% and specificity was 94% leading to an accuracy of 65%.

DISCUSSION

Recent literature is posing specific attention to the clinical implications of having a comorbid ASD and ADHD with a focus on investigating the extension to which the two co-occurring conditions contribute to a different phenotype expression compared to ASD and ADHD alone. Although clinical data support the presence of a more severe outcome and impairment in the quality of life, evidence from the current standardized clinical measures is still inconsistent in detecting, with sufficient accuracy, the neuropsychological differences between individuals with ASD, individuals with a comorbid ADHD (ASD+) and individuals with an ADHD alone. One of the reasons for this clinical challenge, is that, for example, many individuals with ADHD, as well as individuals with ASD, show significant impairments in the social interaction and communication area, as displayed by higher scores on the social affect domain at the Ados Diagnostic Observation Schedule, second edition (ADOS-2), and the main domain that allows to distinguishing between

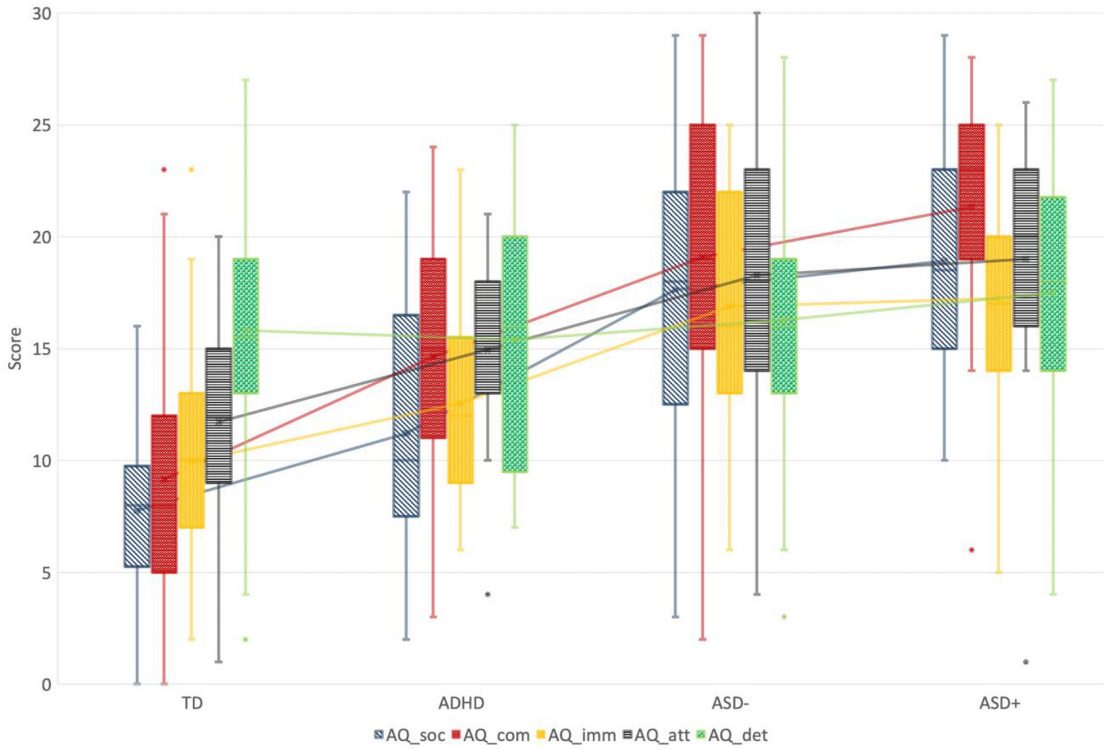

FIGURE 2 | Group differences on the C-AQ subscales scores. TD, Typical Development Group; ADHD, Attention Deficit and Hyperactivity Group; ASD–, Autism Spectrum Disorder Group; ASD+, ASD with comorbid ADHD group; AAQ_soc, Autism Quotient Children—Social Skills Subscale; AQ_com, Autism Quotient Children—Communication Subscale; AQ_soc, Autism Quotient Children—Imagination Subscale; AQ_imm, Autism Quotient Children—Attention Shifting Subscale; AQ_soc, Autism Quotient Children—Attention to Details Subscale.

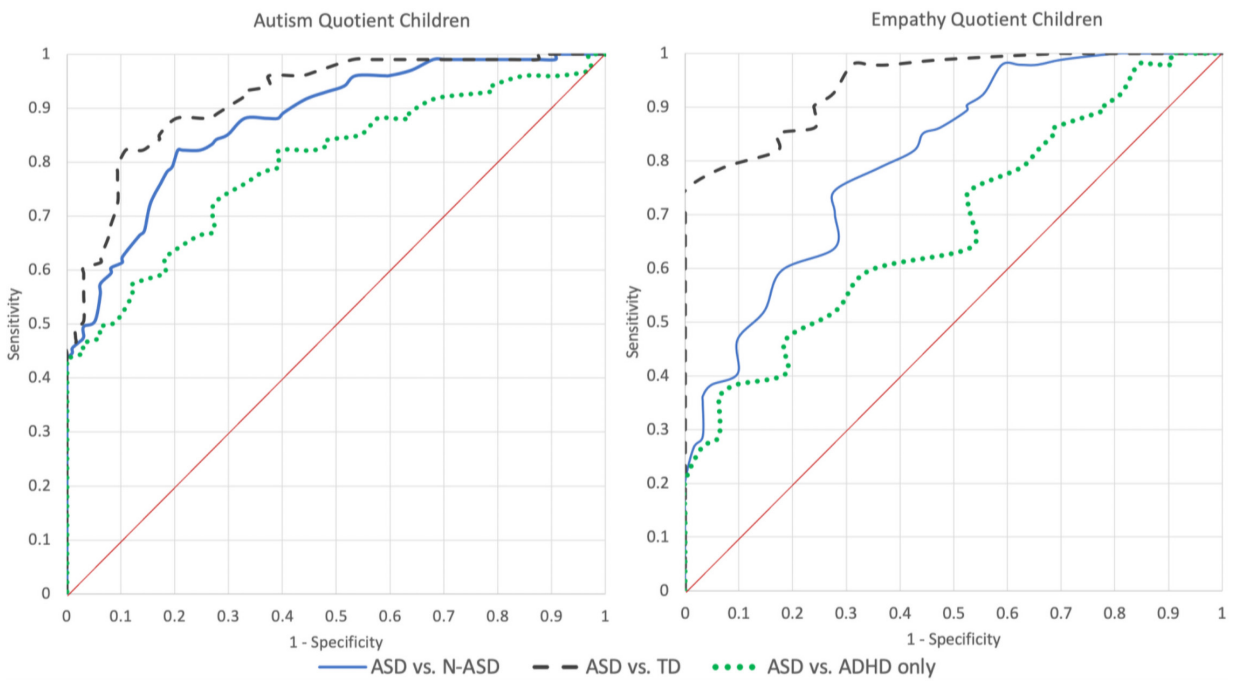

FIGURE 3 | ROC curves of the C-AQ and C-EQ for different combinations of groups. TD, Typical Development Group; ADHD, Attention Deficit and Hyperactivity Group; ASD, Autism Spectrum Disorder with or without comorbid ADHD Group; N-ASD, TD and ADHD grouped together.

TABLE 2 | ROC analysis and accuracy for different combination of groups.

Comparison	Scale	# Pos.	# Neg.	AUC	S.E.	Sig.	95% C.I.	Cut-off	Sensibility	Specificity	Accuracy
ASD vs. N-ASD	AQ	101	97	0.877	0.024	<0.001	0.830–0.923	73	0.822	0.794	0.808
	EQ	94	61	0.806	0.035	<0.001	0.737–0.874	26	0.745	0.721	0.733
ASD vs. ADHD	AQ	101	33	0.795	0.039	<0.001	0.719–0.872	77	0.723	0.727	0.725
	EQ	94	32	0.680	0.051	0.002	0.581–0.779	18	0.362	0.937	0.650
ASD vs. TD	AQ	101	64	0.918	0.021	<0.001	0.877–0.959	73	0.822	0.891	0.857
	EQ	94	29	0.945	0.019	<0.001	0.907–0.983	26	0.745	1.000	0.873

ASD and ADHD individuals at the ADOS-2 is based on the repetitive and restricted behaviors that remain a specific core domain for ASD (Grzadzinski et al., 2016). Similar findings have been reported when individuals with ASD and ASD+ have been compared in their social affect profile at the ADOS-2 (Harkins et al., 2021). However, other measures, based on parent-reports, such as the Social Responsiveness Scale, 2nd Edition (SRS-2) (Constantino and Gruber, 2012), demonstrated a significant group difference between children with ASD and children with ASD+, the latter group scoring significantly higher. Overall, these findings underline that there is still inconsistency in the psychological measures sensitivity and specificity to disentangle the behavioral phenotype of the two conditions both separately and even more, when associated.

To address this gap, in our study, we explored whether two quantitative measures of autism traits and empathy such as the C-AQ and C-EQ were able to distinguish children with ASD with or without ADHD, from children with ADHD alone, and children with typical development. For this reason, we used a ROC analysis to determine the threshold score that maximized classification accuracy among the conditions for each measure (**Figure 3**). We found that the C-AQ has good accuracy at distinguishing between ASD and N-ASD children (80%), and between ASD and TD (86%) and a satisfactory accuracy at discriminating between ASD and ADHD (73%). Our results confirm that the C-AQ is a reliable quantitative measure not only to discriminate between an ASD vs. a non-ASD condition as well as a typical development, but is also able to put apart, with a satisfactory discriminative power, an ASD from an ADHD condition. Furthermore, a cut-off of 73 was able to reliably distinguish between ASD from non-ASD conditions, while a cut-point value of 77 was the best score for discriminating ASD from ADHD. These threshold values are very similar to those reported by Auyeung et al. (2008) (cut-off of 76 for ASD vs. TD groups) confirming the cross-cultural stability of the instrument.

Conversely, the C-EQ demonstrated good accuracy at distinguishing between ASD and TD (87%) but not a sufficient accuracy in the distinction between ASD and ADHD (65%). In line with previous evidence, one possible explanation for the latter finding is that also children with ADHD may show a specific deficit in empathy (especially the affective component) as it has been reported by several studies (Abdel-Hamid et al., 2019; Harmsen, 2019; Cristofani et al., 2020; Fantozzi et al., 2021; Lasmono et al., 2021). Furthermore, this result is not unexpected, being that empathy is a trans-categorical psychological trait, which implies many aspects of social cognition, prosocial behavior, emotion regulation and morality, and is involved in different neurodevelopmental and psychiatric conditions (Henry et al., 2016; Lamm et al., 2016; Cotter et al., 2018). To account for this potential bias, from a clinical perspective, it might be considered that the cut-off of 18 (see **Supplementary Table 1**), maximizes specificity (94%) to the detriment of sensitivity (36%). Another possible explanation for a reduced accuracy of the C-EQ in discriminating between ASD and ADHD is that within the autism heterogeneity, a subgroup of children and individuals with ASD, for their specific clinical profile or different mechanisms of compensation and masking, do not actually score significantly lower at the empathy tasks, compared with neurotypical individuals (Rueda et al., 2015; Alkire et al., 2021; Rieffe et al., 2021). Indeed, to reach a sensibility for ASD of 90%, the cut-off on C-EQ should be raised to 32, leading to a specificity of 76% compared to TD and 22% compared to ADHD. As for the C-AQ, we also confirmed for the C-EQ good cross-cultural stability (Baron-Cohen and Wheelwright, 2004).

Univariate analysis (**Figures 1**, **2**) revealed significant group differences between ASD, scoring the highest, ADHD, presenting intermediate scores and TD scoring the lower. This finding supports the robustness of the two measures to detect ASD traits not only in relation to typical development but also vs. other clinical conditions sharing common traits, such as ADHD. Interestingly, in our sample, children with ASD+ did not score significantly higher than children with ASD alone. A recent study by Pehlivanidis et al. (2021) reported similar findings at the AQ, showing that adults with ASD+ and ASD− had comparable scores, in turn significantly higher than the ADHD group. It means that the effect of comorbid ADHD seemed not to be additive in the reported severity of ASD and the reasons should be examined in future studies. We hypothesized two possible reasons, as follows: it might be that within the autism spectrum the psychological measures detected by the two quantitative instruments are fairly independent of ADHD traits; alternatively it could be related to a behavioral overshadowing of ASD on ADHD (for instance, if a child has difficulties in making friends due to impulsivity, but has also due to major social-communication difficulties, the latter can overshadow the first one).

Limitations

The study has some limitations. The first limitation concerns the demographic characteristics of the sample. Furthermore, average IQ in ASD+ children was higher than in the other groups. Previous studies demonstrated that C-AQ and C-EQ

are independent of age and IQ (Baron-Cohen et al., 2001, 2006; Chapman et al., 2006; Auyeung et al., 2008), Also a *post hoc* stratified boot-strapped analysis led to similar results. Secondly, we have not been able to collect ADHD-related specific measures, therefore, for the purpose of the study, we focused on the discriminative ability of quantitative, parent-report, quick screening measures such as the C-AQ and the C-EQ to detect, relatively early, an ASD condition vs. other clinical overlapping conditions such as ADHD. Furthermore, in future larger studies, cluster analysis and single item resolution analysis would be worthy to explore the specificity and transdiagnostic domains of autism traits distribution and empathy in a hybrid dimensional approach.

CONCLUSION

In our study we found that the C-AQ and C-EQ are reliable and robust instruments to quantify ASD traits and empathy in children with normal intelligence and fluent language with and without comorbid ADHD as compared to children with an ADHD condition alone and TD children. Specifically, within the autism spectrum, the presence of a comorbid ADHD does not influence the severity and distribution of autistic traits and empathy, while children with a diagnosis of ADHD displayed an intermediate phenotype, with higher levels of autistic traits and lower empathy compared to TD children. The results of our study will also contribute to the translational efforts in developing better tailored treatments and preventive strategies.

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 Ethics Committees of CNR (ethical clearance, 01.08.2018). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SA contributed to the study design and to the manuscript draft. DV conducted the statistical analysis and statistical data interpretations and participated in the manuscript draft. ACe contributed with statistical data interpretations and to the manuscript draft. EL, CC, FF, ACa, and FlM enrolled and tested the ASD+, ASD−, and TD participants. RS, MA, and EG enrolled and tested the ADHD participants. FrM and GT contributed with statistical analysis and statistical data interpretations. GP contributed to the coordination of the study. AG participated in the study design, coordinated the enrollment of ADHD participants and contributed to the manuscript draft. LR designed and supervised the study and drafted the manuscript. All authors read and approved the final manuscript.

FUNDING

This research was partially supported by the grant from the Sicilian Region of Italy (Assessorato Regionale dell'Istruzione e della Formazione Professionale, Avviso 11/2017 Rafforzare l'occupabilità nel sistema R&S e la nascita di spin off di ricerca in Sicilia—P.O. FSE 2014/2020, project no. 2014.IT.05.SFOP.014/3/10.4/9.2.10/0011—CUP G47B17000100009, entitled SANi) and partially supported by the grant from Azienda Sanitaria Provinciale di Catania (deliberations nos. 2005 24/06/2016 and 3304 04/10/2017).

ACKNOWLEDGMENTS

We acknowledge all the families who volunteered to participate in the study. We thank the Spazio Asperger No-Profit Association for helping in the recruitment of participants.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.734177/full#supplementary-material

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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.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 25 November 2021
doi: 10.3389/fnins.2021.751465

Adaptive Behavior, Emotional/Behavioral Problems and Parental Stress in Children With Autism Spectrum Disorder

Francesca Felicia Operto†, Grazia Maria Giovanna Pastorino†, Chiara Scuoppo, Chiara Padovano, Valentina Vivenzio, Ilaria Pistola, Gilda Belfiore, Rosetta Rinaldi, Valeria de Simone and Giangennaro Coppola*

Child and Adolescent Neuropsychiatry Unit, Department of Medicine, Surgery and Dentistry, University of Salerno, Salerno, Italy

OPEN ACCESS

Edited by:
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***Correspondence:**
Francesca Felicia Operto
opertofrancesca@gmail.com

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: *01 August 2021*
Accepted: *04 November 2021*
Published: *25 November 2021*

Citation:
Operto FF, Pastorino GMG, Scuoppo C, Padovano C, Vivenzio V, Pistola I, Belfiore G, Rinaldi R, de Simone V and Coppola G (2021) Adaptive Behavior, Emotional/Behavioral Problems and Parental Stress in Children With Autism Spectrum Disorder. Front. Neurosci. 15:751465. doi: 10.3389/fnins.2021.751465

Background: The aim of our study was to compare adaptive skills, emotional/behavioral problems, and parental stress among children with different severity levels of Autism Spectrum Disorder (ASD) symptoms.

Methods: This study included a sample of 88 subjects with ASD (mean age = 6.00 ± 2.70). All subjects underwent standardized neuropsychological tests for the assessment of symptoms of the autism spectrum (Autism Diagnostic Observation Schedule-Second Edition), adaptive level (The Vineland Adaptive Behavior Scales, Survey Interview, 2nd edition), behavioral and emotional problems (Child Behavior CheckList CBCL), and parental stress (Parental Stress Index Short Form-PSI-SF). Non-parametric statistical methods (Kruskal-Wallis test and Mann-Whitney *U*-test for *post hoc* analysis) and linear regression analysis were used in this study.

Results: Children who had higher severity levels of ASD symptoms had less adaptive functioning; younger children showed more severe symptoms of ASD; older children had better communication skills. The presence of greater adaptive difficulties was related to a greater presence of internalizing problems. An increase in parental stress levels was related to an higher severity of ASD symptoms, fewer adaptive skills, and a greater presence of internalizing and externalizing problems.

Conclusion: This study suggests that the adaptive behavior should be considered in order to planning a habilitation intervention in children with autism. It is also important to monitor emotional/behavioral problems and parental stress levels in order to provide parenting support and improve the family quality of life.

Keywords: autism spectrum disorder, adaptive functioning, emotional/behavioral problems, parental stress, neurodevelopmental disorders

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impaired social interaction/communication and the presence of restricted or repetitive behaviors (American Psychiatric Association [APA], 2013). Symptoms are not best explained by intellectual disability and must manifest in the early stages of development. This disorder has very heterogeneous clinical features and, according to the Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-5), the severity of this condition ranging from mild to severe, according to three severity levels, from 1 to 3.The severity level is based on impaired social communication and restricted and repetitive patterns of behavior. In level 1, in the absence of support, social communication deficits cause significant impairment and the inflexibility of behavior causes significant interference with functioning in one or more contexts. In level 2 there are marked deficits in social communication skills and the restricted and repetitive behaviors interfere with functioning in different contexts. Level 3 is characterized by severe deficits in social communication skills and the restricted and repetitive behaviors interfere markedly with all areas of functioning (American Psychiatric Association [APA], 2013).

Research in the literature shows that in subjects with ASD there is an impairment of adaptive abilities which can increase with growth and can be present regardless of intellectual level (Kanne et al., 2011; Chatham et al., 2018; Precenzano et al., 2020; Pastorino et al., 2021).

Adaptive functioning is a construct that includes the skills necessary to live in everyday life (Matthews et al., 2015). A widely used clinical and research tool (Balboni et al., 2001; Schalock et al., 2010) to evaluate adaptive functioning is the Vineland Adaptive Behavior Scales II (VABS II; Sparrow et al., 2005). Through this structured interview, adaptive behaviors from birth up to adulthood are assessed, starting from reaching the first stages of development up to the most complex requests in the various areas. In autism studies using the Vineland II test, deficits in communication, socialization, daily living skills, and motor skills emerged (Balboni et al., 2016; Chatham et al., 2018). Difficulty in adaptive skills adversely affects the lives of individuals with ASD, impacting on daily living skills, social experiences, and school activities (Baker et al., 2008; O'Donnell et al., 2012).

In addition to adaptive difficulties, there are several studies in the literature (Havdahl et al., 2016) which show that children with ASD have a high prevalence of emotional and behavioral problems. In particular, in some researches (Ooi et al., 2010; Kempe et al., 2011) it was found that subjects with ASD had significantly higher scores than children of a mixed clinical control group in the following scales of the CBCL test: social problems, withdrawal problems/depression, attention problems, and thinking problems.

Another much investigated topic is parental stress (Operto et al., 2019a,b). The parental stress in mothers and fathers of children with ASD seem to be higher than in parents of normotypic children (Bonifacci et al., 2016; Craig et al., 2016), and this can adversely affect the general well-being of the whole family. We speak of parental stress when a parent has a psychological reaction of aversion to the request to perform their parental role and generally the parent does not perceive that there are resources available to satisfy this request (Giovagnoli et al., 2015). Many studies have shown that there are various factors that influence each other and determine parental stress, including characteristics of the parent, child, family, and ecology (Lazarus and Folkman, 1984; McCubbin, 1989). A test designed to measure various parental stressors is the Abidin Parental Stress Index (PSI) (Abidin, 1995).

Some characteristics of patients that could increase parental stress are the severity of autism spectrum symptoms, the adaptive level and emotional problems of their children, but there do not appear to be studies in the literature that have analyzed the interaction of the adaptive level with the severity of symptoms in individuals with ASD, also considering emotional/behavioral problems and parental stress level.

Therefore, the aim of our study was to evaluate adaptive skills, emotional/behavioral problems, and parental stress and compare them among children with different severity levels of ASD symptoms.

MATERIALS AND METHODS

Participants

Our sample consisted of children and adolescents diagnosed with ASD (n = 88; males = 57; mean age = 6.00 $\pm$ 2.70). The participants and their parents (mother = 88; father = 88) were recruited at the Child and Adolescents Neuropsychiatry Unit—University Hospital of Salerno (Italy). The diagnosis of ASD, according to the DSM-5 criteria, were made by a team of neuropsychiatrists and psychologists, with the support of standardized and validated neuropsychological tests (Autism Diagnostic Observation Schedule Second Edition—ADOS-2 and Autism Diagnostic Interview Revised—ADI-R). The ADOS-2 modules 1–3 were used for the assessment. Patients with different levels of severity were included, calculated by means of the comparison score from 4 to 10 of ADOS-2 (Gotham et al., 2009; Fiore et al., 2020). In particular the children were divided into "low severity" level for those who obtained comparison score 4, "moderate severity" level for those with a comparison score from 5 to 7, "high severity" level for those with comparison scores of 8–10.

We also consider the following variables: age of mothers and fathers; socioeconomic status measured as educational level of the parents (in years of schooling).

The exclusion criteria were the presence of comorbidities for neurological (cerebral palsy, epilepsy, migraine), psychiatric (anxiety, depression, and psychosis), and other relevant medical conditions. All the participants performed a neuropsychological assessment using standardized tests, as in our clinical practice. All the subjects recruited agreed to participate in our study. The parents provided their written informed consent after receiving a description about the objective and the protocol of the study. The study design was approved by the Campania Sud Ethics Committee (protocol number 0144996—July, 28 2021) and it was

conducted according to the rules of good clinical practice, in line with the Declaration of Helsinki.

Measures

The evaluation of all the participants involved the following neuropsychological standardized tests:

The Autism Diagnostic Observation Schedule Second Edition (ADOS-2) is a standardized and semi-structured measure that assess communication, social interaction, play, and the imaginative use of materials in individuals with ASD (Lord et al., 2012). It represents the gold standard for the evaluation and diagnosis of children and young people with suspicion or diagnosis of ASD. This test consists of different Modules, according to age and language skills of the subject evaluated: Module 1 for subjects aged 31 months or older who do not have a language consistent with the sentences; Module 2 for individuals of any age who use sentence language but are not yet verbally fluent; Module 3 for verbally fluent topics. The ADOS-2 provides two separate domain categories: "Social Affect" and "Restricted, Repetitive Behaviors" from which a single "Total Score" is derived. The "Total Score" is converted into a "Comparative Score" ranging from 1 to 10. This numerical range is divided into four interpretative categories that correspond to the levels of symptoms related to the autism spectrum: levels 1 and 2 for minimum or absence of symptoms associated with the autism spectrum; 3 and 4 for a low level of symptoms; from 5 to 7 for an average level; 8 to 10 for a high level. In modules 1, 2, and 3 Cronbach's alpha values were highest for the AS domain (0.87–0.92 for evolutionary subgroup) and ranged from 0.51 to 0.66 in the CRR domain.

The Autism Diagnostic Interview—Revised (ADI-R) is a semi-structured interview employed by trained examiners and used with caregivers to gather information on the development of behaviors and skills in the first years of the patient's life (Lord et al., 1994). The interviewer codes behavioral descriptions given by the caregiver as 0 (no abnormality), 1 (possible abnormality), 2 (definite autistic type abnormality), and 3 (severe autistic type abnormality). In the present study, scores of 3 were recorded to 2, as recommended by Lord et al. (1994). Internal consistency estimates are 0.95 in the communication domain and 0.69 in the restricted and repetitive behavior domain (Lord et al., 1994). Used together, ADI-R and ADOS are considered the "gold standard" for diagnosing ASD (Falkmer et al., 2013).

The Vineland Adaptive Behavior Scales, Survey Interview, 2nd edition (VABS) is a parent-report questionnaire of adaptive behavior for individuals aged birth to 99 years (Sparrow et al., 2005). In this tool there is an "Adaptive Behavior Composite Score" which derives from 4 scales that, respectively, evaluate "Communication," "Daily life skills," "Socialization," and "Motor skills." These domains consist of 2 or 3 subscales each. In the single items the scores range from 0 to 2 (0 = never, 1 = sometimes, or partially, 2 = usually) through the interview with the parents, which convert into raw scores from which scores on the v-scale are obtained. Scores on the v-scale have a mean of 15 and a standard deviation of 3. Standard scales/scores were used for data analysis to allow comparison between different age groups. The motor abilities are assessed in children from birth to 6 years 11 months of age and were not considered for this study. The reliability coefficients of the Vineland subscales are between 0.80 and 0.90.

The Child Behavior Checklist (CBCL) is an evidence-based questionnaire (Achenbach and Rescorla, 2001) used to assess behavioral, emotional, and social problems and functioning in children up to 18 years. There are two versions compiled by the caregivers: one is used for children from 1 and a half to 5 years old and another from 6 to 18 years old. The tool consists of 113 statements and there are three possible answers recorded on a Likert scale: 0 Not true, 1 Sometimes or Fairly True, 2 Often True or Very True. The results are distributed in subscales as t-scores. The normative data are divided as follows: a t-score $\leq$ 64 is normal, an interval at the limits is indicated by a t-score between 65 and 69 and a t-score $\geq$ 70 indicates clinical symptoms. For the subscales of the "internalization problems," "externalization problems," and "total problems," a t-score $\leq$ 59 indicates normal scores, a t-score between 60 and 64 indicates a score that is within a boundary range and high levels of maladaptive behavior are indicated by t-score $\geq$ 65. The CBCL have an adequate internal consistency, as emerged from the study of D'Orlando et al. (2010).

The *PSI Short Form (PSI/SF)* arises from the Parenting Complete Stress Index (PSI) test (Abidin, 1995) and is composed of 36 sentences. The parents must indicate how much they agree with each of the 36 sentences, according to a 5-point Liker scale, ranging from "strongly agree" to "strongly disagree." In this questionnaire there are three subscales: Parental Distress (PD), which indicates the level of discomfort that a caregiver is experiencing as a parent, also taking into consideration personal factors directly related to that role, Dysfunctional Parent-Child Interaction Scale (P-CDI), which assesses the level of satisfaction linked to the relationship with the child, and finally Difficult Child Scale (DC) which assesses the parent's perception of having a difficult child (Abidin, 2012). In PSI/SF the raw score is converted to t-scores; the higher the t-scores the higher the stress levels, in particular a t-score $\geq$ 85 indicates clinically significant parental stress (Abidin, 1995). The test also includes a defensive response scale (DF) useful for verifying the validity of the protocol as it indicates whether the parent tends, for example, to give a better self-image or to minimize problems and perceived stress in the relationship the child.

STATISTICAL ANALYSIS

All the measures were expressed as mean and standard deviation (SD). The mean score of ADOS-2, VABS-II, PSI, and CBCL was compared among ASD groups (levels low-moderate-high) using the non-parametric Kruskal-Wallis test and with the subsequent *post hoc* analysis using the Mann-Whitney *U*-test. The comparison between the age groups, based on the two versions of the CBCL (<6 years; $\geq$ 6 years), and sex groups, was made using Mann–Whitney *U*-test.

The linear regression analysis was performed in order to evaluate the relationships between different variables (age, ADOS-2 scores, VABS-II scores, CBCL scores, and PSI scores). The strength of relationship ranging from

1 to 1, with −1 indicating a perfect negative linear relation, 1 indicating a perfect positive linear relation, and 0 indicating no linear relation between variables. A *p*-value of less than 0.05 was considered as statistically significant. For statistical processing, we used the data processing program the Statistical Package for Social Science version 23.0.

RESULTS

The three group based on severity level of ASD symptoms did not significantly differ in all the main socio-demographic characteristics (**Table 1**).

The analysis conducted to compare VABS, PSI, and CBCL mean scores among ASD levels showed a significant difference in the three following scales of VABS questionnaire: Communication, Daily Living Skills, Socialization and Adaptive Behavior Composite. No other differences were found. Average scores and statistical analysis were reported in **Table 2**.

Post hoc analysis revealed that children ASD level low had a significant higher score in all subscales of VABS than children ASD level moderate and level high; in addition, subjects ASD level moderate showed higher significant score than subjects ASD level high in all the VABS subscales, except Socialization scale. *Post hoc* analysis was reported in **Table 3**.

The comparison between Age groups among ADOS, VABS, PSI, and CBCL scores showed that the ADOS Social Affect and ADOS Total Score of group Age < 6 was significantly higher than group Age ≥ 6, while in VABS Communication and in PSI Difficult Child and PSI Parental Distress the group Age ≥ 6 exhibited significant higher scores than group Age < 6. The mean scores and statistical analysis were reported in **Table 4**.The comparison between Sex groups among ADOS, VABS, PSI, and CBCL didn't show statistical differences.

Linear Regression Analysis

Linear regression analysis revealed a significant relationship between several factors (**Table 5**).

We found negative relationship between Age and ADOS Social Affect, ADOS Total Score, and positive relationship among Age and VABS Communication, and PSI Difficult Child.

All the ADOS scores correlate negatively with all VABS subscale scores. In addition we found positive relationship among ADOS Social Affect and ADOS Total Score with PSI Dysfunctional Parent-Child Interaction. Moreover ADOS Comparison scores was positively related with CBCL Total problems.

Negative relationship was found between VABS Communication, VABS Socialization, VABS Adaptive Behavior Composite, and PSI Dysfunctional Parent-Child Interaction; furthermore VABS Socialization and CBCL Internalizing problems was negatively related.

The CBCL Internalizing problems, CBCL Externalizing problems, and CBCL Total problems scores correlate positively with PSI Dysfunctional Parent-Child Interaction, PSI Difficult Child and PSI Total Stress. No other relationships were found. All the results are summarized in **Table 5**.

DISCUSSION

The goal of this study was to compare adaptive skills, emotional/behavioral problems, and parental stress among children with different severity levels of ASD. The results of this study may help the clinician to delineate a more specific neuropsychological profile of children with ASD and helping to further differentiate some specific characteristics of ASD individuals with different severity level of symptoms. Furthermore, knowing the adaptive functioning of children with ASD can be useful for planning targeted interventions and for assessing adaptive abilities over time, considering their strengths and weaknesses.

The first result that emerged from the analysis among the three groups, was that a higher level of ASD symptoms was correlated to less adaptive skills. In fact, the performance obtained in all VABS subscales is higher in children with level low of ASD symptoms than in those with moderate and high level. Our finding agreed with a previous study (Tillmann et al., 2019) in which the authors showed that higher levels of ASD symptoms, specifically social communication symptoms, were associated with lower adaptive functioning. Furthermore, children with level moderate ASD obtained better results in all VABS subscales than those of level high, except in Socialization, in which the two groups do not significantly differed. This finding agrees with previous studies (Yang et al., 2016; Chatham et al., 2018) in which the socialization skills were the most compromised domain in individuals with autism; again, autism severity was negatively correlated with the adaptive behavior in daily living, communication, and global adaptive functioning score (Yang et al., 2016).

Another finding that emerged from our study was that older ASD subjects (>6 years) had better communication skills than younger children (<6 years), as shown by the higher score in the VABS communication scale.

From linear regression analysis, we found a negative relationship between age and ASD symptoms. Indeed, in our sample younger children showed higher level of ASD symptoms, especially in social and communicative skills (measured by ADOS Social Affect and ADOS Total Score scales). This result is in line with the study by Yang et al. (2016), who reported a negative correlation between age and the ADOS severity score.

We also found a positive relationship between age and adaptive skills, particularly in communication competences (VABS Communication). This result could suggest that children and adolescents with ASD can obtain an improvement in some aspects of their communication skills over time, probably also due to the rehabilitation interventions, while the difficulties in socialization skills and the daily living skills persist. The study by Tillmann et al. (2019), on 417 participants aged 6–31 years, showed that older age was associated with lower adaptive functioning in all VABS subscales. The discrepancy with our study is probably due to the different range of age considered.

TABLE 1 | Sample characteristics.

	Total ASD ***n*** **= 88**	**Level low** ***n*** **= 28**	**Level moderate** ***n*** **= 34**	**Level high** ***n*** **= 26**	**Statistics**
Male	57 (65%)	19 (68%)	23 (68%)	15 (58%)	Chi square test $\chi^2 = 0.8076$; $p = 0.6677$
Female	31 (35%)	9 (32%)	11 (32%)	11 (42%)	
Age in years (M ± SD)	6.00 ± 2.70	7.07 ± 3.07	5.62 ± 2.51	5.35 ± 2.24	Kruskall-Wallis *U*-test $\chi^2 = 5.558$; $p = 0.063$
Mother age in years (M ± SD)	32.48 ± 3.51	33.21 ± 4.02	32.50 ± 3.41	31.71 ± 3.12	Kruskall-Wallis *U*-test $\chi^2 = 0.960$; $p = 0.619$
Father age in years (M ± SD)	34.93 ± 3.99	35.07 ± 4.51	35.14 ± 3.23	34.57 ± 4.38	Kruskall-Wallis *U*-test $\chi^2 = 0.053$; $p = 0.974$
Maternal educational level* (M ± SD) *n* = 88	12.40 ± 3.39 *n* = 88	12.43 ± 3.32 *n* = 28	11.79 ± 3.24 *n* = 34	13.00 ± 3.72 *n* = 26	Kruskall-Wallis *U*-test $\chi^2 = 2.298$; $p = 0.317$
Paternal educational level* (M ± SD) *n* = 62	12.33 ± 3.16 *n* = 62	11.93 ± 2.13 *n* = 19	11.71 ± 3.67 *n* = 24	13.36 ± 3.43 *n* = 19	Kruskall-Wallis *U*-test $\chi^2 = 2.339$; $p = 0.310$

ASD, Autism Spectrum Disorder; M, mean; SD, Standard Deviation.
**Expressed in age of schooling.*

TABLE 2 | Mean scores comparison among the three ASD severity levels.

	ASD Level low ***n*** **= 28**	**ASD Level moderate** ***n*** **= 34**	**ASD Level high** ***n*** **= 26**	**Statistic Kruskall-Wallis test**
VABS_COM (M ± SD)	78.37 ± 16.03	55.73 ± 18.04	44.23 ± 10.85	**$\chi^2 = 39.357$; $p = < 0.05$**
VABS_DLS (M ± SD)	79.15 ± 15.14	64.18 ± 17.63	54.96 ± 9.82	**$\chi^2 = 28.089$; $p = < 0.05$**
VABS_SOC (M ± SD)	73.44 ± 13.73	61.52 ± 15.38	55.69 ± 10.17	**$\chi^2 = 22.234$; $p = < 0.05$**
VABS_ABC (M ± SD)	74.63 ± 14.22	56.33 ± 19.75	47.63 ± 14.50	**$\chi^2 = 29.092$; $p = < 0.05$**
PSI_PD (M ± SD)	72.88 ± 19.30	59.03 ± 28.48	71.00 ± 23.50	$\chi^2 = 4.034$; $p = 0.133$
PSI_PCDI (M ± SD)	66.35 ± 18.45	76.55 ± 20.18	77.68 ± 16.51	$\chi^2 = 5.356$; $p = 0.069$
PSI_DC (M ± SD)	69.81 ± 21.28	69.31 ± 24.92	71.00 ± 18.43	$\chi^2 = 0.089$; $p = 0.956$
PSI_TS (M ± SD)	71.35 ± 18.34	67.76 ± 26.34	79.20 ± 16.75	$\chi^2 = 3.282$; $p = 0.198$
CBCL_INT (M ± SD)	55.72 ± 10.38	57.88 ± 10.25	56.92 ± 10.00	$\chi^2 = 0.505$; $p = 0.777$
CBCL_EXT (M ± SD)	53.44 ± 10.52	54.25 ± 9.48	52.52 ± 9.34	$\chi^2 = 0.472$; $p = 0.790$
CBCL_TP (M ± SD)	56.16 ± 11.89	57.53 ± 11.01	57.00 ± 9.48	$\chi^2 = 0.023$; $p = 0.989$

M, mean; SD, Standard Deviation; VABS_COM, Vabs Communication; VABS_DLS, Vabs Daily Living Skills; VABS_SOC, Vabs Socialization; VABS_ABC, Vabs Adaptive Behavior Composite; PSI_PD, parental distress; PSI_PCDI, dysfunctional interaction parent-child; PSI_TS, Total Stress; PSI_DC, difficult child; CBCL_INT, cbcl internalization problems; CBCL_EXT, cbcl externalization problems; CBCL_TP, cbcl total problems.
$p < 0.05$ are in bold.

TABLE 3 | *Post Hoc* analysis (Mann-Whitney *U*-test).

	VABS_COM	**VABS_DLS**	**VABS_SOC**	**VABS_ABC**
1 vs. 2	***U* = 122.00 *p* < 0.001**	***U* = 234.50 *p* = 0.002**	***U* = 237.50 *p* = 0.002**	***U* = 182.50 *p* < 0.001**
1 vs. 3	***U* = 28.00 *p* < 0.001**	***U* = 51.50 *p* < 0.001**	***U* = 93.00 *p* < 0.001**	***U* = 71.50 *p* < 0.001**
2 vs. 3	***U* = 258.00 *p* = 0.009**	***U* = 280.00 *p* = 0.023**	*U* = 306.00 *p* = 0.060	***U* = 289.00 *p* = 0.032**

1, low level group; 2, moderate level group; 3 high level group, VABS_COM, Vabs Communication; VABS_DLS, Vabs Daily Living Skills; VABS_SOC, Vabs Socialization; VABS_ABC, Vabs Adaptive Behavior Composite.
Significant p-values are in bold.

Parental Stress related to the child characteristics (PSI Difficult Child) were positively related to the age of child, so the parents of older children reported higher stress levels. This data underlines that parents perceive greater difficulties in family management with the increasing age of their children.

The ASD symptoms were negatively related to adaptive functioning. As showed in our analysis, children with higher severity of ASD symptoms (ADOS Social Affect, ADOS Restricted, and Repetitive Behavior, ADOS Total Score) had fewer communicative, social, and daily living skills (VABS Communication, VABS Daily Living Skills, VABS Socialization,

TABLE 4 | Mean scores comparison between Age groups.

	Age < 6 (*n* = 41)	Age ≤ 6 (*n* = 47)	Mann– Whitney *U*-test
ADOS_AS (M ± SD)	12.38 ± 4.62	9.83 ± 3.92	***U* = 630.00; *p* = 0.008**
ADOS_RRB (M ± SD)	3.48 ± 1.26	3.17 ± 1.52	*U* = 821.00; *p* = 0.298
ADOS_TOT (M ± SD)	15.85 ± 5.04	13.00 ± 4.93	***U* = 637.00; *p* = 0.010**
ADOS_Comp (M ± SD)	6.10 ± 1.37	6.15 ± 1.69	*U* = 909.00; *p* = 0.947
VABS_COM (M ± SD)	54.28 ± 16.66	63.78 ± 22.82	***U* = 677.00; *p* = 0.035**
VABS_DLS (M ± SD)	66.35 ± 14.37	65.87 ± 20.11	*U* = 896.00; *p* = 0.835
VABS_SOC (M ± SD)	64.48 ± 13.46	62.65 ± 16.53	*U* = 874.50; *p* = 0.693
VABS_ABC (M ± SD)	61.90 ± 18.41	57.33 ± 20.81	*U* = 800.50; *p* = 0.301
PSI_PD (M ± SD)	59.46 ± 27.05	74.00 ± 20.62	***U* = 541.00; *p* = 0.014**
PSI_PCDI (M ± SD)	72.03 ± 17.46	74.93 ± 20.34	*U* = 695.00; *p* = 0.329
PSI_DC (M ± SD)	63.64 ± 22.19	75.47 ± 19.75	***U* = 544.00; *p* = 0.015**
PSI_TS (M ± SD)	68.92 ± 23.40	75.58 ± 16.62	*U* = 663.00; *p* = 0.119
CBCL_INT (M ± SD)	56.51 ± 10.00	57.27 ± 10.33	*U* = 822.00; *p* = 0.922
CBCL_EXT (M ± SD)	52.35 ± 8.85	54.40 ± 10.30	*U* = 766.50; *p* = 0.538
CBCL_TP (M ± SD)	55.27 ± 9.43	58.33 ± 11.62	*U* = 694.50; *p* = 0.198

ADOS_SA, Social Affect; ADOS_RRB, Restricted and Repetitive Behaviors; ADOS_TOT, Ados Total Score; ADOS_Comp, Comparison Scores; VABS_COM, Vabs Communication; VABS_DLS, Vabs Daily Living Skills; VABS_SOC, Vabs Socialization; VABS_ABC, Vabs Adaptive Behavior Composite; PSI_PD, parental distress; PSI_PCDI, dysfunctional interaction parent-child; PSI_DC, difficult child; PSI_TS, Total Stress; CBCL_INT, cbcl internalization problems; CBCL_EXT, cbcl externalization problems; CBCL_TP, cbcl total problems.
$p < 0.05$ are in bold.

VABS Adaptive Behavior Composite). Our results are in agreement with several previous literature studies, in which it was reported that greater communication-relational impairment was associated with greater difficulties in adaptive skills (Yang et al., 2016; Chatham et al., 2018; Tillmann et al., 2019).

The presence of higher ASD symptom (ADOS Social Affect and ADOS Total Score) was related to higher Parental Stress, generating difficulties in the parent-child relationship (PSI Dysfunctional Parent-Child Interaction). Also low adaptive skills in socialization and communication (VABS Communication, VABS Socialization, VABS Adaptive Behavior Composite) generated higher levels of parental stress (PSI Dysfunctional Parent-Child Interaction). Several chronic conditions often generate increased parental stress (Craig et al., 2016; Operto et al., 2019a,b). Neurodevelopmental disorders are associated with an increase in parental concerns about the child's clinical condition, core symptoms, comorbidities, the child's integration into social contexts, future independence (Operto et al., 2021).

Higher severity of ASD symptoms (ADOS Comparison scores) was also associated to more emotional and behavioral problems in children (CBCL Total problems). The difficulties in adapt to the social context (VABS Socialization) were also related to more internalizing problems in children (CBCL Internalizing Problems). Internalizing and externalizing problems are very common in children and adolescents with ASD, such as anxiety, depression, attention problems, behavioral problems (Craig et al., 2016; Guerrera et al., 2019; Operto et al., 2021). However, to the best of our knowledge there is no previous work analyzing the emotional and behavioral profile in relation to adaptive skills.

Finally, another important result of our study was that the higher presence of internalizing and externalizing problems in children (CBCL Internalizing problems, CBCL Externalizing problems, and CBCL Total problems) were positively related with higher level of parental stress, leading to the perception of having a difficult child and a complicated parent-child interaction (PSI Difficult Child, PSI Dysfunctional Parent-Child Interaction, and PSI Total Stress). Our results agree with recent studies (Operto et al., 2021) in which the parents of these children experience high levels of stress in their parental role and have the perception of having a difficult child; this could be due to the parents' difficulty in obtaining the child's cooperation or managing her behavior.

The main limitation of our work is the cross-sectional design that does not give information about causal relationship between the tested variables. Future prospective studies will be important to test statistical and clinical relevance of the selected variables. Furthermore, another weaknesses of the study are that, although PSI and CBCL have good psychometric properties and are important for assessing internalizing states, they are self-correlated subjective measures that could lead to possible bias.

One of the main strengths of our study is the sample size. In addition, another important strength is that our results further contribute to underline the importance of an even more patient-centered approach, that practitioners and clinicians could try to adopt when working with ASD children. As already showed in a previous review of Weitlauf et al. (2014), our study also suggests the need to evaluate different clinical aspects such as adaptive skills or emotional and behavioral features, that may differ among the ASD subjects in the same ASD severity level, contributing to personalized interventions.

The results of our study could help to better understand some specific characteristics of the different severity levels of ASD, highlighting the strengths, and weaknesses of the each individual subject and proposing more targeted treatments. Our study suggests to emphasize an individualized treatment that, in addition to the ASD symptoms severity level, also take into account the adaptive level (personal, social, and daily life skills)

TABLE 5 | Linear regression analysis.

		ADOS_SA	ADOS_RRB	ADOS_TOT	ADOS_COMP	VABS_COM	VABS_DLS	VABS_SOC	VABS_ABC	PSI_PD	PSI_PCDI	PSI_DC	PSI_TOT	CBLB_INT	CBCL_EXT	CBCL_TP
Age	F	4.870	0.193	4.029	1.943	10.799	0.218	2.519	1.094	2.333	1.278	7.894	1.279	0.623	0.117	1.028
	Beta	−0.223	−0.048	−0.213	0.150	0.338	−0.051	−0.171	−0.113	0.170	0.127	0.303	0.127	0.088	0.038	0.113
	p	0.030*	0.662	0.048*	0.167	0.001*	0.642	0.116	0.299	0.131	0.262	0.006*	0.262	0.432	0.734	0.314
ADOS_SA	*F*					41.784	18.234	21.486	21.590	0.019	4.003	0.298	1.772	2.232	0.201	1.461
	Beta					−0.576	−0.422	−0.451	−0.361	0.016	0.221	0.062	0.149	0.165	0.050	0.134
	p					0.000*	0.000*	0.000*	0.001*	0.889	0.049*	0.587	0.187	0.139	0.655	0.230
ADOS_RRB	*F*					17.552	12.008	9.002	10.654	3.316	1.202	0.922	0.164	0.015	0.248	0.212
	Beta					−0.416	−0.354	−0.311	−0.335	−0.202	0.123	0.108	−0.046	0.014	0.056	0.051
	p					0.000*	0.001*	0.004*	0.002*	0.72	0.276	0.340	0.686	0.901	0.620	0.646
ADOS_TOT	*F*					49.610	22.520	24.241	16.197	0.130	4.108	0.532	1.067	1.704	0.272	1.346
	Beta					−0.609	−0.460	−0.473	−0.402	−0.041	0.224	0.082	0.116	0.144	0.058	0.129
	p					0.000*	0.000*	0.000*	0.000*	0.719	0.046*	0.468	0.305	0.195	0.603	0.249
ADOS_Comp	*F*					7.831	7.936	16.470	6.128	0.180	1.433	3.031	0.173	3.723	1.474	4.142
	Beta					−0.294	−0.295	−0.407	−0.262	−0.048	0.135	0.195	0.047	0.212	0.135	0.223
	p					0.006*	0.006*	0.000*	0.015*	0.673	0.235	0.086	0.678	0.057	0.228	0.045*
VABS_COM	*F*									0.214	5.321	0.043	2.349	2.198	1.308	2.108
	Beta									0.052	−0.253	−0.023	−0.171	−0.164	−0.127	−0.160
	p									0.645	0.024*	0.836	0.129	0.142	0.256	0.150
VABS_DLS	*F*									0.185	2.403	1.698	2.488	2.730	1.183	2.363
	Beta									−0.049	−0.173	−0.146	−0.176	−0.182	−0.121	−0.169
	p									0.668	0.125	0.196	0.199	0.102	0.280	0.128
VABS_SOC	*F*									0.065	6.399	1.941	1.976	4.452	0.618	2.258
	Beta									−0.029	−0.275	−0.156	−0.157	−0.230	−0.088	−0.166
	p									0.799	0.013*	0.168	0.164	0.038*	0.434	0.137
VABS_ABC	*F*									0.411	5.204	1.577	3.604	1.228	0.569	1.446
	Beta									−0.072	−0.250	−0.141	−0.210	−0.123	−0.084	−0.133
	p									0.532	0.025*	0.213	0.061	0.271	0.453	0.233
PSI_PD	*F*													2.577	1.939	2.923
	Beta													0.181	0.158	0.192
	p													0.113	0.168	0.091
PSI_PCDI	*F*													10.214	3.373	8.221
	Beta													0.344	0.297	0.312
	p													0.002*	0.008*	0.005*
PSI_DC	*F*													11.297	14.346	12.769
	Beta													0.360	0.398	0.379
	p													0.001*	0.000*	0.001*
PSI_TS	*F*													8.251	10.609	11.201
	Beta													0.313	0.350	0.358
	p													0.005*	0.002*	0.001*

ADOS_SA, Social Affect; ADOS_RRB, Restricted and Repetitive Behaviors; ADOS_TOT, Ados Total Score; ADOS_Comp, Comparison Scores; VABS_COM, Vabs Communication; VABS_DLS, Vabs Daily Living Skills; VABS_SOC, Vabs Socialization; VABS_ABC, Vabs Adaptive Behavior Composite; PSI_PD, parental distress; PSI_PCDI, dysfunctional interaction parent-child; PSI_DC, difficult child; PSI_TS, Total Stress; CBCL_INT, cbcl internalization problems; CBCL_EXT, cbcl externalization problems; CBCL_TP, cbcl total problems.
**Significant p-value.*

and the emotional and behavioral problems of the children. In fact, identifying and treating early these children's weaknesses, we could help to improve their quality of life and prevent emotional-behavioral symptoms. Furthermore, given the level of stress that emerged in the parents of children with ASD, the importance of extending the intervention to the whole family to favor a better development and adaptation of the child must be considered.

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 Campania Sud Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

FO conceptualized the work. CS, CP, and VV performed psychometric measurements and analyzed the data. IP, GB, and VS drafted the manuscript and revised the language. RR researched the data in the literature. GP analyzed the data, drafted the manuscript, involved in planning, and supervised the work. GC was involved in planning and supervised the work. All authors have agreed to this final version and participated in a meaningful way in the preparation of the manuscript.

ACKNOWLEDGMENTS

We thank all the patients and their families who participated in the study.

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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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frontiers in Neuroscience

ORIGINAL RESEARCH
published: 17 December 2021
doi: 10.3389/fnins.2021.747273

Atypical Neural Responses of Cognitive Flexibility in Parents of Children With Autism Spectrum Disorder

Xin Cheng[1,2], Yu Li[1], Xiwen Cui[1], Hong Cheng[3], Chunyan Li[1], Linyan Fu[1], Jiying Jiang[1], Zhenyu Hu[2] and Xiaoyan Ke[1]**

[1] The Child Mental Health Research Center, Nanjing Brain Hospital Affiliated to Nanjing Medical University, Nanjing, China, [2] Department of Child Psychiatry, Ningbo Kangning Hospital, Ningbo, China, [3] Physical Diagnostic Department, Nanjing Brain Hospital Affiliated to Nanjing Medical University, Nanjing, China

Impaired cognitive flexibility has been repeatedly demonstrated in autism spectrum disorder (ASD). There is strong evidence for genetic involvement in ASD. First-degree relatives of individuals with ASD may show mild deficits in cognitive inflexibility. The present study investigated cognitive flexibility and its neuroelectrophysiological mechanisms in first-degree relatives of individuals with ASD to assess its potential familiality. Forty-five biological parents of individuals/children with ASD (pASD) and thirty-one biological parents of typically developing individuals/children (pTD), matched by gender, age, and IQ, were enrolled. The broad autism phenotype questionnaire (BAPQ) and cognitive flexibility inventory (CFI) were used to quantitatively assess autistic traits and cognitive flexibility in daily life, respectively. The task-switching paradigm was used to evaluate the behavioral flexibility in a structured assessment situation. Event-related potentials (ERPs) induced by this paradigm were also collected. Results showed that compared with the pTD group, the pASD group had lower CFI scores ($t = -2.756$, $p < 0.01$), while both groups showed an equivalent "switch cost" in the task-switching task ($p > 0.05$). Compared with the pTD group, the pASD group induced greater N2 amplitude at F3, F4, Fz, and C4 ($F = 3.223$, $p < 0.05$), while P3 amplitude and latency did not differ between the two groups. In addition, there was a significant negative correlation between the CFI total scores and BAPQ total scores in the pASD group ($r = -0.734$, $p < 0.01$). After controlling for age and IQ, the N2 amplitude in the frontal lobe of pASD was negatively correlated with the CFI total scores under the repetition sequence ($r = -0.304$, $p = 0.053$). These results indicated that pASD had deficit in cognitive flexibility at the self-reported and neurological levels. The cognitive flexibility difficulties of parents of children with ASD were related to autistic traits. These findings support that cognitive flexibility is most likely a neurocognitive endophenotype of ASD, which is worthy of further investigation.

Keywords: autism spectrum disorder, first-degree relatives, cognitive flexibility, N2, P3

OPEN ACCESS

Edited by:
Mirko Uljarevic,
The University of Melbourne, Australia

Reviewed by:
Ru Ying Cai,
Autism Spectrum Australia, Australia
Farnaz Ghassemi,
Amirkabir University of Technology, Iran

***Correspondence:**
Zhenyu Hu
hzy86690952@163.com
Xiaoyan Ke
kexiaoyan@njmu.edu.cn

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: *26 July 2021*
Accepted: *01 December 2021*
Published: *17 December 2021*

Citation:
Cheng X, Li Y, Cui X, Cheng H, Li C, Fu L, Jiang J, Hu Z and Ke X (2021) Atypical Neural Responses of Cognitive Flexibility in Parents of Children With Autism Spectrum Disorder. Front. Neurosci. 15:747273. doi: 10.3389/fnins.2021.747273

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by persistent difficulties in social communication and interaction, as well as restricted, repetitive patterns of behavior. A number of studies have found that unaffected family members of ASD individuals share some behavioral and cognitive traits with probands, but to a lesser extent, known as the broader autism phenotype (BAP) (Piven et al., 1997), which indicates that the core autistic traits can be passed from generation to generation. However, even with heritability estimated as high as 74∼93% (Tick et al., 2016), our understanding of the underlying pathophysiological mechanism and their relationship to autistic characteristics remains unclear. This is partly due to the lack of well-established biomarkers associated with core clinical features. Therefore, characterizing the brain profiles of unaffected first-degree relatives of ASD individuals may be helpful to understand the characteristic patterns of intergenerational inheritance, identify endophenotypes of ASD, and bridge etiological processes and clinical phenotypes.

Cognitive flexibility is one of the core executive functions, referring to the ability to adjust behaviors appropriately to environmental changes (Dajani and Uddin, 2015). It is very important for goal-oriented and adaptive behaviors. In the laboratory environment, cognitive flexibility is usually measured using the Wisconsin Card Sorting Test (WCST), task switching and set-shifting paradigms. Impaired cognitive flexibility in ASD individuals of different ages has been repeatedly demonstrated (D'Cruz et al., 2013; Rosenthal et al., 2013; Van Eylen et al., 2017; Johnston et al., 2019; Schmitt et al., 2019; Xie et al., 2020). Also, multiple studies have also shown that cognitive flexibility is closely related to stereotypical behaviors (Lopez et al., 2005; Miller et al., 2015; Faja and Nelson Darling, 2019; Iversen and Lewis, 2021). Thus, cognitive flexibility is regarded as one of the neurocognitive dimensions associated with the core clinical features of ASD, closely related to the underlying neurobiological processes.

Several studies have shown deficit in behavioral flexibility in unaffected first-degree relatives of individuals with ASD, suggesting that it may serve as neurocognitive traits linked to familiality (Hughes et al., 1999; Moazzen et al., 2015; Li et al., 2017; Schmitt et al., 2019; Shalani et al., 2019). However, some studies have reported that unaffected first-degree relatives of individuals with ASD retained intact cognitive flexibility (Wong et al., 2006; McLean et al., 2014; Rosa et al., 2017). Mixed findings may result from differences in subject characteristics, such as age and sample size. For example, a study demonstrated significant differences between 124 parents and siblings of autistic children and 124 parents of typically developing children in WCST (Moazzen et al., 2015), whereas another study found 22 unaffected siblings of ASD individuals performed similarly to control participants in WCST (Rosa et al., 2017). In addition, there are inconsistencies between measures. For example, a study showed neither parents nor siblings of individuals with ASD displayed significant difficulties in set-shifting (Wong et al., 2006).

Previous behavioral findings suggested that there may be abnormalities in the executive control networks in ASD, especially those involving cognitive flexibility. It is reported that the lateral frontal parietal network (L-FPN) and the middle cingulate gyrus-insular network (M-CIN) play a central role in supporting executive function and cognitive flexibility (Uddin, 2021). The literature has showed aberrant patterns in these brain regions related to cognitive flexibility in ASD, including frontal and parietal lobes (Shafritz et al., 2008; Yerys et al., 2015; Lynch et al., 2017). However, little is known about more precise cognitive processing and the underlying pathobiological mechanism of cognitive flexibility in ASD. Event-related potentials (ERPs) are time-locked measures of event-related electrical activity in the brain, providing neural processes underlying specific cognitive and behavioral responses. The N2 is a late negative fluctuation observed approximately 200 ms after a stimulus onset (Cremone-Caira et al., 2020). The P3, a late positive waveform that occurs at a latency of approximately 300 ms after a stimulus onset, is known to reflect executive and attentional function, working memory, event categorization, and attentional resource allocation (Polich, 2007). Previous studies in healthy people have shown that N2 and P3 are frequently observed during task switching (Karayanidis et al., 2010; Kopp et al., 2020). It is well known that P3 shows a maximum amplitude in the parietal lobe, and the inferior and posterior parietal regions are associated with P3 amplitude modulation during task switching (Petruo et al., 2019). The N2 is strongly related to frontocentral regions (Kopp et al., 2020) and it reflects attentional control and inhibition, and its amplitude varies with changes in conflict and the need for cognitive control. As far as we know, only a few studies have reported changes in the ERPs of cognitive flexibility in ASD. Moreover, differences in ERP patterns between ASD and typically developing individuals vary depending on task types and developmental levels. For example, a study found that when ASD adolescents over 16 years had larger N2 during a Go/NoGo task, compared with the control group, there was no significant difference in P3 (Høyland et al., 2017). Another study reported that there was no significant difference in P3 between the ASD group and the control group during task switching (Hoofs et al., 2018).

As described above, several studies have reported deficit in behavioral flexibility in first-degree relatives of autistic children, although findings were inconsistent. What's more, neuroimaging studies have shown that first-degree relatives of autistic children had abnormal activation patterns in the frontal lobes, cingulate gyrus and parietal lobe, which were core brain areas supporting cognitive flexibility (Spencer et al., 2012; Dajani and Uddin, 2015; Moseley et al., 2015; Mehdizadehfar et al., 2020; Peng et al., 2020). Therefore, it is reasonable to assume that cognitive flexibility in unaffected first-degree relatives of autistic individuals may be impaired and manifested at the neurological level. However, to the best of our knowledge, no studies have specifically explored the neuroelectrophysiological mechanism of cognitive flexibility in first-degree relatives of ASD probands.

Therefore, the present study aimed to compare and analyze the differences in cognitive flexibility between parents of children with ASD and typically developing children and use

the ERPs technique to accurately analyze the neural activity changes in parents of children with ASD during task-switching paradigm. We also investigated the relationship between ERPs (N2, P3) and cognitive flexibility to determine whether such neuroelectrophysiological differences might affect cognitive flexibility. This study also assessed the extent to which cognitive flexibility deficits covaried with subclinical autistic traits in unaffected relatives to better understand the intergenerational transmission of behavioral traits associated with ASD. Based on previous findings, we hypothesized that the cognitive flexibility in the parents of individuals with ASD (pASD) group was worse than that in the control group. We also predicted higher ERPs amplitudes generated in the pASD group, reflecting increased efforts at the task switching process. Finally, we expected to find a positive covariant relationship between cognitive flexibility deficits and subclinical autistic traits.

MATERIALS AND METHODS

Participants

Parents of children with ASD were recruited in the outpatient and rehabilitation department of Nanjing Brain Hospital and the control group were recruited through advertisements. Biological parents of 31 children with ASD (30 boys; mean age: 5.38 ± 2.11 years), diagnosed by two senior child psychiatrists according to the diagnostic criteria for ASD in the *Diagnostic and Statistical Manual of Mental Disorders*, Fifth Edition (DSM-5), and 23 typically developing children (15 boys; mean age: 6.48 ± 2.73 years) participated in the study. In total, forty-five biological parents (20 fathers, 25 mothers) of children with ASD (pASD) participated in the study. Thirty-one biological parents (15 fathers, 16 mothers) of typically developing children (pTD) were recruited to serve as the control group. It is worth noting that pTD did not give birth to children with neurodevelopmental disorders, such as ASD. All participants were right-handed and had normal or corrected vision. All participants had an IQ score greater than 80. The IQ was estimated using the short form of the Wechsler Adult Intelligence Scale. In addition, participants who had a history of psychiatric illness, serious physical disease, taking psychotropic drugs in the past month or EEG examination contraindication were excluded.

This study has been reviewed and approved by the Medical Ethics Committee of Nanjing Brain Hospital Affiliated to Nanjing Medical University (2020-KY104-01). According to the Declaration of Helsinki, after all participants were given informed consent to this study and signed informed consent, they first completed a series of questionnaires and assessments (see section "Assessment" for details), and then underwent an EEG recording while performing task-switching task in Nanjing Brain Hospital. Demographic information of participants was summarized in **Table 1**.

TABLE 1 | Comparison of demographic characteristics between the two groups.

	pASD	pTD	Statistics	*p*
Male/female	20/25	15/16	χ^2 (0.115)	0.735
Age, years	35.29 ± 3.89	36.84 ± 4.37	*t*(−1.622)	0.109
Intelligence quotient (IQ)	113.23 ± 11.67	113.68 ± 8.12	*t*(−0.176)	0.861
BAPQ total scores	92.99 ± 23.95	92.37 ± 13.56	*t*(0.143)	0.887
CFI total scores	72.38 ± 11.28	79.33 ± 9.78	*t*(−2.756)	0.007**

*BAPQ, Broad Autism Phenotype Questionnaire; CFI, Cognitive Flexibility Inventory. **$p < 0.01$.*

Assessment

(1) Wechsler Abbreviated Scale of Intelligence (WASI) (Dumont et al., 2013): It was used to assess general intelligence in this study. It consisted of knowledge (I), similarity (S), mapping (PC), and block (BD). The short form yields an IQ score with a mean of 100 and a standard deviation of 15.

(2) Broad Autism Phenotype Questionnaire (BAPQ) (Hurley et al., 2007): It has a total of 36 items and is used to quantify the level of autistic traits in non-ASD people. It consists of three subscales, including aloof, rigid, and pragmatic language. The questionnaire is based on a 6-point Likert scale, which ranges from rarely (1) to always (6). Higher scores on the BAPQ indicate greater severity of autistic traits.

(3) Cognitive Flexibility Inventory (CFI) (Dennis and Wal, 2010): This is a 20-item, two-subscale self-reported questionnaire designed to assess aspects of cognitive flexibility that enables individuals to think adaptively rather than maladaptively when encountering stressful life events. The questionnaire is based on a 5-point Likert scale, which ranges from rarely (1) to always (5). Lower scores indicate worse cognitive flexibility. The Chinese version of the CFI has good reliability and validity (Wang et al., 2016). CFI has been used in parents of ASD individuals (Moradi et al., 2021).

Experimental Tasks

In this study, a task-switching paradigm was adopted (see **Figure 1**). The task was implemented in E-Prime 2.0 software. Participants were instructed to switch between two different types of tasks [odd–even (OE) vs. high–low (HL) task] based on external cues (color of digits).

Stimuli and Design

The stimuli were composed of the digits 1∼9, excluding 5, and each digit had two colors of red and blue. The number's colors cued the tasks. The presentation forms of stimuli included repetition sequences and switch sequences. If the cue was the same as the previous cue, it was called a repetition sequence. If it differed from the previous one, it was called a switch sequence. The two types of sequences appeared randomly. In the task, repetition sequences accounted for 40% and switch sequences accounted for 60%. Participants had their performance evaluated on two task types: the odd–even task and the high–low task. The OE task required participants to classify the stimulus number as either "odd" or "even" when a red number appeared centered. The HL task required participants to classify the stimulus number as either "lower than 5" or "higher than 5"

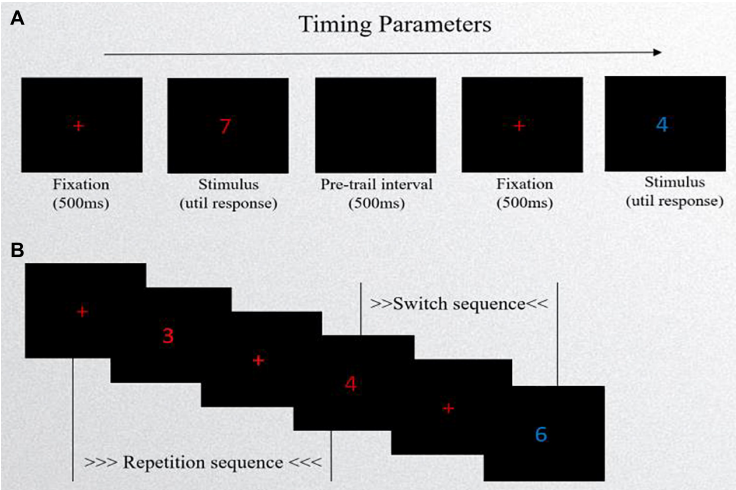

FIGURE 1 | Schematic of task-switching task. **(A)** Experimental procedure: first, a red "+" fixation point occurred in the center of the computer screen, lasting for 500 ms, and then a target stimulus (a number in red or blue) was presented with no time limit. Lastly, the next trail appeared after a blank screen lasting 500 ms. **(B)** The presentation forms of stimuli. If the cue (the color of number) is the same as the previous cue, it is called a repetition sequence. If it is different from the previous one, it is called a switch sequence.

when a blue number appeared centered. Response box templates were created for the task so that the "F" button had a label of Odd/Low and the "J" button had a label of Even/High above the corresponding buttons. The experiment consisted of practice and formal sessions. The practice session consisted of pure-OE tasks (16 trials), pure-HL tasks (16 trials), and 32 mixed-condition trials with feedback. Then, they completed three mixed-condition blocks of 80 trials (without feedback) each, with a short break between the two blocks. Each number was presented in a random manner, appearing at the same frequency.

Experimental Procedure

First, a red " + " fixation point occurred in the center of the computer screen and lasted 500 ms, and then a number in red or blue was presented with no time limit. Lastly, the next trail appeared after a blank screen lasting 500 ms. The subjects were required to respond quickly and accurately when the stimulus presented. During the experiment, reaction time (RT) and errors were recorded. Data were cleaned of the first trials of each block, error trials, and trials from practice sessions. Next, trials with RT and error rates exceeding three standard deviations from the mean (considered per condition of each participant) were not included in the analysis.

EEG Data Recording

EEG signals were continuously recorded while the subjects performed the task-switching task in a quiet room with dim lighting. The EEG signal was recorded with a 32-channel system produced by Brain Product, with the active electrodes situated on a standard cap according to the 10–20 system digitalized at 500 Hz. The reference electrode was placed at FCz, with a grounding electrode on AFz, and an electrode was placed under the right eye to record vertical electrooculography signals. Impedance of all electrodes were below 10 kΩ. The online filter was set at 0.016–100 Hz.

Event-Related Potentials Analysis

Off-line EEG data were analyzed using EEGLAB v13.0.0 toolbox that operates within the MATLAB R2013b framework. Raw EEG signals were referenced to the average of the two earlobe electrodes and filtered between 0.5 and 30 Hz with a 50 Hz notch filter using a FIR filter. Trial epochs were extracted from −200 ms to +1000 ms with respect to target stimulus onset. Baseline correction was performed with the mean EEG signals 200 ms before the target stimulus. Artifacts such as eye movements and blinking were removed by independent component analysis (ICA). In addition, segments with amplitudes greater than ± 100 μV were eliminated. The ERPs for each individual were based on averaging the trials of the respective task condition after artifact correction. The ERPs were measured by the average amplitude method. The N2 (220–260 ms) and P3 (330–390 ms) at nine electrode points, including F3, F4, Fz, C3, C4, CZ, P3, P4, and Pz, were measured. The mean value in the F3, F4, and Fz electrodes was considered the mean amplitude within the frontal region.

Statistical Analysis

The SPSS 23.0 software package was used for statistical analysis. Normality of the distributions was checked by the Shapiro–Wilk test. Categorical variables were investigated with χ^2 tests, whereas normally distributed continuous variables, such as age, IQ, scale scores, were investigated with parametric test. Taking into account gender may affect cognitive flexibility (Zeestraten et al., 2017; Van't Westeinde et al., 2020), we set it as a covariable. Accuracy and RT were analyzed by 2 (groups) × 2 (sequence types) repeated measures analysis of variance (RMANOVA). The Mann–Whitney U test was used to compare the difference in

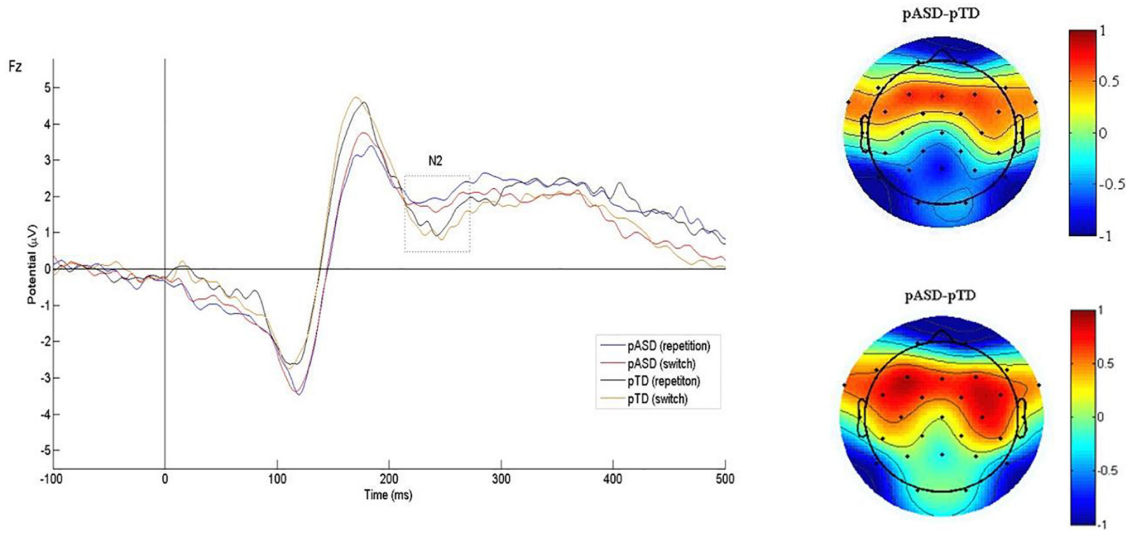

FIGURE 2 | Grand average N2 waveforms and difference topographical maps (in μV). Grand average event-related N2 waveform measured at Fz for both conditions (switch/repetition) in the two groups (left). Group difference scalp topographical maps at Fz for both repetition (top right panel) and switch condition (bottom right panel).

switch costs between the two groups (non-normally distributed data). A 2 (groups) × 2 (sequence types) × 9 (electrodes) RMANOVA was performed for the mean amplitude and latency of N2 and P3, respectively. Independent sample *t*-tests were used for post-tests, and Geisser-Greenhouse *P* value correction was used for multiple comparisons. Pearson correlation was used to investigate the correlations between CFI total scores and N2/P3 amplitude and BAPQ total scores. The test level was $\alpha = 0.05$ (two-tailed).

RESULTS

Demographic Characteristics in the Two Groups

There were no significant differences in sex, age, IQ, or BAPQ total scores between the two groups ($p > 0.05$). Parents of children with ASD had significantly lower CFI total scores than that of the controls ($p < 0.01$) (see **Table 1**).

Behavioral Performances in the Task

Accuracy

The results of RMANOVA showed that the sequence types had a main effect [$F(1, 73) = 12.398$, $p = 0.001$], and the accuracy in the switch sequence was lower than that of repetition sequence (0.935 ± 0.10 vs. 0.947 ± 0.10). There was no main effect between groups, and the interaction between groups and sequence types was not significant ($p > 0.05$).

Reaction Time

The results of RMANOVA showed that the sequence types had a main effect [$F(1, 73) = 10.487$, $p = 0.002$], RT of the switch sequences was longer than that of the repetition sequences (1309.90 ± 37.11 vs. 1082.10 ± 23.67 ms). There was no main effect between groups, and the interaction between groups and sequence types was not significant ($p > 0.05$).

Switch Cost

There was no significant difference in switch cost ($Z = -0.682$, $p > 0.05$) between the two groups.

In conclusion, there were no significant differences in accuracy, response time or switch cost between the two groups.

Event-Related Potential Data

N2

The N2 waveform induced by the task is shown in **Figure 2**, and the mean N2 amplitude and latency of two groups are shown in **Table 2**.

In the latency, RMANOVA results showed that the main effects of groups and sequence types were not statistically significant, and there was no interaction between groups, electrodes and sequence types ($p > 0.05$). The main effect of electrodes was significant [$F(3.189, 8) = 3.344$, $p = 0.018$].

In the amplitude, the main effects of electrodes, groups and sequence types were not significant, and there was no interaction between groups, electrodes and sequence types ($p > 0.05$). The interaction between electrodes and groups was

TABLE 2 | Mean amplitude and latency of N2 in the two groups.

	Amplitude (μV)		Latency (ms)	
	Repetition sequences	Switch sequences	Repetition sequences	Switch sequences
pASD	2.681 ± 0.278	2.674 ± 0.286	237.574 ± 1.705	236.883 ± 1.638
pTD	2.232 ± 0.334	1.925 ± 0.344	237.091 ± 2.055	237.644 ± 1.974

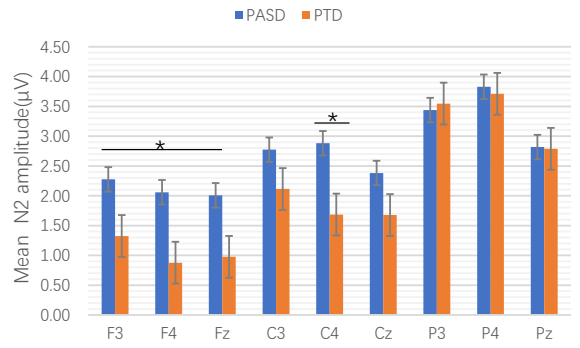

FIGURE 3 | Comparison of mean N2 amplitude at each electrode point between the two groups. *$p < 0.05$.

statistically significant [$F(2.510, 183.255) = 3.223$, $p = 0.031$, $\eta^2 p = 0.042$]. Simple effect analysis showed that there were statistically significant differences in amplitude at F3 ($p = 0.054$, $\eta^2 p = 0.050$), F4 ($p = 0.014$, η^2p $= 0.079$), Fz ($p = 0.048$, $\eta^2 p = 0.052$), and C4 ($p = 0.019$, $\eta^2 p = 0.073$) between the two groups (see **Figure 3**).

P3

The P3 waveform induced by the task is shown in **Figure 4**, and the mean P3 amplitude and latency of the two groups are shown in **Table 3**.

In the latency, RMANOVA results showed that the main effects of groups and electrodes were not statistically significant, and there was no interaction between groups, electrodes and sequence types ($p > 0.05$). The main effect of sequence types was significant [$F(1, 73) = 4.717$, $p = 0.033$].

In the amplitude, the main effects of groups and sequence types were not significant, and there was no interaction between groups, electrodes and sequence types ($p > 0.05$). The interaction between electrodes and groups was also not significant. The main effect of electrodes was significant [$F(1.964, 143.381) = 4.133$, $p = 0.019$].

In summary, there were no significant differences in P3 latency and amplitude between the two groups.

Brain-Behavior Correlation Analysis

The CFI total scores of pASD were negatively correlated with BAPQ total scores ($r = -0.734$, $p < 0.001$).

The mean amplitude of N2 in the frontal lobe was negatively correlated with the CFI total scores ($r = -0.278$, $p = 0.016$). After controlling for age and IQ, the mean N2 amplitude in the frontal lobe of ASD parents was negatively correlated with the CFI total scores under the repetition condition ($r = -0.304$, $p = 0.053$).

There was no correlation between the mean amplitude of P3 and CFI total scores ($p > 0.05$).

DISCUSSION

The current study evaluated cognitive flexibility in biological parents of individuals with ASD and typically developing individuals and further investigated the neuroelectrophysiological characteristics of cognitive inflexibility in the two groups. The results were partially consistent with our hypotheses. As expected, this study showed that parents of children with ASD had self-reported cognitive flexibility difficulties. In contrast, in a laboratory setting, their performance was comparable to the controls in task accuracy, response time, and switch costs. The present study is the first to investigate the neuroelectrophysiological characteristics of cognitive flexibility in pASD. We found that pASD induced significantly larger N2 amplitudes in the frontal lobe and right central region than the controls. However, there was no significant difference in P3 between the two groups. This study also found associations between self-reported cognitive flexibility difficulties and BAPQ total scores and N2 amplitude in the frontal lobe.

Individuals with ASD of different ages have been reported to have cognitive flexibility difficulties in daily life (Granader et al., 2014; Leung and Zakzanis, 2014; McLean et al., 2014). Our results showed that the CFI total scores of parents of children with ASD were significantly lower than those of parents of typically developing children, suggesting that parents of autistic children had cognitive flexibility difficulties in daily life. Our results provide new evidence that cognitive flexibility may be a neurocognitive endophenotype of ASD. However, this study did not find impairment of cognitive flexibility on the task-switching task in parents of children with autism. This contrasted with the findings of Moazzen et al. (2015); Li et al. (2017), and Schmitt et al. (2019) and it is interesting that Wong et al. (2006) and McLean et al. (2014) did not report any evidence of a deficit in cognitive flexibility in parents of children with ASD using the intra-dimensional/extra-dimensional (ID/ED) shifting task and Delis–Kaplan Executive Functioning System. These inconsistencies may be due to different task paradigms. First, the broad range of cognitive abilities required to complete some tasks may interfere with the assessment of specific areas. Thus, poorer performance may be the result of executive function deficits rather than a specific impairment of cognitive flexibility (Lange et al., 2017). For example, poor performances on the WCST may not only result from cognitive flexibility, but due to various additional cognitive processes (like the high social demands, high working memory, inhibitory control, and generativity load) (Eylen et al., 2011; Albein-Urios et al., 2018). Furthermore, task difficulty is an factor in explaining the mixed findings (Geurts et al., 2009). For example, examiners may set a longer stimulus presentation time and interstimulus interval to ensure the high level of behavioral performance (Dirks et al., 2020). Another explanation is that in tests tapping executive functions explicitly providing a high degree of task instructions (like the task-switching paradigm), the examiner provides the necessary structure and organization to act as external executive control for the subject and reducing the requirement for executive functions (including cognitive flexibility) (Eylen et al., 2011). Thus, even if they do have deficit on the cognitive flexibility, they are able to compensate for these impairments with highly explicit task instructions. Finally, the use of lab-based neurocognitive tasks to measure cognitive flexibility may be limited by their limited ecological validity, which hinders their predictive value

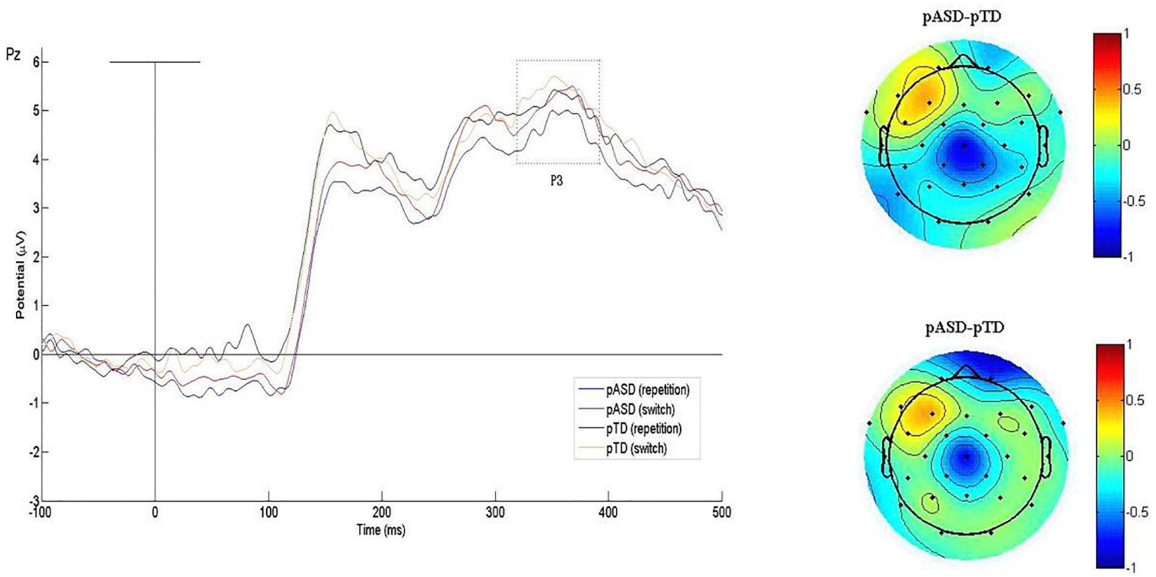

FIGURE 4 | Grand average P3 waveforms and difference topographical maps (in μV). Grand average event-related P3 waveform measured at Pz for both conditions (switch/repetition) in the two groups (left). Group difference scalp topographical maps at Pz for both repetition (top right panel) and switch condition (bottom right panel).

for everyday function. In any case, we did observe a dissociation between behavioral performance and self-reported impairment of cognitive flexibility in parents with ASD. Our findings showed the possibility that self-reported measures of cognitive flexibility may be more sensitive than lab-based neurocognitive measures.

Cognitive flexibility is impaired in ASD individuals (Granader et al., 2014; Leung and Zakzanis, 2014; McLean et al., 2014). In addition, there is also evidence that cognitive inflexibility is strongly associated with clinical outcomes and is important predictors of the severity of symptoms in ASD children (Kenworthy et al., 2008, 2014). The present study also showed difficulty in cognitive flexibility in parents of children with ASD and found that self-reported cognitive flexibility difficulty in parents of children with ASD was significantly negatively correlated with autistic traits, which is similar to previous findings in ASD individuals. Thus, all these findings suggested that subclinical individuals with higher autistic traits show a similar, but milder cognitive flexibility profile as individuals with ASD. However, these results should be interpreted with caution because we cannot rule out the possibility that this is not a true association. For example, CFI and BAPQ may be positively associated in part due to shared methodological effects (i.e., both are self-reported measures), since individuals may exhibit a consistent style of response (Albein-Urios et al., 2018).

TABLE 3 | Mean amplitude and latency of P3 in the two groups.

	Amplitude (μV)		Latency (ms)	
	Repetition sequence	Switch sequence	Repetition sequence	Switch sequence
pASD	3.683 ± 0.413	3.865 ± 0.377	355.162 ± 2.240	357.314 ± 2.233
pTD	3.956 ± 0.497	3.919 ± 0.455	357.237 ± 2.700	354.637 ± 2.690

The amplitude and latency of P3 were used to measure attention resource allocation and information processing speed, respectively. This study found no significant difference in P3 between the pASD and control group, indicating that both groups of subjects allocated the same amount of attention during the task. In addition, we found that the pASD group induced significantly larger N2 amplitudes, suggesting that parents of children with ASD needed to mobilize more neurocognitive resources to monitor and adapt to new changes. The N2 component is closely associated with cognitive flexibility (Kopp et al., 2020), which is supported by the findings that the N2 amplitude in the frontal lobe is significantly negatively correlated with self-reported cognitive flexibility. It has been reported that individuals with ASD induced larger N2 amplitudes under different task conditions than normally developing individuals (Faja et al., 2016; Høyland et al., 2017). These findings suggest that ASD individuals and parents of children with ASD exhibited similar atypical N2 responses. We also found a significant positive correlation between N2 amplitude in the frontal lobe and autistic traits in parents of children with ASD. We interpreted these findings as abnormal brain activity from genetic traits in first-degree relatives of ASD. In conclusion, N2 may be a neuroelectrophysiological endophenotype reflecting cognitive flexibility impairment in ASD.

The frontal lobe shows the most sustained development of any brain region (Sousa et al., 2018), which plays a vital role in executive functions involved in planning, monitoring, attention, and cognitive flexibility (D'Cruz et al., 2016; Sallet et al., 2020). Most previous neuroimaging studies of cognitive flexibility in ASD have reported atypical frontal activity (Schmitz et al., 2006;

Shafritz et al., 2008; Doesburg et al., 2013; D'Cruz et al., 2016; Yeung et al., 2016; Lukito et al., 2020; May and Kana, 2020). We found that the atypical N2 responses in the parents of children with ASD were mainly in the frontal lobe. These results provide further evidence that frontal lobe dysfunction is the neural basis of cognitive flexibility impairment in individuals with autistic traits. Atypical N2 responses in the right central region in parents of children with ASD may be related to the lateralization of the brain. There is a general increase in activation in the right hemisphere and a decrease in activation in the left hemisphere with age (Rubia et al., 2006; Taylor et al., 2012). The spatial requirements of most cognitive flexibility tasks may preferentially recruit the right hemisphere as a result of development.

There are also some limitations in this study. First, we did not collect EEG data from ASD children in the early stage and only proposed hypotheses based on previous findings. If similar findings can be replicated in our own ASD cases, the research will be more systematic, and is thus planned for our future research. Second, parenting stress, anxiety, and depression level are higher in parents of children with ASD than that in parents of typically developing children (Ansari et al., 2021). Although subjects with mental illness (including anxiety and depression) were excluded, we did not take into account the possible influence of subclinical stress levels on EEG signal. In addition, future studies using lab-based neurocognitive tasks may consider a more ecological measure to provide stronger relations to everyday behaviors.

In summary, our results show that cognitive flexibility is reduced in parents of children with ASD. Impaired cognitive flexibility may be an endophenotype of ASD. In addition, self-reported measures of cognitive flexibility are sensitive. Impairment of cognitive flexibility can significantly affect the daily function and quality of life of patients with ASD and increase existing difficulties in social interaction (Albein-Urios et al., 2018). Exploring the neuropathophysiological mechanism of cognitive flexibility is helpful to further understand the neuropathophysiological mechanism of cognitive flexibility in ASD and explore effective intervention strategies to improve flexibility.

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.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Nanjing Brain Hospital Affiliated to Nanjing Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XK designed the study and revised the draft. XC contributed to the design of the study, data collection and analysis, and wrote the draft of the manuscript. XWC, CL, LF, JJ, and HC contributed to the data collection. YL contributed to the data collection and analysis. ZH contributed to the manuscript revision. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the "special disease cohort" research project of Nanjing Medical University (NMUC2018010A).

ACKNOWLEDGMENTS

We are thankful for the support of the funding mentioned above and acknowledge all subjects for their support and participation, as they made this study possible.

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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.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 22 December 2021
doi: 10.3389/fnins.2021.796437

Alteration of Effective Connectivity in the Default Mode Network of Autism After an Intervention

*Han Yu[1], Hang Qu[1], Aiguo Chen[2], Yifan Du[1], Zhimei Liu[2] and Wei Wang[1]**

[1] Department of Radiology, Medical Imaging Center, The Affiliated Hospital of Yangzhou University, Yangzhou University, Yangzhou, China, [2] College of Physical Education, Yangzhou University, Yangzhou, China

Neuroimaging has revealed numerous atypical functional connectivity of default mode network (DMN) dedicated to social communications (SC) in autism spectrum disorder (ASD), yet their nature and directionality remain unclear. Here, preschoolers with autism received physical intervention from a 12-week mini-basketball training program (12W-MBTP). Therefore, the directionality and nature of regional interactions within the DMN after the intervention are evaluated while assessing the impact of an intervention on SC. Based on the results of independent component analysis (ICA), we applied spectral dynamic causal modeling (DCM) for participants aged 3–6 years (experimental group, N = 17, control group, N = 14) to characterize the longitudinal changes following intervention in intrinsic and extrinsic effective connectivity (EC) between core regions of the DMN. Then, we analyzed the correlation between the changes in EC and SRS-2 scores to establish symptom-based validation. We found that after the 12W-MBTP intervention, the SRS-2 score of preschoolers with ASD in the experimental group was decreased. Concurrently, the inhibitory directional connections were observed between the core regions of the DMN, including increased self-inhibition in the medial prefrontal cortex (mPFC), and the changes of EC in mPFC were significantly correlated with change in the social responsiveness scale-2 (SRS-2) score. These new findings shed light on DMN as a potential intervention target, as the inhibitory information transmission between its core regions may play a positive role in improving SC behavior in preschoolers with ASD, which may be a reliable neuroimaging biomarker for future studies.

Clinical Trial Registration: This study registered with the Chinese Clinical Trial Registry (ChiCTR1900024973) on August 05, 2019.

Keywords: default mode network, social communication, dynamic causal modeling (DCM), effective connectivity, exercise intervention

OPEN ACCESS

Edited by:
Mirko Uljarevic,
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Reviewed by:
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Sebastian Klich,
University School of Physical Education in Wrocław, Poland

***Correspondence:**
Wei Wang
waywang@126.com

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: *16 October 2021*
Accepted: *08 December 2021*
Published: *22 December 2021*

Citation:
Yu H, Qu H, Chen A, Du Y, Liu Z and Wang W (2021) Alteration of Effective Connectivity in the Default Mode Network of Autism After an Intervention. Front. Neurosci. 15:796437. doi: 10.3389/fnins.2021.796437

INTRODUCTION

Autism spectrum disorder (ASD) is a complex neurodevelopmental disability (Lord and Bishop, 2015) that emerges in the early stages of life, often severely impairs daily life functions, and is generally associated with life-long disability (Howlin et al., 2004). The prevalence of ASD has currently risen to one in 54 children and ASD was 4.3 times as prevalent among boys as among

girls (Maenner et al., 2020). ASD is primarily manifested as persistent impairments in social communications (SC) and the presence of restricted, repetitive patterns of behaviors, interests, or activities. Despite heterogeneity in behavioral manifestations across sensory and other domains, impaired SC is a core feature of autism (Pelphrey et al., 2011; Xiao et al., 2021).

In general, SC deficits in individuals with ASD often result in exclusion from social interactions (Bolling et al., 2011), contributing to a perception of being "lost in one's narrow world" due to the lack of initial motivation for social communications and interactions and initiating a vicious cycle. Furthermore, the terrible suffering of individuals and families also contributes to ASD being a major public health concern (Maenner et al., 2020). Therefore, timely intervention is particularly important for the healthy development of preschoolers with ASD who are in a critical period of brain development.

Various evidence-based ASD interventions aimed at improving SC ability (Roane et al., 2016), have been applied successfully. Physical exercise interventions (being low-cost, easy-to-implement, and acceptable multimodal intervention methods) may be more suitable for children and are extensively used in the improvement of brain cognitive function (Ketcheson et al., 2018; Reinders et al., 2019). Previous studies (Tse, 2020) together with our colleagues study (Cai et al., 2020) have indicated that autism receiving various exercise interventions have different degrees of improvement in SC-related outcomes. Furthermore, for preschool children with a critical brain development period, early intervention may play a particular role in facilitating the development of the nervous system, including the formation of synapses and myelination (Khundrakpam et al., 2016). In addition, a select study suggested that the earlier age of starting intervention was a statistically significant predictor of better developmental functioning and/or diagnostic status outcome in children with ASD (Towle et al., 2020). Hence, in the current context, we have implemented the 12W-MBTP, a multipath and multimodal theoretical exercise program specially designed for children with ASD, to improve the SC deficits via improving the neurophysiological state and elevating the ability of scenarios interactions.

Of note, although a deficit called "social communication" is considered one of the most universal and specific characteristics of autism in the DSM-5 diagnostic criteria (Tager-Flusberg, 2010), there is a lack of universal consensus about the underlying neural mechanism of social disorders in ASD (Venkataraman et al., 2015). This uncertainty is due to the etiological complexity and phenotypic heterogeneity as well as environmental assimilation effects of ASD. One theory (Gallagher and Frith, 2003) attempts to explain the social disorder of ASD, that is, theory of Mind (ToM) that relates to the ability of individuals to predict the behavior of others on the basis of their own mental states (goals, feelings and beliefs) and enables the identification of others' intentions, emotions, and self-awareness. Perhaps it is not surprising that the DMN, a core brain system for processing information about the "self" and "other" (Mars et al., 2012; Molnar-Szakacs and Uddin, 2013), has been linked to both social cognition and the mentalizing process. Analysis of the extant literature in neurotypical individuals reveals that the core nodes in DMN involved in the ToM process [e.g., medial prefrontal cortex (mPFC), posterior cingulate cortex (PCC), bilateral temporoparietal junction (TPJ)] (Hyatt et al., 2015) play distinct and interacting roles in both the monitoring of the psychological state of self and the evaluation of others (Padmanabhan et al., 2017).

Synthesizing the previous literature, a universal consensus has recently been reached that the abnormal functional connectivity between the core regions of the DMN contribute to the SC impairments in ASD (Yerys et al., 2015; Padmanabhan et al., 2017). Furthermore, there is convergent evidence that SC impairments of ASD reflect a pattern of dual impairment, that is hyper/hypo-connectivity, which results in reduced specialization, flexibility, and processing efficiency of social network (e.g., DMN) (Uddin et al., 2013).

Overall, research increasingly indicates that, as a core network for mentalizing process (Buckner et al., 2008), the atypical connectivity between key regions of the DMN (Molnar-Szakacs and Uddin, 2013) affects both social network integration and differentiation (Yerys et al., 2015), resulting in deficits in SC of ASD (Molnar-Szakacs and Uddin, 2013). Unfortunately, the nature, directional and extent of this atypical functional connectivity remain sharply debated (Uddin et al., 2015), leading to difficulties in accurately understanding the potential mechanisms and intervention targets of ASD social disorders. Moreover, to the best of our knowledge, research has rarely explored the underlying neurophysiological mechanism in ASD after an intervention. Therefore, to examine the directionality and nature of the specific influence of one region on another region, we established a DCM (Friston et al., 2003) for resting-state functional magnetic resonance imaging (fMRI) cross-spectral densities (Friston et al., 2014) among different brain regions of preschoolers with ASD based on the results of ICA. Finally, applying Pearson correlation analysis to ask whether any directed connections are related to symptom severity to establish the symptom-based validation. Compared with other methods of causality analysis, the DCM not only is more suited to disclose the causality and directed nature of the coupling between intrinsic modes of brain activity but also can effectively avoid the suspicious error caused by "lag-based" causality in Granger causality analysis (Webb et al., 2013; Zhou et al., 2018).

This original research aims to investigate the underlying neural mechanisms of exercise interventions in enhancing social communication (SC) skills for preschoolers with autism by using the spectra DCM. Therefore, we hypothesized the exercise-induced effects may alter the imbalance of local excitatory/inhibitory in the DMN feedback loop, improving the influence of secondary pathological processes caused by chronically elevated metabolic stress and relieving the symptoms of social deficits in preschoolers with ASD.

MATERIALS AND METHODS

Study Design

This research design with a two-factor (time and group) repeated measurements was conducted between October and

December 2018 in Yangzhou, China. The current research is mainly aimed at preschool (3–6 years old) with ASD to reduce the deviation caused by age development (Vander Wyk et al., 2014). Research of young children is also particularly critical for developing neuroimaging biomarkers of the disorder (Padmanabhan et al., 2017).

Participants Selection

All participants were outpatients diagnosed by pediatricians in a tertiary hospital based on the Diagnostic and Statistical Manual of Mental Disorders 5th-edition (DSM-5) and assisted by Childhood Autism Rating Scale (CARS) (Schopler et al., 1980). Potential participants were excluded if they met any of the following criteria: (1) involvement in a structured exercise program in the past 6 months; (2) a history of substance abuse or dependence in the last 6 months; (3) co-morbid psychiatric or neurological disorders; (4) visual or auditory impairments; (5) intracranial lesions that affect image analysis; (6) exercise contraindications in medical rehabilitation; (7) contraindications for magnetic resonance imaging (MRI) scanning.

Ninety-four participants were recruited from Chuying Child Development Center and Starssailor Education Institution (Yangzhou, China). Of the initially diagnosed ASD preschoolers, participants who did not meet study criteria (n = 36) or declined to participate in this study (n = 18) were excluded so that 40 participants were finally eligible and randomly equally distributed into two groups by using simple randomization with a random number table: the experimental group (n = 20) and the control group (n = 20). Of note, participants from the two different places and their parents had no prior social interactions with one another to minimize differential expectancies. Because some subjects were unable to complete the MRI scans post-intervention (n = 6), and inferior images were not allowed for subsequent analysis (n = 3). The final data analysis: a total of 31 participants between the experimental group (n = 17) and the control group (n = 14). Before the research, explain the research including contents and precautions in detail to the parents or guardians of the participants and obtain written authorization. The study was approved by the Ethics and Human Protection Committee of the Affiliated Hospital of Yangzhou University, and complied with the ethical standards of the Helsinki declaration. Meanwhile, this study retrospectively registered with the Chinese Clinical Trial Registry (ChiCTR1900024973) on August 05, 2019.

Behavioral Measurements

Apart from gathering information about demographics (age, sex, and body mass index) at baseline, the CARS and clinical assessment report were used to evaluate the symptom severity of ASD. The CARS is a behavior rating scale consisting of 15 items for assisting in ASD diagnosis and determine the severity. The SRS-2 (Constantino et al., 2003) is a reliable and valid 65-item teacher or parent questionnaire used to measure social disorder symptoms as they occur in natural social settings for ASD. In this work, the SRS-2 scale was filled out by parents according to the specific performance of the participants in daily life. Of note, the questionnaire must be filled in by the same person before and after intervention.

Mini-Basketball Training Program

Besides routine behavior rehabilitation training according to the standard rehabilitation program set by the institutions, the experimental groups also received an additional 12W-MBTP, while controls maintained a consistent lifestyle and did not participate in other sports-related activities.

The MBTP was conducted by two certified physical educators, adopting the mode of combining the basic movements of a mini-basketball with sports games to design projects of varying degrees of difficulty and a collective teaching model to facilitate social interactions and communications among participants while parents were strongly inspired to accompany them throughout the curriculum. The 12W-MBTP contents (40 min × 5 sessions per week × 12 weeks, fixed time, location, and physical educators) can be simply summarized as a three-phase stage, with four small parts for each course including first 2 min classroom routine preparation, then 8 min warm-up activities, followed by a 25 min mini-basketball training program, and finally 5 min cool-down activities. More details of the intervention program, please refer to the articles published by our colleagues (Cai et al., 2020) and **Supplementary Tables 1, 2**. After the first phase of 12W-MBTP, participants established interest in training through some simple mini-basketball games. Although some ASD individuals lacked sufficient interest or motivation for further training, they could still complete the entire training process with the help of parents and physical educators. The average heart rate during the intervention was monitored (MD = 136.97, SD = 7.45) using a heart rate meter (POLAR M430) to keep the activity at a moderate intensity. Most importantly, assessments were performed after each course, and participants were removed from the study if they asked for leave for more than 2 days consecutively or more than 7 days cumulatively.

Magnetic Resonance Imaging Acquisition Protocol

The participants were deprived of sleep with the consent of the family members or guardians and sedation with 10% chloral hydrate, a safe way for children was administered before the scan to avoid excessive head motion (Doria et al., 2010; Nordahl et al., 2016). Two groups were both scanned within 3 days before and after the intervention and were completed with the company of a guardian. If the participants began to wake up, MRI acquisition was paused.

Neuroimaging data were acquired using a 3.0 T GE scanner (GE Discovery MR750w 3.0 T, Chicago, United States) located in the Affiliated Hospital of Yangzhou University. Functional images were collected in 28 axial slices using echo-planar imaging (EPI) with T2* weighted contrast sequence sensitive to blood-oxygen-level-dependent (BOLD) contrast [voxel size: 3.5 mm × 3.5 mm × 4 mm; repetition time (TR) = 2000 ms, echo time (TE) = 30 ms; flip angle (FA) = 90°, matrix = 64 × 64, field of view (FoV) = 256 mm × 256 mm; slice thickness = 4 mm; slice gap = 1 mm]. Each fMRI session lasted 8 min and thus contained 240 volumes. High-resolution T1-weighted structural images were acquired in a sagittal orientation using a three-dimensional magnetization-prepared

rapid acquisition with gradient-echo (MPRAGE) sequence (voxel size: 1 mm × 1 mm × 1 mm, no gap, TR/TE = 7.2/3.1 ms; FA = 12°; FoV = 256 mm × 256 mm; 166 slices).

Functional Magnetic Resonance Imaging Data Processing

Conventional functional imaging preprocessing was performed using Statistical Parametric Mapping software (SPM12 version 7771) implemented in MATLAB 2013b (MathWorks, Inc., Natick, MA, United States). The initial ten volumes of each dataset were discarded, then the remaining images were corrected for differences in slice time. Motion correction was performed by aligning each participant's time-series to the mean image, and 6 motion parameters were calculated during realignment. Participants' data were excluded if movement in the translational or rotational planes exceeded 2.5 mm or 2.5°, respectively. Functional images were registered to the standard MRI template, unbiased age-specific structural brain atlases specially provided for the Chinese pediatric population (Zhao et al., 2019), with specific operations to be processed using advanced normalization tools (ANTs) in individual subject space and then resampled to 3 mm × 3 mm × 3 mm. Resultant images were smoothed with a Gaussian kernel full width at a half-maximum of 6 mm and high-pass filtered (128 s, ∼0.008 Hz) to remove low-frequency drifts and Higher frequencies (>0.1 Hz) were not removed, since they are known to contain meaningful information in resting-state studies (Csukly et al., 2020). These images were then used as an input for group ICA.

Selection of Regions of Interest: Independent Component Analysis

To identify regions for subsequent DCM, ICA was performed by the Group ICA of fMRI Toolbox (GIFT). First, the optimal number of components was estimated to generate 33 independent spatial maps by using a minimum description length approach. Meanwhile, the corresponding components for each subject were calculated via a back-reconstruction step. After visually inspecting all highly relevant components, we determined the peak coordinates of each region of interest in the group level while excluding all components related to head motion, physiological noise, or cerebrospinal fluid fluctuations. Finally, subject-specific coordinates were identified as the peaks in subject-specific ICA maps within 8 mm of the group-level coordinates (Zhou et al., 2018).

General Linear Model for Time-Series Extraction

To control motion and physiologic noise effects and correct for the influence of interindividual differences, a general linear model (GLM) containing a discrete cosine basis set and the nuisance regressors that included 6 head motion parameters and WM and CSF signals (Almgren et al., 2020), age, sex and handedness was used to model the resting-state data. The mean time series over the regions identified in ICA were extracted with the use of the principal eigenvariates (principal components) of voxels within 8 mm of the subject-specific coordinates adjusted for the confounding regressors.

Effective Connectivity: Spectral Dynamic Causal Modeling With Parametric Empirical Bayesian

Generally, DCM analysis requires the specification of a model space (Friston et al., 2014). Due to the rarity of research on resting state in autism under intervention, we adopted an approach starting with a fully connected model. This means that all 4 identified ROIs based on ICA were connected, generating a total of 16 connectivity parameters, including inhibition of self-connections. We take advantage of the latest developments in the use of spectral DCM to model endogenous activities in the framework of parametric empirical Bayesian (PEB) (Friston et al., 2016) analysis to inform whether increases or decreases in extrinsic and intrinsic connections were present within each region at the group level. After the model specification, the full model was estimated and inverted for each subject with a hierarchical empirical Bayesian inversion, which allowed the variability in an individual subject's connection strengths to influence the second-level analysis, thereby eliminating the between-subject degree of variability. As opposed to classical inference such as ANOVA, PEB analysis takes not only the means but also the uncertainty of individual connection strengths into account, which means that participants with more uncertain parameter estimates will be down-weighted, while participants with more precise estimates receive greater influence.

After performing the first-level analysis, we created a second-level analysis by specifying a design matrix with four regressors, including the effect of time interacting with the group that best described the effects of the intervention of the 12W-MBTP.

To finally utilize the model, we used Bayesian model reduction (BMR) (Friston et al., 2016), an automatic search method to quickly prune parameters from the second-level PEB model that do not contribute to the model evidence, to infer connections best describing the interaction effect (group × time). Bayesian model averaging (BMA) was performed for the PEB models after obtaining the probability for all possible PEB models (nested and full) separately from BMR and weighted by their model evidence rather than choosing one final model. The parameters best describing interaction effects are reported not as p-values but instead in terms of posterior probability values (PPs), so connections that survived a non-zero criterion with posterior probability PPs > 0.95 are considered significant.

Correlation Analysis

Finally, we asked whether any directed connections are related to symptom severity to establish the symptom-based validation. Following our hypothesis, we then analyzed the correlation between the changes in EC and SRS-2 scores. The alterations here refer to post-intervention minus the baseline level unless otherwise specified. The analysis of the correlation was limited to the final model following second-level analysis by PEB. If a correlation was found between them, then the correlations

TABLE 1 | Demographic and clinical characteristics of the participants.

Characteristics	Control group	Experimental group	*p*-value
Number	14	17	NaN
Sex (M/F)	13/2	15/2	1.000
Age (years)	4.75 ± 0.62	4.89 ± 0.80	0.520
BMI (height/weight^2)	15.96 ± 1.85	15.68 ± 1.10	0.609
CARS (baseline)	40.50 ± 4.65	39.76 ± 6.38	0.722
SRS-2 *T*-score (baseline)	86.07 ± 20.55	93.94 ± 28.56	0.396

BMI, body mass index; CARS, Childhood Autism Rating Scale; M/F, male/female; SRS-2, Social Responsiveness Scale – Second Edition.

between the connectivity strength and the items of the given SRS-2 sub-score were also analyzed.

Statistical Analysis

The demographic analysis were performed using SPSS Version 21.0 (IBM, Armonk, NY, United States), with two-tailed independent sample *t*-tests for continuous variables and χ^2 tests for categorical variables. We conducted two-factor (time and group) repeated measurements ANOVA analysis to evaluate the positive role of the 12W-MBTP in SC on preschoolers with ASD. Once we found significant group × time interaction effects, *post hoc* tests were performed. We calculated the mean and standard deviations of all variables and adopted the traditional cutoff of $p < 0.05$ to determine significance. The Bonferroni correction for multiple testing was applied in Pearson correlation analysis, resulting in a corrected α-value of (0.05/5 = 0.01).

RESULTS

Demographic Analysis

Demographic characteristics including sex, age, and body mass index do not differ significantly between the two groups. CARS and SRS-2 scores at baseline revealed no significant differences across groups, with $p = 0.722$ and $p = 0.396$, respectively (see **Table 1** for more details).

Social Communication Performance

For the SRS-2 total score (as shown in **Figure 1** and **Table 2**), not surprisingly, a group × time interaction effect was observed [$F_{(1,29)} = 8.785$, $p = 0.006$]. Follow-up simple effect analysis indicated that the SRS-2 total score of the experimental group at the post-test were significantly lower relative to baseline [$F_{(1,29)} = 4.586$, $p = 0.041$], whereas such a positive effect was not found in the control group [$F_{(1,29)} = 4.240$, $p = 0.049$]. Notably, a greater SRS-2 total score indicates worse SC performance.

Group × time interaction effect was only observed in the three subscales (social cognition, social communication, and autistic mannerisms: $F_{(1,29)} = 8.258$, 9.537, 4.993, $p = 0.008$, 0.004, 0.033, respectively]. Follow-up simple effect analysis results for the subscales of SRS-2 as follows: (1) the pre-test social cognition score was significantly higher than that at post-test in the experimental group [$F_{(1,29)} = 7.206$, $p = 0.012$], where no significant change from the post to pre-test was observed in the control group; (2) the post-test social communication score was significantly lower than that at baseline in the experimental group [$F_{(1,29)} = 4.715$, $p = 0.038$], whereas a higher score was observed in the control group from the post-test to pre-test [$F_{(1,29)} = 4.839$, $p = 0.036$; higher scores indicate severe symptoms]; (3) there was no significant difference between baseline and post-test in the experimental group [$F_{(1,29)} = 0.659$, $p > 0.05$] in terms of autistic mannerisms, whereas the post-test score was significantly higher than that at baseline in the control group [$F_{(1,29)} = 5.202$, $p = 0.03$]. All the above simple effect analysis uses Bonferroni correction to correct multiple comparisons.

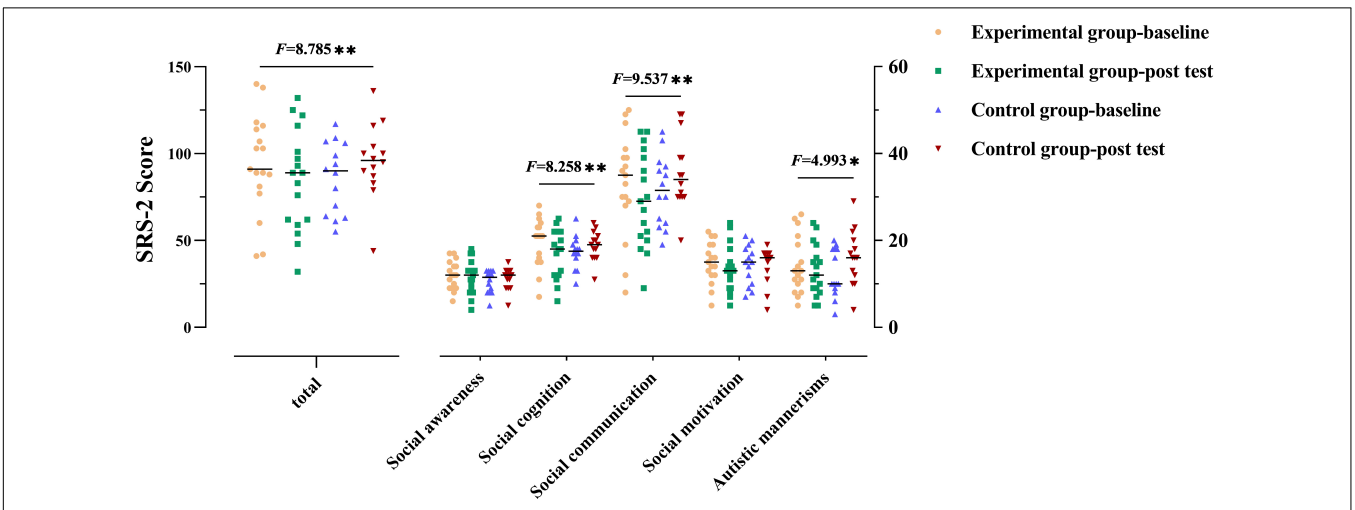

FIGURE 1 | Social communication performance of the two groups. The performance for social communication symptoms of the participants in two groups before and after the intervention. The higher the score, the more severe the deficits in social communication. Numbers presented are *F* statistics representing interaction effects of groups over time. * and ** means $p < 0.05$ and $p < 0.01$, respectively.

TABLE 2 | Analysis of two groups for social communication variables (mean ± standard deviation).

Characteristics	Control group (n = 15)		Experimental group (n = 17)		F
	Baseline	Post-test	Baseline	Post-test	
SRS-2 *T* score	86.07 ± 20.548	95.86 ± 21.343	93.94 ± 28.575	84.71 ± 29.146	8.785**
Social awareness	10.50 ± 2.534	11.14 ± 2.476	12.00 ± 3.240	11.65 ± 3.904	1.461
Social cognition	17.29 ± 3.688	18.79 ± 3.378	19.71 ± 5.520	17.18 ± 5.670	8.258**
Social communication	31.43 ± 8.055	35.50 ± 8.364	33.06 ± 11.798	29.41 ± 10.932	9.537**
Social motivation	14.36 ± 4.551	14.29 ± 4.232	14.94 ± 4.723	13.41 ± 5.363	0.972
Autistic mannerisms	12.50 ± 5.488	16.14 ± 6.298	14.24 ± 6.524	13.06 ± 5.910	4.993*

*F statistics representing tests of interaction effect of group by time. ** and * means $p < 0.01$, $p < 0.05$, respectively.*

The Dynamic Causal Modeling Analysis and Effective Connectivity of Default Mode Network

The ICA was successfully selected four ROIs: mPFC (3,49,0), PCC (0,−52,23), LTPJ (−48,−63,10), and RTPJ (50,−55,11) in the DMN that specifically engaged by in the ToM process, which were selected as nodes for subsequent DCM.

The EC parameters of each specific effect obtained following group-level analysis were listed in **Table 3**. Please refer to **Supplementary Table 3** for the specific connection strength of each connection of participants before and after the exercise intervention. Note that self-connection is the log of scaling parameters that multiply up or down the default value −0.5 Hz such that the positive self-connection values represent increased self-inhibition relative to the prior (Zeidman et al., 2019).

For the effect of group × time interaction, parameters best describing the effect included the inhibitory directional connections mediated by PCC between bilateral TPJ (from left to right). Besides, the positive self-connection values observed in mPFC that showed no overall mean effect (PPs = 0) were instead the best discriminative parameters of an interaction effect, which can be explained as the size of the positive effect means an increase in self-inhibition (all PPs > 0.95, **Figure 2**).

TABLE 3 | Strength of effective connectivity in each specific effect.

Source	Target	Strength	PPs
Effect of the interacions			
mPFC	mPFC	0.138	1
mPFC	RTPJ	0.042	0.6406
PCC	RTPJ	−0.051	1
LTPJ	PCC	−0.066	1
Effect of the group			
mPFC	mPFC	0.212	1
mPFC	PCC	−0.177	1
mPFC	RTPJ	0.036	0.5782
PCC	LTPJ	−0.077	1
RTPJ	mPFC	−0.149	1
RTPJ	LTPJ	−0.034	0.5438
RTPJ	RTPJ	0.067	0.6775
Effect of the time			
mPFC	PCC	−0.141	1
mPFC	LTPJ	−0.11	1
mPFC	RTPJ	−0.069	1
PCC	RTPJ	−0.028	0.616
LTPJ	PCC	−0.073	1
RTPJ	mPFC	0.05	0.6658
RTPJ	PCC	0.14	1
RTPJ	LTPJ	−0.048	0.6734

The self-connection with the same source and target is the log of scaling parameters that multiply up or down the default value −0.5Hz. PPs, posterior probability values.

Correlations Between Effective Connectivity and SRS-2 Scores

As illustrated in **Figure 3**, for the experimental group, the changes in the strength of the self-connection in mPFC correlated significantly with a change in the SRS-2 total score (Pearson $r = -0.6549$, $n = 17$, $p = 0.0043$). To further explore these results, the correlation between each of the five sub-items of the SRS-2 and self-connectivity in the mPFC was analyzed. Changes in EC of mPFC correlated significantly with the changes in social cognition and social communication sub-scores (Pearson $r = -0.7418$ and -0.7250, respectively; $n = 17$; $p = 0.0007$ and 0.0010, respectively), while the correlation with autistic mannerisms (Pearson $r = -0.5750$, $n = 17$, $p = 0.0158$) sub-score did not survive the correction for multiple tests. Unquestionably, there was no significant correlation between changes of EC strength (in the final model) and SRS-2 total scores in the control group, p value was 0.3011, 0.6348, and 0.5344, respectively.

DISCUSSION

Since the absence of assessment about the influence of one region to another region in functional connectivity analysis, it is impossible to reasonably explain the information exchange between brain regions in the context of physical exercise intervention. Therefore, this research aims to investigate the underlying neural mechanisms of exercise interventions in enhancing social communication (SC) skills for preschoolers with autism by using the spectra DCM in the framework of Parametric Empirical Bayesian to characterize the longitudinal changes in intrinsic and extrinsic effective connectivity in DMN core regions. Following the exercise intervention, we discovered noteworthy inhibitory directed connections in the default

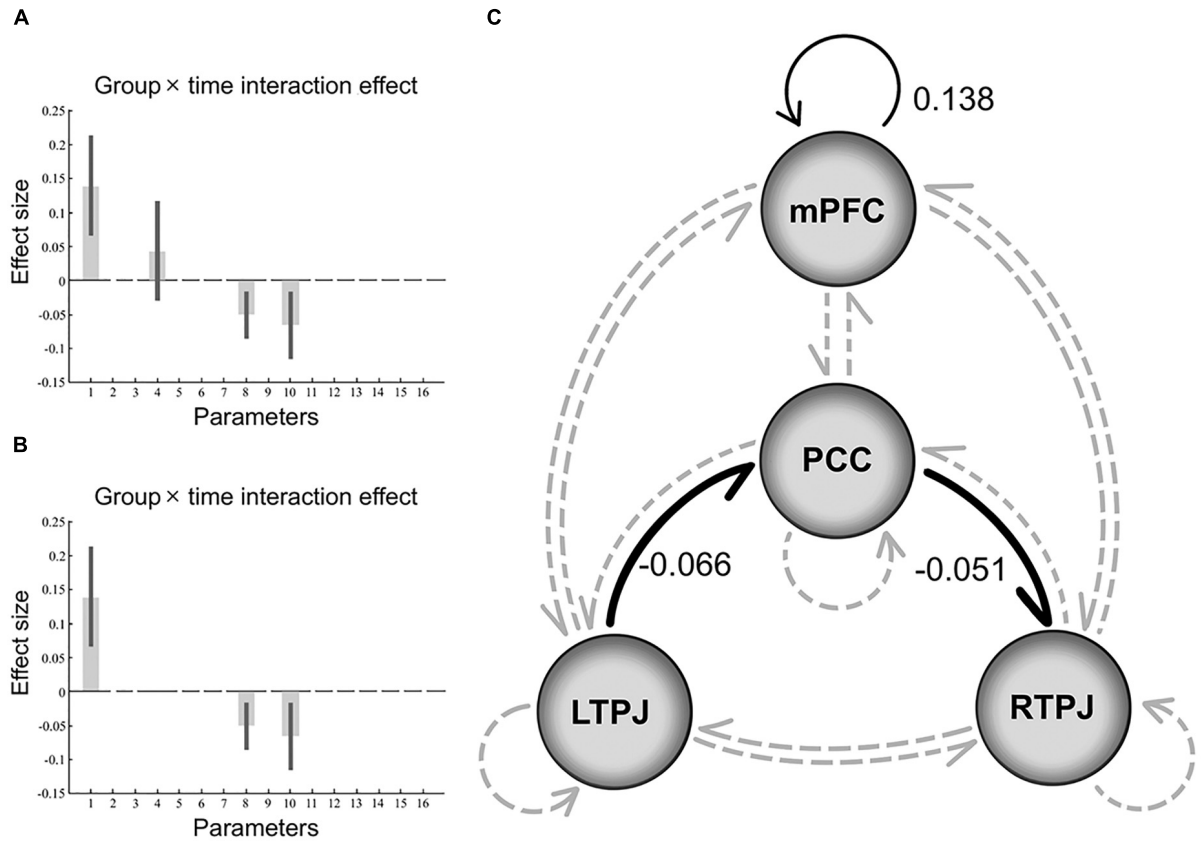

FIGURE 2 | Results of the dynamic causal modeling in the group × time interaction. Panels **(A,B)** shows the effect size of each parameter in the final model that represents the interaction effect, after Bayesian model averaging, the corresponding DCM parameters with no threshold (PPs ≥ 0, graph **A**), and parameters that survived a non-zero criterion with a PPs > 0.95 (graph **B**). The black error bars are 90% credible intervals derived from the posterior variance of each parameter. Panel **(C)** depicts a schematic diagram representing the final model that includes significant negative (inhibitory) extrinsic between-regions connections in bold black line and positive intrinsic self-connections in thin black line, while non-significant connections in gray dotted line. Note that the self-connection parameter is the log of scaling parameters with no units that scale up or down the default value –0.5 Hz. So, such the positive self-connectivity values indicate increased self-inhibition than the default. PCC, posterior cingulate cortex; mPFC, medial prefrontal cortex; TPJ, temporoparietal junction.

mode network in the experimental group, which predicted an improvement in SC skills.

Differently, previous research focused more on assessing the changes in the effective connectivity of ASD during various task states about psychology, but rarely assessed the impact of endogenous neuron fluctuations under the resting state. However, earlier effective connectivity study by multivariate Granger causality analysis with blind deconvolution have also indicated the impaired mPFC pathway and its association with SC deficits in children with ASD, which is consistent with our findings using resting state data (Li et al., 2021).

The mPFC, as the core region of the social brain network (Bzdok et al., 2013), is engaged in inferring others' mental states, and has consistently been shown to have an abnormal overgrowth in young children with ASD from about the age of 2–5 years (Carper and Courchesne, 2005; Libero et al., 2019), with its dysfunction being closely related to the SC deficits in autism (Padmanabhan et al., 2017). Interestingly, concerning the abnormal activation of mPFC and hyper-connectivity of the intrinsic or task-related between mPFC and other regions, previous reports (Smith et al., 2013; White et al., 2014) assumed that such aberrant results may reflect a compensatory response (thus not necessarily representing an improvement of the performance of ToM) or misuse of cognitive reserve. This abnormal activation or hyper-connectivity may even be a state of underlying neuropathology or overload, which may be indicative of impending cognitive decline.

Therefore, the final model of interaction effect found strong evidence of greater self-inhibition in mPFC, indicating a decrease in the gain or excitability of this structure (Zeidman et al., 2019). Similarly, in the final model of group and time effect, mPFC also exhibited an inhibitory effect on other ROIs. To our knowledge, the DCM parameters about intrinsic self-connections lend self-inhibitory properties to regions to preclude any run-away excitation (Csukly et al., 2020), as possibly mediated by increase in postsynaptic inhibition. Specifically, it may cause mPFC to enhance the inhibition of output activity transmitted to others, which leads to changes in the connectivity of the brain circuits.

Interestingly, Prat et al. (2016) found that the non-selective replication of signals in the basal ganglia of individuals with ASD

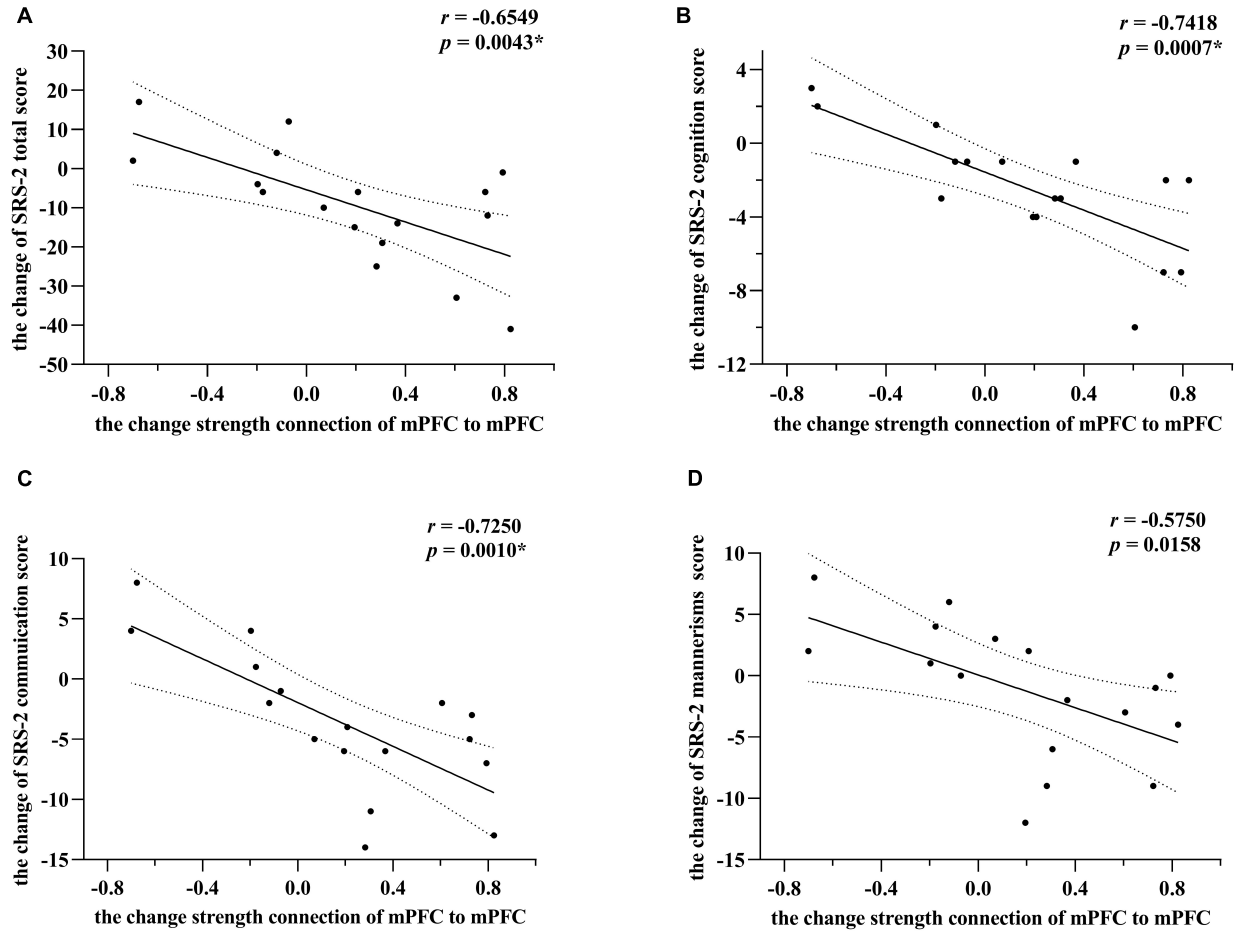

FIGURE 3 | Correlations between changes of effective connectivity and SRS-2 scores in the experimental group. Panel **(A)** indicates alterations of SRS-2 total scores correlate strongest with changes in self-connection in mPFC. The alterations here mainly refer to post-intervention minus the baseline level. *Post hoc* tests showed that among SRS-2 items, social cognition and social communication scores alterations correlated significantly with changes in self-connection of mPFC in panels **(B,C)**, respectively. Panel **(D)** showed that the correlation between the alterations of autistic mannerisms scores and changes in self-connection of mPFC did not survive the correction for multiple tests. However, * means passed the multiple comparison correction. Linear regression lines and 95% confidence intervals were obtained with linear models, and statistical results are based on Pearson correlation analysis. All the results point toward that the increasing inhibitory self-connection in mPFC can predict improvement of social communications for preschool with ASD.

resulting in the information (including some that is irrelevant to the task at hand) overload of the prefrontal cortex. So, such an increase in mPFC self-connection inhibition as mediated by increasing postsynaptic inhibition in our results predicates the improvement of SC.

Furthermore, our findings are also consistent with a prominent neurobiological theory that an excitatory/inhibitory (E/I) imbalance (Rubenstein and Merzenich, 2003; Vattikuti and Chow, 2010; Yizhar et al., 2011) in local neural circuits alters local and global brain signals. These abnormal patterns are critically likely to affect brain function, especially if they occur in highly interconnected hubs, such as mPFC (Padmanabhan et al., 2017), leading to an obstacle in social cognition. A rodent study showed that the elevated *E*/*I* ratio in mPFC is related to an SC impaired, and this effect can be improved by increasing the inhibitory function (Yizhar et al., 2011; Zhang et al., 2020). Taken together, similar to our results, enhancing the self-inhibition of mPFC after the 12W-MBTP intervention exerts a positive effect on the SC of preschoolers with ASD.

From the significant negative correlations between changes in EC and SRS-2 score (as illustrated in **Figure 3**), greater mPFC self-inhibition changes (including the conversion from disinhibition to inhibition, the decrease of disinhibition and the increase of inhibition) indicated more extreme significant improvement in SC. This exciting result also confirms our previous conjectures and provides preliminary evidence for possible neural correlates of SC. That is, as a potential target for physical exercise intervention, the increased mPFC self-connection inhibition can predict better improvement of SC.

Another finding supporting the theory is that in the final model of interaction effect, the inhibitory directional connection from left TPJ to right TPJ was mediated by PCC. This result may seem a counterintuitive finding at first, and it does reflect profound and complex changes in these brain regions and

underscores their importance for restoring and maintaining SC among preschoolers with ASD. Recently a new perspective proposes that there may be atypical maturational trajectories in ASD (Nomi and Uddin, 2015; Uddin et al., 2015; Muller and Fishman, 2018), that is, early hyperconnectivity followed by decreased connectivity in adulthood (Assaf et al., 2010), which may be caused by the imbalance of *E*/*I* ratio during the development of neural systems. One likely reason is the gene-, receptor-, and enzyme-level deficits in inhibitory signaling pathways involving gamma-aminobutyric acid (GABA) (Baroncelli et al., 2011; Pizzarelli and Cherubini, 2011). Not surprisingly, enhance GABAergic signaling can bridge the gaps and obtain better social cognition (Han et al., 2014; Zhang et al., 2020).

Taken together, we speculate that the inhibitory information transmission between bilateral TPJ caused by exercise intervention altered the imbalance of local E/I, improving the influence of secondary pathological processes caused by chronically elevated metabolic stress (Hillary and Grafman, 2017). Such a shocking effect can rehabilitate the homeostatic aspects of the DMN on the nervous system through increasing postsynaptic inhibition (Laughlin and Sejnowski, 2003), predicting the improvement in SC skills for autism.

Concerning the high-level cluster in the social brain, most core regions (left TPJ instead of right) exhibited a left-favored lateralization pattern of functional connectivity (Tomasi and Volkow, 2012; Alcala-Lopez et al., 2018). Unsurprisingly, autism exhibits remarkably reduced left lateralization in connections involving regions from the DMN essential for SC and maybe predicting more severe SC deficits (Nielsen et al., 2014). Combined with results of the overall mean effect, the left-sided brain function of preschoolers with ASD regained the dominant position in the process of social cognition and undoubtedly showed better SC symptoms after the 12W-MBTP intervention.

Behaviorally, the classical role of PCC is to quickly switch between different cognitive processes to coordinating external and internal-oriented cognition (Leech et al., 2012). After the 12W-MBTP, the similar regulatory effect of PCC, namely mediates the inhibitory information transmission, changed the status of so-called network isolation (Uddin et al., 2015), which is also reflected in the drops in SRS-2 scores of experimental group participants.

We presume the impact of intervention is primarily because of the exercise-induced neuroplasticity caused by the multipath effect. Evidence-based investigations have confirmed continuous moderate-intensity aerobic exercise will increase basal peripheral brain-derived neurotrophic factor (BDNF) concentrations (Knaepen et al., 2010). BDNF is generally thought to be a key neurotrophin in supporting neuroplasticity. Not only was neuromotor activity significantly improved, but the release of endogenous neurotrophins was also related to the improvement of social cognition (Baker et al., 2010). Apart from that, the exercise-induced cholinergic effects may increase cerebral perfusion, possibly affecting the neurodynamics of the BOLD fMRI signal (Smith et al., 2010) that may reflect altered brain connections and improved network efficiency.

Limitations

There are a few limitations of our work must be taken into consideration and addressed by future studies. The lack of ADOS or ADI-R assessment might also be a potential limitation of the study. For preschoolers with autism, it is difficult to successfully recruit more participants on the premise of successful completion of the entire intervention process and two MRI scans. On the other hand, given the male preponderance in the prevalence of autism (Maenner et al., 2020), the profiles of SC impairments in ASD likely differ between sex (Muller and Fishman, 2018). Although there is a good sex matching between groups in our research, the existence of a high male/female ratio limits the applicability of our results to all populations. Larger scale studies, especially with the same proportion of males to females, are required to replicate and validate these findings. Furthermore, analyzing the EC of complex network model (the triple network model) (Menon, 2011) interaction not only is a top priority but may also provide a brand-new perspective for understanding the potential neuropathological mechanism of patients with ASD in SC deficits.

CONCLUSION

Taken together, this study provides sufficient evidence that exercise intervention can improve SC skills for preschoolers with ASD. Such inspiring results also emphasize the importance of early and timely intervention for the neurodevelopmental of ASD and improvement of core symptoms and quality of life. By applying spDCM in the framework of PEB to characterize the longitudinal changes in intrinsic and extrinsic effective connections between the DMN, we conclude that the inhibitory information transmission in DMN may be an underlying neurophysiological mechanism for improving SC behaviors among preschoolers with ASD who delay or stunt maturational processes. Finally, these findings provide further factual evidence that the directional effectivity connection between key regions of the DMN has become one of the well-validated targets for exercise intervention to improve SC behaviors, which may be a reliable neuroimaging biomarker or endophenotype in subsequent studies.

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 studies involving human participants were reviewed and approved by The Ethics and Human Protection Committee of the Affiliated Hospital of Yangzhou University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

HY and WW drafted the manuscript, performed all administrative tasks required for submission, and contributed to the identification of research topics and preparation of study design. HY and HQ took part in planning, supervision, brainstorming the manuscript, designed the study, and contributed to the statistical analysis and interpretation of results. HY, AC, YD, and ZL designed the intervention method and guided the intervention process. All authors critically revised the manuscript and approved the final version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.796437/full#supplementary-material

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frontiers in Neuroscience

MINI REVIEW
published: 17 January 2022
doi: 10.3389/fnins.2021.786220

The Autism Phenome Project: Toward Identifying Clinically Meaningful Subgroups of Autism

Christine Wu Nordahl[1,2], Derek Sayre Andrews[1,2], Patrick Dwyer[3,4], Einat Waizbard-Bartov[1,2], Bibiana Restrepo[1,5], Joshua K. Lee[1,2], Brianna Heath[1,2], Clifford Saron[1,3], Susan M. Rivera[1,3,4], Marjorie Solomon[1,2], Paul Ashwood[1,6] and David G. Amaral[1,2]*

[1] MIND Institute, University of California, Davis, Davis, CA, United States, [2] Department of Psychiatry and Behavioral Sciences, School of Medicine, University of California, Davis, Davis, CA, United States, [3] Center for Mind and Brain, University of California, Davis, Davis, CA, United States, [4] Department of Psychology, University of California, Davis, Davis, CA, United States, [5] Department of Pediatrics, School of Medicine, University of California, Davis, Davis, CA, United States, [6] Department of Medical Microbiology and Immunology, School of Medicine, University of California, Davis, Davis, CA, United States

OPEN ACCESS

Edited by:
Eugenia Conti,
Stella Maris Foundation, Scientific Institute for Research, Hospitalization and Healthcare (IRCCS), Italy

Reviewed by:
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Commonwealth Scientific and Industrial Research Organization (CSIRO), Australia
Valentina Riva,
Eugenio Medea, Scientific Institute for Research, Hospitalization and Healthcare (IRCCS), Italy

***Correspondence:**
Christine Wu Nordahl
cnordahl@ucdavis.edu

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: 29 September 2021
Accepted: 20 December 2021
Published: 17 January 2022

Citation:
Nordahl CW, Andrews DS, Dwyer P, Waizbard-Bartov E, Restrepo B, Lee JK, Heath B, Saron C, Rivera SM, Solomon M, Ashwood P and Amaral DG (2022) The Autism Phenome Project: Toward Identifying Clinically Meaningful Subgroups of Autism. Front. Neurosci. 15:786220. doi: 10.3389/fnins.2021.786220

One of the most universally accepted facts about autism is that it is heterogenous. Individuals diagnosed with autism spectrum disorder have a wide range of behavioral presentations and a variety of co-occurring medical and mental health conditions. The identification of more homogenous subgroups is likely to lead to a better understanding of etiologies as well as more targeted interventions and treatments. In 2006, we initiated the UC Davis MIND Institute Autism Phenome Project (APP) with the overarching goal of identifying clinically meaningful subtypes of autism. This ongoing longitudinal multidisciplinary study now includes over 400 children and involves comprehensive medical, behavioral, and neuroimaging assessments from early childhood through adolescence (2–19 years of age). We have employed several strategies to identify sub-populations within autistic individuals: subgrouping by neural, biological, behavioral or clinical characteristics as well as by developmental trajectories. In this Mini Review, we summarize findings to date from the APP cohort and describe progress made toward identifying meaningful subgroups of autism.

Keywords: autism, MRI, heterogeneity, immune, development, gastrointestinal, ERP, females

INTRODUCTION

Autistic individuals present with a broad continuum of social communication difficulties as well as non-social characteristics such as repetitive behaviors, intense focused interests and sensory experiences. Co-occurring medical, developmental, and mental health conditions are common (American Psychiatric Association, 2013; Soke et al., 2018). This heterogeneous presentation has led to inconsistency in research findings and challenges with identifying etiological causes and optimal treatments or interventions. One approach to constraining heterogeneity is to restrict research samples (e.g., all male or IQ cut offs). However, this has led to underrepresentation of certain portions of the autism spectrum, including autistic females (Lai et al., 2015) and individuals with intellectual disability (Russell et al., 2019), thus limiting generalizability of findings.

Another approach to addressing heterogeneity is to conduct comprehensive evaluations of all autistic individuals and then stratify based on one or more salient characteristics. These subgroups can then be evaluated further for shared etiology and mechanistic underpinnings. The ultimate goal is to decrease variability in treatment response among autistic individuals by identifying individualized interventions specific to the phenotypic and biological commonalities of given subgroups. Here, we summarize findings from the Autism Phenome Project (APP), a large, longitudinal, multidisciplinary study that has utilized this approach to identify autism subgroups at the behavioral, neural, and biological levels.

The APP was initiated in 2006 to integrate behavioral, neuroimaging, and other biological and medical data in a large cohort of autistic children with the overarching goal of identifying clinically meaningful subgroups that share common biological or behavioral features. Children with autism are enrolled at 2–3.5 years. Age and sex-matched non-autistic children with no developmental delay are enrolled as typically developing (TD) controls. Thus far, APP participants have been followed for 4 time points through early and middle childhood, with the oldest participants currently returning for a fifth time point during adolescence. Initial recruitment was conducted from 2006 to 2011 with over 300 children enrolled during that timeframe, which clarifies that enrollment did not stop at 300 participants: enrollment of new participants is ongoing.

Diagnostic confirmation for autism is conducted by licensed psychologists using the Autism Diagnostic Observation Schedule (ADOS) (Lord et al., 2000, 2012) and Autism Diagnostic Interview -Revised (Lord et al., 1994). TD control participants are screened for autism using the Social Communication Questionnaire (Rutter et al., 2003) and developmental delay using the Mullen Scales of Early Learning (MSEL) (Mullen, 1995). Initial recruitment reflected the male predominance of autism diagnoses, and about 4–5 times more boys than girls were enrolled.

To increase female representation within the APP cohort, we initiated the Girls with Autism—Imaging of Neurodevelopment (GAIN) study in 2014. The study protocol is identical to the APP, and all participants in the GAIN study are automatically included in the APP dataset. Thus far, GAIN participants have completed three early childhood time points and are scheduled to return for a 4th middle childhood timepoint. With almost 100 autistic females in the cohort, evaluation of similarities and differences across sexes are now possible and included in all analyses.

Participant demographics across all timepoints are summarized in **Figure 1**. Importantly, the cohort includes children with all levels of intellectual functioning, including 30% of participants with IQs in the range of intellectual disability at the middle childhood time point (9–12 years of age). **Figure 2** depicts the longitudinal assessment battery, which includes comprehensive behavioral assessments, medical exams, and magnetic resonance imaging (MRI). Auditory event related potentials (ERPs) were conducted at Time 1. Medical exams are conducted by developmental pediatricians and include assessment of pubertal status at later time points and characterization of gastrointestinal symptoms and family histories of autoimmune conditions. Blood specimens from the child and biological parents are used to evaluate immune function and genomic sequencing. MRIs are conducted during natural nocturnal sleep for the first three time points (Nordahl et al., 2008), resulting in inclusion of children with all levels of developmental ability. To ensure that all could be followed longitudinally, we developed strategies to acquire MRI scans in children with intellectual disability while awake at the older time points (Nordahl et al., 2016).

Below, we highlight efforts from 30 published studies utilizing this comprehensive, multidisciplinary dataset to identify subgroups based on neural, other biological, behavioral/clinical characteristics, and developmental trajectories. In many instances, subgroups ascertained on the basis of one characteristic (e.g., neural) are cross validated using characteristics from other disciplines (e.g., behavioral) to identify multidisciplinary commonalities and increase clinical significance.

SUBGROUPING BY NEURAL CHARACTERISTICS

Brain Volume

Because of multiple reports of early brain enlargement (Courchesne et al., 2001; Sparks et al., 2002; Hazlett et al., 2005), we were initially surprised by the degree of overlap in brain volume between autistic children and controls. It was clear early on that not all autistic children have enlarged brains and that group mean differences were driven by a small subset of autistic children with cerebral volumes outside the range of their age-matched TD peers. We began evaluating clinical characteristics of this subset of children with larger brain volumes (i.e., megalencephaly) and found that 22% of males who had a regressive onset of autism had megalencephaly compared to only 5% of males without regression (Nordahl et al., 2011).

Subsequent efforts to define this subgroup accounted for height in order to distinguish brain enlargement from generalized somatic overgrowth (Klein et al., 2013; Campbell et al., 2014). Autism with disproportionate megalencephaly (ASD-DM) was defined as a ratio of cerebral volume to height greater than 1.5 standard deviations above age- and sex-matched TD controls. In the APP, 12.6% of autistic boys and 6% of autistic girls were characterized as ASD-DM at Time 1. Clinical characteristics of this subgroup include lower language ability at age 3 and higher rates of intellectual disability at age 6 (Amaral et al., 2017). Further, ASD-DM is not simply a uniformly bigger brain, but rather has distinct regional expansion of surface area (Ohta et al., 2016) and gyrification patterns (Libero et al., 2019) that differ from autistic children without DM. The rate of cerebral gray and white matter growth in ASD-DM does not differ from other autistic children, and cerebral volume remains elevated in this subgroup throughout early and middle childhood (Libero et al., 2016; Lee et al., 2021). The ASD-DM subgroup also has differentially altered auditory ERP responses, exhibiting a different pattern of loudness-dependent electrophysiological responses than other autistic children (De Meo-Monteil et al., 2019).

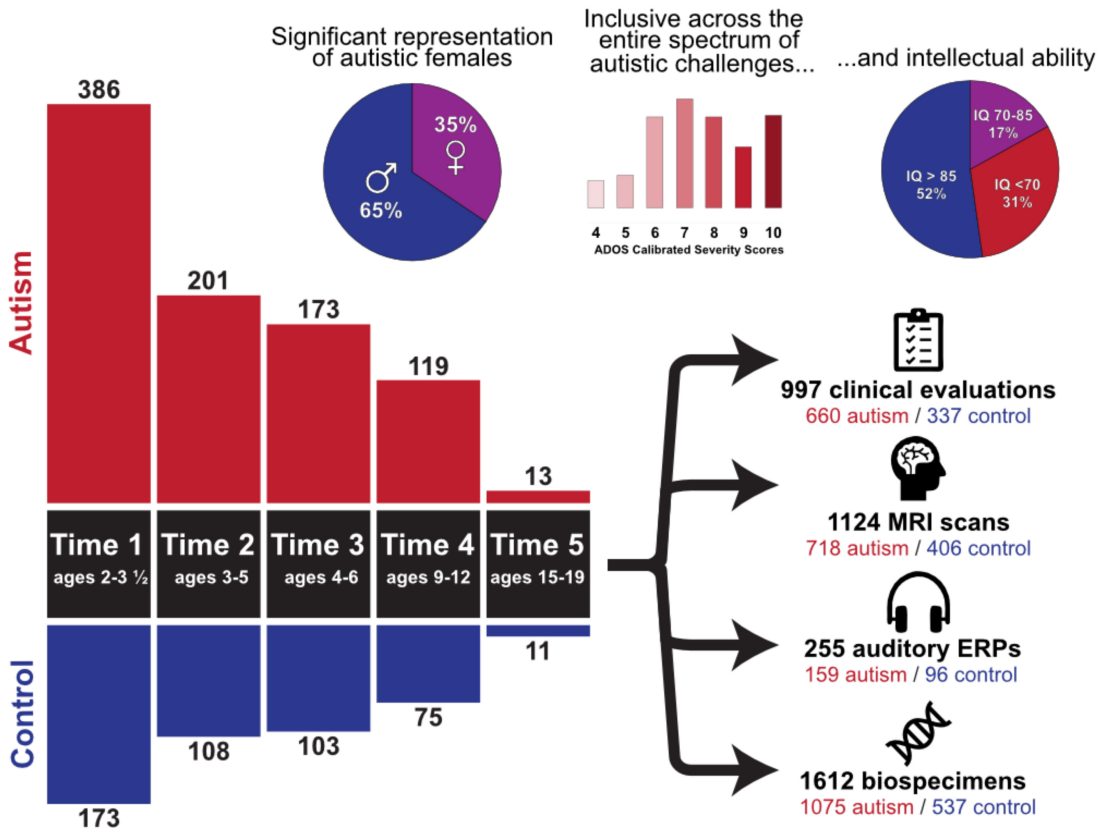

FIGURE 1 | Overview of the Autism Phenome Project cohort. Longitudinal behavioral, neuroimaging, and medical data has been acquired since 2006 in over 500 children from early childhood through adolescence. The cohort includes children across the entire autism spectrum, including understudied groups such as females and children with co-occurring intellectual disability. Data collection at all-time points is ongoing. ADOS Calibrated Severity Scores at Time 1 and IQ scores at Time 4 are depicted.

Extra-Axial Cerebrospinal Fluid

The role of cerebrospinal fluid (CSF) in neurodevelopment and disease is an area of increased focus (Lehtinen et al., 2013; Shen, 2018). Elevated extra-axial CSF, sometimes referred to as external or communicating hydrocephalus, is characterized by excessive CSF in the subarachnoid space between the brain and dura mater. Although commonly considered benign in children under 2 years, recent evidence from two independent infant sibling cohorts suggests that elevated extra-axial CSF during infancy is associated with increased likelihood for autism diagnosis at age 3 (Shen et al., 2013, 2017). In the APP cohort at Time 1, 13% of autistic children had elevated levels extra-axial CSF (Shen et al., 2018). This subset also had increased sleep problems compared to autistic children without elevated extra-axial CSF.

Auditory Event-Related Potentials

Prior studies have examined whether, on average, autistic and TD groups differ in latencies and amplitudes of auditory ERPs (reviewed by Williams et al., 2021). However, few have examined whether different sub-populations within autism show differential auditory responses cf. (Salmond et al., 2007; De Meo-Monteil et al., 2019; Roberts et al., 2019). In the APP, autistic participants exhibit—at the group mean level—diminished amplitudes of the N2, a negative-going cortical response to auditory stimuli over frontocentral channels ∼200–350 ms post-stimulus, at Time 1 (Dwyer et al., 2021a), consistent with prior studies (Williams et al., 2021). However, examination of inter-individual differences in ERP morphologies suggests this pattern is driven by a subset of participants with atypical positive-going ERP responses over the spatiotemporal window associated with the N2 (Dwyer et al., 2021c). Although the behavioral implications of this ERP positivity are unclear, this finding illustrates how group averages can distort and occlude patterns at the individual and subgroup levels.

We have also examined how ERPs in autism are affected by differences in auditory stimulus intensity. We clustered autistic and TD participants based on the relative strengths of ERP global field power responses to 50 through 80 dB tones (Dwyer et al., 2020). Autistic participants with disproportionately strong responses to loud (80 dB) tones were reported by caregivers to struggle with auditory distractibility, which we interpret as a neurophysiologic reflection of hyperacusis or noise distress. Intriguingly, relative to TD controls, a disproportionate number of autistic participants with higher cognitive ability scores than other autistic participants formed part of another

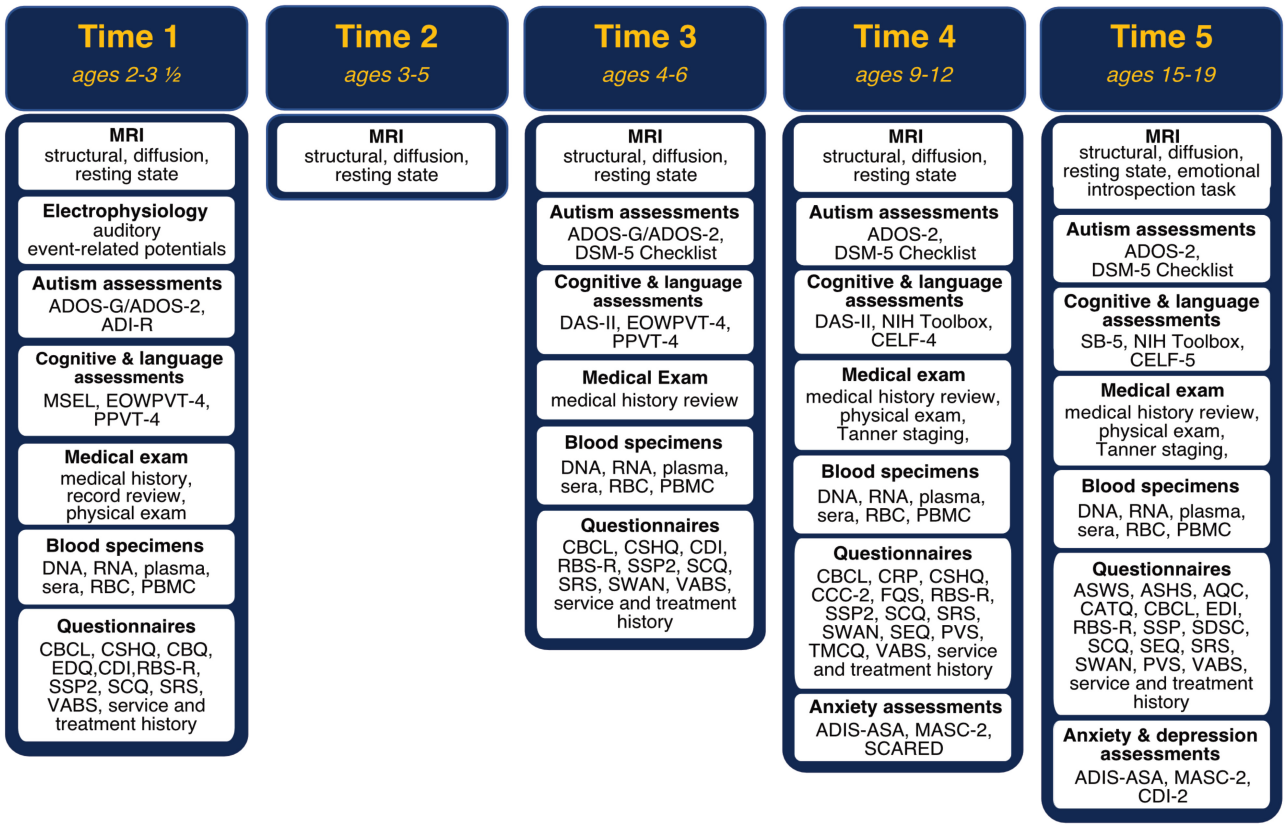

FIGURE 2 | Autism Phenome Project longitudinal study design and assessment battery at each time point from early childhood through adolescence. ADI-R, Autism Diagnostic Interview; ADIS-ASA, Anxiety Disorders Interview Schedule with Autism Addendum; ADOS, Autism Diagnostic Observation Schedule; AQC, Alexithymia Questionnaire for Children; ASWS, Adolescent Sleep Wake Scale; ASHS, Adolescent Sleep Hygiene Scale; CATQ, Camouflaging Autistic Traits Questionnaire; CBCL, Child Behavior Checklist; CBQ, Children's Behavior Questionnaire; CCC-2, Children's Communication Checklist; CDI-2, Child Depression Inventory; CDI, MacArthur-Bates Communicative Development Inventories; CELF, Clinical Evaluation of Language Fundamentals; CRP, Child Rearing Practices; CSHQ, Children Sleep Habits; DAS-II, Differential Ability Scale—2nd Edition; EDI, Emotional Dysregulation Inventory; EDQ, Early Development Questionnaire; EOWPVT, Expressive One Word Picture Vocabulary Test; FQS, Friendship Quality Scale; MASC-2, Multidimensional Anxiety Scale for Children; MSEL, Mullen Scales of Early Learning; PBMC, peripheral blood mononuclear cells; PPVT, Peabody Picture Vocabulary Test; PVS, Self-Perception Profile for Children and Peer Victimization Scales; RBC, red blood cells; RBS-R, Repetitive Behavior Scale—Revised; SB-5, Stanford Binet—Fifth Edition; SCARED, Screen for Child Anxiety Related Disorders; SCQ, Social Communication Questionnaire; SDSC, Sleep Disturbances Scale for Children; SEQ, Sensory Experiences Questionnaire; SRS, Social Responsiveness Scale; SSP2, Short Sensory Profile 2; SWAN, Strengths and Weaknesses of Attention-Deficit/Hyperactivity-symptoms and Normal-behaviors; TMCQ, Temperament in Middle Childhood; VABS, Vineland Adaptive Behavior Scale.

cluster characterized by gradual increases in response amplitude from 50 to 70 dB.

SUBGROUPING BY OTHER BIOLOGICAL CHARACTERISTICS

Biological Sex

Biological sex is an important source of variability in autism. As a first step, we have evaluated autistic females as a biologically defined subgroup based on sex assigned at birth. We recognize, however, that autistic females do not represent a singular, distinct phenotype of autism. Additional studies are needed to explore heterogeneity within autistic females and gender diverse populations.

Thus far, we have identified sex differences in diffusion-weighted properties and in the organization of the corpus callosum (Nordahl et al., 2015; Andrews et al., 2019). At Time 1, autistic females had a smaller callosal region projecting to anterior frontal regions compared to TD females. In contrast, autistic males had a smaller callosal region projecting to orbitofrontal cortex than TD males (Nordahl et al., 2015). More globally, autistic females exhibited a slower rate of cerebral gray and white matter growth across early childhood relative to sex-matched controls, but no difference in overall cerebral volume at any time point (Lee et al., 2021). In contrast, autistic males did not differ in rate of cerebral growth from TD male controls, but they had larger cerebral brain volumes across early childhood than TD males, an effect driven by the subgroup with disproportionate megalencephaly, which is much less common in autistic females.

Behaviorally, autistic males and females in the APP do not differ on measures of core autistic traits or developmental ability at Time 1. Autistic females were, however, more highly represented in a subgroup with clinically significant levels of co-occurring mental health symptoms (40% of females compared to 22% of autistic males) (Nordahl et al., 2020). Moreover, amygdala volume was associated with internalizing and externalizing problems in autistic girls, but not boys, suggesting sex differences in the role of the amygdala in autism. Sex differences in amygdala functional connectivity are also apparent during early childhood (Lee et al., 2020).

Immune Factors

Evidence supporting dysregulation of the immune system in autism includes a higher prevalence of familial autoimmunity, gestational immune influences, as well as altered innate and adaptive immune responses in some autistic individuals (reviewed in Hughes et al., 2018). We have investigated several immune-related factors in the APP, including evaluation of specific immune cell types and response to immunological stressors. In one study, peripheral blood mononuclear cells from Time 1 plasma samples were stimulated with the bacteria product lipopolysaccharide (LPS). A subset of 44% of autistic children had an increased pro-inflammatory profile that was associated with lower developmental scores and increased sleep problems and aggression (Careaga et al., 2017b). Altered cell signaling in autistic children was also related to immune activation and repetitive behaviors (Onore et al., 2017). In a related study of the innate immune system, which includes cell types that respond to LPS, the frequency of myeloid dendritic cells was increased by 25% in autistic children and associated with gastrointestinal problems, repetitive behaviors, and amygdala volumes (Breece et al., 2013).

We also investigated levels of cell adhesion molecules that immune cells use to tether to endothelial cells before gaining access to tissue, including brain parenchyma. There were decreased levels of platelet endothelial adhesion molecule-1 (PECAM-1) in plasma from autistic children. In support of the notion that altered trafficking of immune cells may have implications in brain homeostasis, PECAM-1 levels were positively correlated with head circumference in TD controls, but not in autistic children (Onore et al., 2012).

Maternal immune factors have also been linked to neurodevelopmental disabilities (Careaga et al., 2017a). In the APP, 8% of autistic children were born to mothers with a specific set of maternal IgG autoantibodies that bind to fetal brain tissue, compared to none of the TD controls. This subset also exhibited 12% larger brain volume than controls and 7% larger brain volume than other autistic children (Nordahl et al., 2013). More recently we found that maternal immune conditions such as autoimmunity, asthma and allergies that occurred during pregnancy were predictors of externalizing behaviors in autistic children (Patel et al., 2020). Maternal asthma was the most commonly reported condition and was twice as common in mothers of autistic males (20%) than autistic females (11%).

Gastrointestinal Symptoms

Gastrointestinal (GI) concerns are frequently reported by parents of autistic children and may be related to immune dysregulation (Buie et al., 2010). This is particularly concerning because it may be more challenging for autistic children to verbalize or communicate physical pain, leading to lack of appropriate medical care. Within the APP, we evaluated parent-reported GI symptoms at Time 1 and identified a subgroup comprising 48% of autistic children who experience significant GI problems (Restrepo et al., 2020). Children with significant GI problems also had higher levels of self-injurious behaviors, restricted stereotyped behaviors, sensory sensitivities, aggressive behavior, attention problems, as well as sleep problems such as shorter sleep duration, night awakenings, and parasomnia.

SUBGROUPING BY BEHAVIORAL AND CLINICAL CHARACTERISTICS

Anxiety

In the APP, children are assessed for clinical anxiety disorders at middle childhood (Time 4) and adolescence (Time 5) using the Anxiety Disorders Interview Schedule-IV-Parent Interview (ADIS) (Albano and Silverman, 1996) to identify traditional DSM forms of anxiety including generalized anxiety disorder (GAD), separation anxiety, specific phobia, and social phobia. The ADIS Autism Spectrum Addendum (ADIS-ASA) (Kerns et al., 2017) is administered to identify anxieties distinctly related to autism, including idiosyncratic fears, fear relating to social confusion, intense interest fears, and fears of change. At Time 4, 69% of autistic children were diagnosed with clinically significant anxiety, with 21% having a DSM anxiety disorder, 17% an ADIS-ASA distinct anxiety disorder, and 31% both (Kerns et al., 2020). Differences in the rates of DSM-anxiety presentations in autistic children with and without intellectual ability were also noted. Autistic children with intellectual disability predominately endorsed specific phobias while other DSM anxieties were less common compared to autistic children without intellectual disability.

Language

While some autistic individuals learn and use language consistent with their chronological age, others experience delayed or impaired language development. At Time 1, we grouped autistic children based on language ability and examined associations with white matter development (Naigles et al., 2017). Autistic children were grouped into a low (48%; language beginning or not yet begun), middle (21%; language included a stable lexicon of nouns), or high (31%; language included a large vocabulary plus some grammar) groups. Subgroup differences were identified in the left and right inferior longitudinal fasciculus (ILF), left superior longitudinal fasciculus, and the left corticospinal tract. In particular, fractional anisotropy in the occipital region of the ILF was correlated with language ability, but not ADOS severity scores. Other efforts utilizing the APP have identified subgroups based interactions between social communication and language

development (Blume et al., 2021) and grammatical language ability (Wittke et al., 2017).

SUBGROUPING BY DEVELOPMENTAL TRAJECTORIES

Cognitive Development

Intellectual functioning is one of the most heterogeneous aspects of autism (Maenner, 2020). In the APP, we identified subgroups based on the trajectory of intellectual functioning across early childhood (Times 1–3) (Solomon et al., 2018). Four distinct trajectories were identified: two groups, comprising 26 and 18% of the sample, respectively, had IQs in the intellectual disability range at both time points; a third group (22%) had IQs in the normal range at both time points. Of particular interest, a fourth group, comprising 35% of the cohort, initially had IQs in the intellectual disability range but made significant gains (34 points) to have IQs in the normal range by age 6–7.

Sensory Behaviors

Atypical sensory experiences and behaviors have been reported in 82–97% of autistic people (Dellapiazza et al., 2018) and are related to diminished quality of life (Lin and Huang, 2019; McConachie et al., 2020). In the APP, the Short Sensory Profile (SSP; McIntosh et al., 1999) was used to examine sensory behaviors at Times 1 and 3. Almost two-thirds of autistic participants showed a stable intense sensory phenotype characterized by high levels of atypical sensory behavior at both time points; these participants also had elevated anxiety levels (Dwyer et al., 2020). Another third of autistic participants and almost all TD participants exhibited milder, more typical sensory behaviors at both time points.

Another study examined how different SSP subscales (from the factor solution of Williams et al., 2018) contribute to overall SSP trajectories in autism and typical development (Dwyer et al., 2021b). Almost 28% of autistic participants showed disproportionately high levels of low energy/weakness, most likely reflecting hypotonia. Interestingly, these participants had higher cognitive ability scores at Time 1 relative to other autistic participants, which suggests that hypotonia might be developmentally protective in autism. Around 13% of autistic participants showed intensely atypical sensory behaviors across all subscales of the SSP as well as ERP hyper-responsivity to loud sounds. Autistic participants in these hypotonic and generalized-intense subgroups had more anxiety and sleep disturbances than the remaining subgroup that exhibited less intense sensory behaviors.

Autism Characteristics

Recent evidence suggests that the intensity or degree of autistic characteristics can vary over time (Gotham et al., 2012). Using change in ADOS calibrated severity scores (CSS) from Time 1 to Time 3, we found that 54% of APP participants had stable autism characteristics while 29% significantly decreased and 17% increased in ADOS-CSS scores over this period. Change groups did not differ by initial ADOS-CSS or hours of intervention received. However, the group with decreasing ADOS-CSS had higher IQ scores and were more likely to be female (Waizbard-Bartov et al., 2021). Follow up MRI analyses between these groups identified that individuals with increasing degree of autism characteristics had slower development of the sagittal stratum fiber bundle (Andrews et al., 2021).

CONCLUSION AND FUTURE DIRECTIONS

The studies described above reveal subpopulations within the broad autism spectrum that are likely obscured when group-level comparisons between autistic and TD control groups are made. Efforts to identify subgroups with more homogenous characteristics provides a deeper characterization of the heterogeneity of autism and co-occurring conditions. Importantly, the identification of subgroups is not meant to divide or marginalize portions of the autism community. Rather, some promising subgrouping efforts may guide clinical care by influencing selection of interventions or access to services and supports. For example, disproportionate megalencephaly at age 3 may provide early clues to parents about children who may require higher levels of support, or individuals with autism distinct forms of co-occurring anxiety may benefit from autism-specific anxiety interventions. In other cases, subgrouping may increase awareness for, and treatment of, debilitating co-occurring medical conditions such as gastrointestinal dysfunction. These subgroups need to be further examined and validated in order to make specific recommendations for clinical care. A recent review of subtyping efforts in autism research provides a checklist for validating subtypes that will be useful for future studies (Agelink van Rentergem et al., 2021).

To fully achieve the goals of the APP, future studies will require cohorts representative of all autistic individuals, including all cognitive abilities, speaking and non-speaking individuals, all racial and ethnic groups, and increased representation of female and gender diverse individuals. The current APP sample size may be an adequate starting point for subgroup identification, but much larger sample sizes are necessary. Developmental considerations are also key, as the trajectory-based subgroups suggest that evaluating individuals at a single time point may not be sufficient. Longitudinal lifespan studies are necessary to identify subgroups that could determine early predictors of later outcomes. Environmental factors, such as individual and sociodemographic variables should also be considered (Modabbernia et al., 2017). Ultimately, the goal of identifying sources of heterogeneity is to increase understanding of the underlying causes of autism and to improve the quality of life for autistic individuals and their families.

AUTHOR CONTRIBUTIONS

CWN, DSA, JL, MS, SR, and DGA contributed to the conception of the review. CWN wrote the first draft of the manuscript. DSA, PD, and PA wrote sections of the manuscript. BH and

DSA conceptualized and designed the illustrations. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was supported by the National Institute of Mental Health (Grant Nos. R01MH104438 to CWN, R01MH103284 to MS, and R01MH103371 to DGA), UC Davis MIND Institute Autism Center of Excellence (Grant No. P50HD093079 to DGA), and the MIND Institute Autism Research Training Program (Grant No. T32MH073124 to DSA). JL was also supported by the Health Resources and Services Administration (HRSA) of the United States Department of Health and Human Services (HHS) under the Autism Intervention Research Network on Physical Health (AIR−P) (Grant No. UT2MC39440). The information, content and/or conclusions are those of the authors and should not be construed as the official position or policy of, nor should any endorsements be inferred by HRSA, HHS or the United States Government.

ACKNOWLEDGMENTS

We wish to thank the families and children who participated in the APP and GAIN studies as well as all members of the research study staff.

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Conflict of Interest: DGA was on the Scientific Advisory Boards of Stemina Biomarkers Discovery, Inc. and Axial Therapeutics.

The remaining 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.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 31 January 2022
doi: 10.3389/fnins.2021.768219

Alterations of Prefrontal-Posterior Information Processing Patterns in Autism Spectrum Disorders

*Hai-Chen Zhao†, Rui Lv†, Guang-Yu Zhang†, Le-Min He, Xiao-Tao Cai, Qiang Sun, Chun-Yan Yan, Xiang-Yuan Bao, Xin-Yue Lv and Bin Fu**

Department of Radiology, Shandong First Medical University & Shandong Academy of Medical Sciences, Tai'an, China

Autism spectrum disorder (ASD) is a heterogeneous disorder characterized by different levels of repetitive and stereotypic behavior as well as deficits in social interaction and communication. In this current study, we explored the changes in cerebral neural activities in ASD. The purpose of this study is to investigate whether there exists a dysfunction of interactive information processing between the prefrontal cortex and posterior brain regions in ASD. We investigated the atypical connectivity and information flow between the prefrontal cortex and posterior brain regions in ASD utilizing the entropy connectivity (a kind of directional connectivity) method. Eighty-nine patients with ASD and 94 typical developing (TD) teenagers participated in this study. Two-sample t-tests revealed weakened interactive entropy connectivity between the prefrontal cortex and posterior brain regions. This result indicates that there exists interactive prefrontal-posterior underconnectivity in ASD, and this disorder might lead to less prior knowledge being used and updated. Our proposals highlighted that aforementioned atypical change might accelerate the deoptimization of brain networks in ASD.

Keywords: autism spectrum disorders, entropy connectivity, predictive coding theory, information processing, rest-state fMRI

OPEN ACCESS

Edited by:
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***Correspondence:**
Bin Fu
fubin03@126.com

†These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: *31 August 2021*
Accepted: *27 December 2021*
Published: *31 January 2022*

Citation:
Zhao H-C, Lv R, Zhang G-Y, He L-M, Cai X-T, Sun Q, Yan C-Y, Bao X-Y, Lv X-Y and Fu B (2022) Alterations of Prefrontal-Posterior Information Processing Patterns in Autism Spectrum Disorders. Front. Neurosci. 15:768219. doi: 10.3389/fnins.2021.768219

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder. According to the data from the Centers for Disease Control and Prevention (CDC) reported in 2020, one out of every 54 children is diagnosed with ASD. The main symptoms of ASD are deficits in social interaction and stereotypical or repetitive behavior (Maenner et al., 2020).

Previous studies have found neural underpinnings of ASD to be heterogeneous. Quantifying the size of the brains of autistic patients revealed early gray matter and white matter hyperplasia (Courchesne et al., 2001) but Lee et al. found that autistic children without megalencephaly had rather comparable gray and white matter development to non-autistic children (Lee et al., 2020), which may suggest cortical development as a heterogeneous condition for ASD. Neuroimaging studies found that ASDs presented atypical functional connectivity patterns in brains, such as underconnectivity (Just et al., 2004; Schipul et al., 2011; Starck et al., 2013), overconnectivity (Delmonte et al., 2013; Li et al., 2020; Seghatol-Eslami et al., 2020) and mixed connectivity (Monk et al., 2009; Chen et al., 2018; Oldehinkel et al., 2019). These altered functional connections affect the functions of multisensory, social communication, and high-level cognitive activities. However, it is not entirely clear how abnormal functional connectivity affects the clinical features of ASD. Some researchers have attempted to use directional functional connectivity to explore the

mechanism by which the changes of brain connectivity leading to atypical symptoms. Weaker effective connectivity from the ventral attention network to the salience-executive network in adolescents with IQs in the normal range was also found by using the Granger causality method (Bernas et al., 2018). In addition, previous studies also detected abnormalities within language networks of ASD which depend on directional connectivity pattern from the precuneus *via* caudate nucleus to interior frontal gyrus rather than the connectivity pattern from the interior frontal gyrus via caudate nucleus to the precuneus by using dynamic causal modeling (DCM) (Radulescu et al., 2013) and verified underconnectivity between brain regions of ASD utilizing transfer entropy and graph theory (Ejman et al., 2017).

Some studies have reported atypical alterations in the structural properties of the prefrontal cortex in ASDs, such as increased gray matter volume in the left frontal and right medial prefrontal cortex (Deramus and Kana, 2015), atypical developmental trajectory in the volume of the dorsolateral prefrontal cortex (Carper and Courchesne, 2005), developmental abnormality of minicolumns in the dorsal and orbital frontal cortices (Buxhoeveden et al., 2006), and weakened asymmetries in the cortical thickness and surface area of the medial orbitofrontal cortex (Postema et al., 2019). It is worth noting that the prefrontal lobe plays a key role in ASD (Damasio and Maurer, 1978; Mundy, 2003). ASDs with dysfunction of the prefrontal cortex display abnormalities in some cognitive functions, such as working memory (Koshino et al., 2005; Vogan et al., 2018), cognitive control (Solomon et al., 2014; Lukito et al., 2020), mentalizing (Spengler et al., 2010), effortful control (Krishnamurthy et al., 2020), and self-referential processing (Burrows et al., 2016; Hashimoto et al., 2017). Additionally, ASDs also presented changes in structure and functions in some posterior brain regions including the parietal, occipital, temporal lobes. For instance, some researchers found the atypical changes in the middle temporal gyrus (Pappaianni et al., 2018), fusiform gyrus (Kuno-Fujita et al., 2020), interior parietal lobes (May and Kana, 2020) and sensorimotor cortex (Sapey-Triomphe et al., 2019).

The predictive coding theory suggested that higher hierarchies are involved in the storage and application of the prior knowledge and lower hierarchies are related to the information integration from body and environment. The higher hierarchies initiate modulation signals to actively infer the state of body and world and lower hierarchies also generate feedback input to revise the prior knowledge (Friston, 2010; Smith et al., 2017). This functional interaction is similar to the information communication between the prefrontal cortex and other brain regions. For instance, Miller (2000) and Duncan (2001) had reviewed much evidence that the prefrontal cortex can broadcast the signals from prior knowledge to other neocortical regions (e.g., the inferior temporal cortex and posterior parietal cortex) to modulate the information in them. Furthermore, the ecological model of the prefrontal cortex proposed that the prefrontal cortex implements niche construction through facilitating the construction of rules and norms that guide learning and behavior, and biasing processing in posterior neural regions to align with currently relevant rules and norms. In turn, the posterior brain regions projects the information from body and environment to the prefrontal cortex to drive it to adapt the changes of information sampling (Werchan and Amso, 2017). Underconnectivity between the frontal and posterior regions in ASD has been reported in previous studies (Fulvia et al., 2002; Kana et al., 2009). However, it is unclear whether there exists an alteration of interactive information flow between the prefrontal cortex and posterior brain regions and its potential effect. We hypothesized that the changes of interactive information flow might influence the deoptimization of the autistic brain networks. In this present study, we explored the hypothesis by using entropy connectivity (a kind of directional connectivity, the entropy connectivity between two brain areas reflects the direction of information flow from one brain area to the other). Our objective is to research the changes of the prefrontal-posterior information processing patterns and its potential influences.

Abbreviations: ASD, autism spectrum disorder; ABIDE I, Autism Brain Imaging Data Exchange I; BA, Brodmann area; CDC, Centers for Disease Control and Prevention; DCM, dynamic causal modeling; DLPFC, dorsolateral prefrontal cortex; DPARSF, Data Processing Assistant for Resting-State fMRI; EEG, electroencephalography; fMRI, functional magnetic resonance imaging; IPL, inferior parietal lobe; IQ, intelligence quotient; LPFC, lateral prefrontal cortex; MEG, magnetoencephalography; PFC, prefrontal cortex; PRT, positive reproduce test; TD, typical developing.

EXPERIMENTAL PROCEDURES

Subjects

Eighty-nine patients (age, 15–20 years; mean = 15.1 ± 5.1 years) and ninety-four well-matched teenagers (age, 16–19 years, mean = 16.0 ± 4.2 years) participated in this study. All data of these participants come from the Autism Brain Imaging Data Exchange (ABIDE), including PITT, SDSU, UM, YALE, CMU, NYU, STANFORD, UCLA, CALTECH, USM, and LEUVEN (Di Martino et al., 2014). All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional Ethics Committee of Shandong First Medical University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards (R202104130149).

Functional Magnetic Resonance Imaging Data

Data used in our study come from eleven sites. Full details are shown in **Table 1**. For more information about scanner types and parameters, please visit the website (http://fcon_1000.projects.nitrc.org/indi/abide/abide_I.html).

Firstly, data were preprocessed by using the DPARSF (Processing Assistant for Resting-State fMRI) software (http://preprocessed-connectomes-project.org/abide/Pipelines.html) and this preprocessing steps included dropping first 4 volumes (scrubbing), slice timing, motion realignment, registration [a transform from original to template (MNI152) space was calculated for each dataset from a combination of functional-to-anatomical and anatomical-to-template transforms. The

TABLE 1 | Summary table of the train and test datasets.

Acquisition site	Nr. ASD's	Nr. TD's	Nr. subjects	Age, ASD	Age, TD	Scanner
CALTECH	3	1	4	20.9 ± 1.3	20.8	SIEMENS MAGNETOM TrioTim syngo MR B17
PITT	10	3	13	15.1 ± 4.9	22.2 ± 7.3	SIEMENS MAGNETOM Allegra syngo MR A30
SDSU	8	13	21	14.9 ± 2.0	14.3 ± 1.6	GE 3T MR750
UM	18	15	33	14.1 ± 2.5	14.2 ± 2.7	3 Tesla GE Signa
YALE	8	10	18	12.9 ± 3.0	14.5 ± 1.2	SIEMENS MAGNETOM TrioTim syngo MR B17
CMU	3	0	3	27.0 ± 6.9	0	SIEMENS MAGNETOM Verio syngo MR B17
NYU	9	19	28	19.1 ± 9.5	15.1 ± 5.0	SIEMENS MAGNETOM Allegra syngo MR 2004A
STANFORD	2	0	2	11.4 ± 0.7	0	GE SIGNA 3T
UCLA	28	12	40	13.4 ± 2.6	13.7 ± 1.2	SIEMENS MAGNETOM TrioTim syngo MR B15
USM	0	10	10	0	17.9 ± 3.5	SIEMENS MAGNETOM TrioTim syngo MR B17
LEUVEN	0	11	11	0	22.1 ± 1.8	LEUVEN-1: PHILIPS INTERA 3T

anatomical-to-template transforms were calculated using a two step procedure that involves (one or more) linear transform that is later refined with a very high dimensional non-linear transform], Gaussian smoothing ($6 \times 6 \times 6$ mm), detrend, nuisance regression (including the removal of realignment parameters, mean white matter and cerebrospinal fluid signals), band-pass temporal filtering (0.01–0.1 HZ), Then, all preprocessed fMRI data were further processed by using the virtual digital brain software package VDB1.7 (https://www.nitrc.org/projects/vdb/). The steps are described as follows: (1) calculate causal connectivity between BAs (Brodmann's areas); (2) statistical analysis; (3) result display.

Entropy Connectivity

The Brodmann area (BA) atlas was normalized as a standard MNI brain template. Each BA is regarded as the seed of entropy connectivity. All brain regions in the BA template were selected as seed regions, however, only those that presented significant changes in the prefrontal cortex were analyzed in this study. The index of BA is defined in **Table 2**. Functional connectivity reflects statistical correlations between brain regions. To describe the direction of the functional connectivity, in the present study, we adopted an entropy connectivity method that has been used in a previous study (Zhang et al., 2016). Entropy connectivity between two brain areas reflects the direction of information flow from one brain area to the other. The steps of entropy connectivity are described as follows: (1) obtain the BOLD signal of a BA X and a BA Y from the time series ($t_1 \rightarrow t_n$); (2) obtain the change directions of the BOLD signal of BA X in a certain time interval Δt_1 and BA Y in the next intermittent time interval Δt_2; (3) compare the direction of changes and repeat the second step to obtain the probability of the same and opposite changes, respectively, which were observed throughout the time series; (4) if Bayesian probability acquired in the third step $[P(Y/X)] > 0.5$ simultaneously Pearson correlation coefficient $r > 0$, it is defined the synchronous entropy connectivity from the BA X to BA Y. Similarly, if the BOLD signal changes in the opposite direction and $r < 0$, it is defined as an asynchronous entropy connectivity from the BA Y to BA X.

Entropy connectivity describes the interregional causality and information flow. The entropy connectivity of the synchronous change of BOLD signals in two brain regions is also called synchronous entropy connectivity, which indicates cooperative relation between two brain regions, that is, these two brain areas work with consistent steps. Similarly, the entropy connectivity of the asynchronous change is also called asynchronous entropy connectivity, which reflects inconsistent work pattern between two brain regions, that is, they work with opposite steps. Synchronous output entropy connectivity indicates the change of BOLD signal in one brain will drive that in the other with the same change trend, and asynchronous output entropy connectivity indicates the change of BOLD signal in one brain will drive that in the other with the opposite change trend. Synchronous input entropy connectivity denotes the change of BOLD signal in one brain is driven by that in the other with the same change trend, and asynchronous input entropy connectivity denotes the change of BOLD signal in one brain is driven by that in the other with the opposite change trend. Increased synchronous entropy connectivity indicates enhanced cooperative work pattern between brain regions; decreased synchronous entropy connectivity indicates reduced cooperative work pattern between brain regions. In contrast, increased asynchronous entropy connectivity indicates enhanced opposite work pattern between brain regions; decreased asynchronous entropy connectivity indicates reduced opposite work pattern between brain regions. A detailed description can be found in a study by Zhang et al. (2016).

Positive Reproducible Test

For any given sample size, the probability of false positives and false negatives is a zero-sum game. To obtain a trade-off between the probability of false positives and false negatives, we used the positive reproducible test (PRT) method (Zhang et al., 2021) to correct statistical results. Decreased type I errors will lead to increased type II errors, the PRT method can obtain a trade-off between the false positive and negative probabilities by randomly selecting samples and repeating this test. This method can obtain low false negative probability and few type I errors through selecting high positive reproducible rate in the

TABLE 2 | Indexes and corresponding brain regions.

Indexes	Brodmann area	Corresponding brain regions in AAL template
BA 1L	Left primary somatosensory cortex	Left precentral gyrus
BA 2L	Left primary somatosensory cortex	Left precentral gyrus
BA 2R	Right primary somatosensory cortex	Right precentral gyrus
BA 7L	Left somatosensory association cortex	Left somatosensory association cortex
BA 7R	Right somatosensory association cortex	Right superior parietal lobule
BA 8R	Right dorsal frontal cortex	Right middle frontal gyrus
BA 9L	Left dorsolateral prefrontal cortex	Left middle frontal gyrus/medial superior frontal gyrus
BA 9R	Right dorsolateral prefrontal cortex	Right middle frontal gyrus/medial superior frontal gyrus
BA 10L	Left anterior prefrontal cortex	Left orbital superior frontal gyrus
BA 11L	Left orbitofrontal cortex	Left rectus
BA 17R	Right primary visual cortex	Right calcarine
BA 20R	Right inferior temporal gyrus	Right inferior temporal gyrus
BA 21L	Left middle temporal gyrus	Left middle temporal gyrus
BA 21R	Right middle temporal gyrus	Right middle temporal gyrus
BA 24L	Left ventral anterior cingulate cortex	Left anterior/middle cingulum
BA 24R	Right ventral anterior cingulate cortex	Right anterior/middle cingulum
BA 32L	Left dorsal anterior cingulate cortex	Left anterior cingulum
BA 33L	Left anterior cingulate cortex	N/A
BA 39L	Left angular gyrus	Left angular gyrus
BA 39R	Right angular gyrus	Right angular gyrus
BA 40L	Left supramarginal gyrus	Left supramarginal gyrus
BA 43L	Left subcentral area	Left postcentral gyrus/Rolandic operculum
BA 45L	Left IFC pars triangularis	Left inferior frontal gyrus triangle
BA 46R	Right dorsolateral prefrontal cortex	Right inferior frontal gyrus triangle
BA 47R	Right inferior prefrontal gyrus	Right orbital inferior frontal gyrus

statistical hypothesis testing. In order to illustrate this issue, we performed the comparison between the PRT correction and the uncorrected results for the synchronous entropy connectivity of the whole brain.

Firstly, we executed a statistical test for synchronous entropy connectivity and selected a high probability of false positives ($p = 0.05$), which is responding to a low probability of false negatives, and then performed the steps below. (a) Execute the statistical test 1,000 times with 70 subjects selected randomly in every group under a certain positive reproducible rate. (b) Repeat step (a) 100 times with the positive reproducible rate selected from 1 to 100% with an interval of 1%. (c) Calculate the mean of those p values responding to every positive reproducible rate for every synchronous entropy connectivity. (d) Obtain the number of positive results for every positive reproducible rate and averaged p value. (e) Calculate the probability of false negatives. Let M and m denote the total number of independent experiments and true positive results in the statistical hypothesis testing respectively. m_i and p_i denote the number of positive results and the probability of false positives in the *i-th* test respectively. Then the probability of false negatives $P_{fn}(i)$ is written as $P_{fn}(i) = \frac{[m-m_i\times(1-p_i)]}{M}$, where $i = 1, 2, \cdots, 100$.

Finally, the statistical analysis and comparison between the PRT correction and the uncorrected are performed, and the results are shown in **Figure 1**. In **Figure 1A**, the curves start from the positive reproducibility rate of 41% due to its responding averaged p value is about 0.049 ($p < 0.05$). We analyzed these results and found that the probability of false negatives obtained by PRT correction was lower than that obtained by the uncorrected for the same positive reproducibility rate (i.e., the same probability of false positive) (**Figure 1A**). Furthermore, we also found that the PRT correction presented lower probability of false positive for the same probability of false negative compared with the uncorrected (**Figure 1B**). To further verify the validity of the PRT correction, we studied the effect of this method. As shown in **Table 3**, the PRT correction presented lower false-positive and false-negative probabilities for the same number of positive results compared with the uncorrected.

Our experimental results indicate that higher positive reproducible rate (i.e., "Ri" in the previously published paper (Zhang et al., 2021) means lower probability of false positive rate (i.e., fewer type I errors). In addition, compared with the uncorrected, the PRT correction presented lower false-negative and false-positive probabilities for the same number of positive results.

It is worth noting that selecting higher positive reproducible rate might cause some true positive results to be removed and increase false-negative probabilities.

Statistical Analyses

Age and sex were analyzed using statistical software (SPSS, version 19.0) to examine whether these demographic characteristics were significantly different. A $p < 0.05$ was regarded as a significant difference.

We selected all BAs as seeds of entropy connectivity and investigated the cross-group differences of entropy connectivity. The PRT correction method was used to correct the results of entropy connectivity. Correction parameters are described as follows. Seventy samples were selected randomly in each group, $p < 0.05$, reproducible rate $= 0.85$, repeated number of PRT $= 1000$.

RESULTS

Demographic and Behavioral Data Test

A chi-squared test revealed no significant difference in sex between the ASD and TD groups. A two-sample t-test was performed to examine whether there was a significant difference

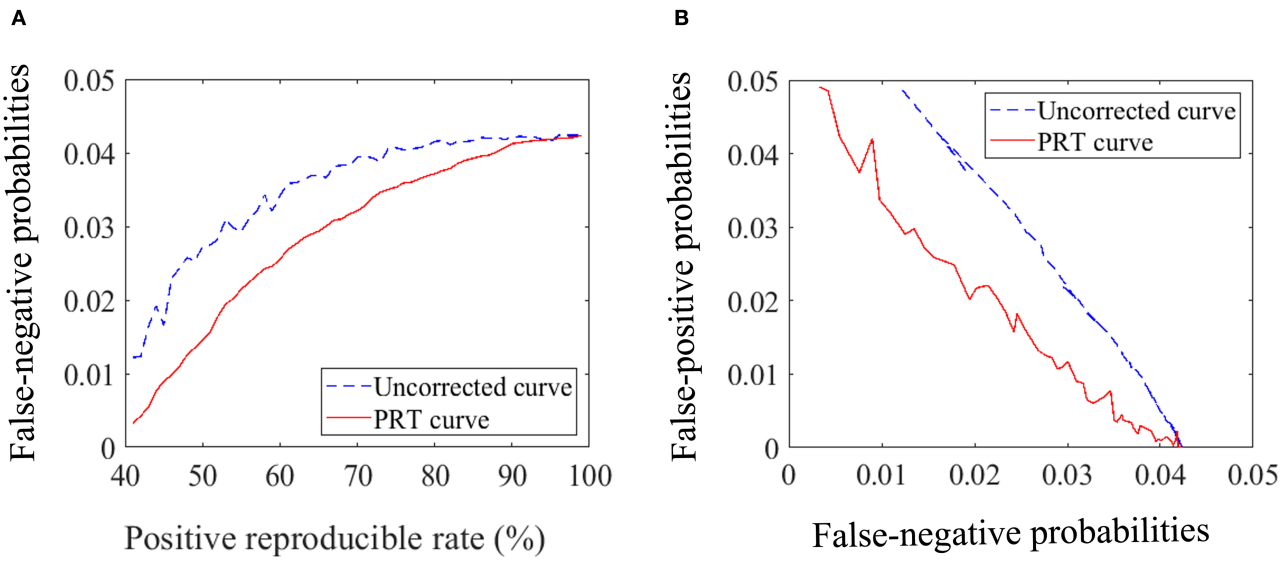

FIGURE 1 | Statistical analysis and comparison. **(A)** Diagram of the relationship between the probability of false negative and the positive reproducible rate. **(B)** Diagram of the relationship between the probability of false positive and negative.

in age across the two groups, and no significant difference was found (**Table 4**).

Cross-Group Differences of Entropy Connectivity From the Prefrontal Cortex to Posterior Brain Regions

The current study also found that ASDs presented weakened synchronous entropy connectivity from the right dorsolateral prefrontal cortex to the right somatosensory association cortex (BA 46 R→ BA 7R) ($t = -3.137608$, $p < 0.05$, PRT corrected; $p < 0.003$, no corrected) (**Figure 2A**); weakened asynchronous entropy connectivity from the right dorsolateral prefrontal cortex to the left primary somatosensory cortex (BA 9R→ BA 2L) ($t = -3.121635$, $p < 0.05$, PRT corrected; $p < 0.003$, no corrected) (**Figure 2B**); weakened asynchronous entropy connectivity from the right dorsolateral prefrontal cortex to the left angular gyrus (BA 46R→ BA 39L) ($t = -3.25226$, $p < 0.05$, PRT corrected; $p < 0.002$, no corrected) (**Figure 2C**); weakened asynchronous entropy connectivity from the right dorsal frontal cortex to the left primary somatosensory cortex (BA 8 R→ BA 1L) ($t = -3.583862$, $p < 0.05$, PRT corrected; $p < 0.0005$, no corrected) (**Figure 2D**).

Cross-Group Differences of Entropy Connectivity From the Posterior Brain Regions to Prefrontal Cortex

Compared with TDs, ASDs represented weakened synchronous entropy connectivity from the left middle temporal gyrus to the left dorsolateral prefrontal cortex (BA 21L→ BA 9L) ($t = -3.406756$, $p < 0.05$, PRT corrected; $p < 0.0009$, no corrected) (**Figure 3A**); weakened synchronous entropy connectivity from the left supramarginal gyrus to the right dorsolateral prefrontal cortex (BA 40L→ BA 46 R) ($t = -3.25226$, $p < 0.05$, PRT corrected; $p < 0.002$, no corrected) (**Figure 3B**); weakened asynchronous connectivity from the right primary somatosensory cortex to the anterior prefrontal cortex (BA 2R→ BA 10L) ($t = -3.80959$, $p < 0.05$, PRT corrected; $p < 0.0003$, no corrected) (**Figure 3C**).

The Differences of Prefrontal-Posterior Connectivity Between ASDs and TDs Within the Male Group

In the male group (45 males with ASD vs. 46 males with TD), the males with ASD presented the weakened synchronous entropy connectivity from the left angular gyrus to the left anterior prefrontal cortex (BA 39L→ BA 10L) ($t = -3.768572$, $p < 0.05$, PRT corrected; $p < 0.0003$, no corrected) (**Figure 4A**); weakened synchronous entropy connectivity from the left dorsolateral prefrontal cortex to the right inferior temporal gyrus (BA 9L→ BA 20R) ($t = -4.194185$, $p < 0.05$, PRT corrected; $p < 0.00008$, no corrected) (**Figure 4B**); weakened synchronous entropy connectivity from the left IFC pars triangularis to the right middle temporal gyrus (BA 45L→ BA 21R) ($t = -3.785974$, $p < 0.05$, PRT corrected; $p < 0.0003$, no corrected) (**Figure 4C**); weakened synchronous entropy connectivity from the right angular gyrus to the left dorsal cingulate cortex (BA 39R→ BA 32L) ($t = -3.81218$, $p < 0.05$, PRT corrected; $p < 0.0003$, no corrected) (**Figure 4D**); weakened synchronous entropy connectivity from the right angular gyrus to the left anterior cingulate cortex ($t = -3.316281$, $p < 0.05$, PRT corrected; $p < 0.002$, no corrected) and weakened synchronous entropy connectivity from the right angular gyrus to the bilateral ventral anterior cingulate cortex (BA 39R→ BA 24L) ($t = -3.649195$, $p < 0.05$, PRT corrected; $p < 0.0008$, no corrected) (BA 39R→ BA 24R) ($t = -3.981538$, $p < 0.05$, PRT corrected;

TABLE 3 | Comparison of statistical analysis results for the uncorrected and PRT correction.

Uncorrected	PRT corrected
(PFP, PFN)	(PFP, PFN)
(0.029055, 0.025729)	(0.015948, 0.025503)
(0.020127, 0.030990723)	(0.009363, 0.030864106)
(0.020432, 0.030994311)	(0.009363, 0.030864106)
(0.012633, 0.035940157)	(0.004472, 0.035885797)
(0.010778, 0.036909172)	(0.003453, 0.036867647)
(0.010877, 0.036909734)	(0.003453, 0.036867647)
(0.006048, 0.039558816)	(0.000804, 0.039543209)
(0.00688, 0.039279796)	(0.001708, 0.039262937)
(0.003484,0.040681022)	(0.001477, 0.040677324)
(0.003536, 0.040681118)	(0.001477, 0.040677324)
(0.002598, 0.041527521)	(0.000802, 0.041525739)
(0.001847, 0.041668237)	(0.000962, 0.041667485)
(0.002573, 0.041527496)	(0.000802, 0.041525739)
(0.002313, 0.041527238)	(0.000802, 0.041525739)
(0.001708, 0.041668119)	(0.000962, 0.041667485)
(0.000804, 0.042092179)	(0.000128, 0.042091891)
(0.001173, 0.041950778)	(0.000061, 0.041950148)
(0.001009, 0.042092266)	(0.000128, 0.042091891)
(0.001477, 0.041809437)	(0.002289, 0.041810012)
(0.000825, 0.042092188)	(0.000128, 0.042091891)
(0.000802, 0.042092178)	(0.000128, 0.042091891)
(0.000962, 0.042092246)	(0.000128, 0.042091891)
(0.002289, 0.041527214)	(0.000802, 0.041525739)
(0.000061, 0.042375292)	(0.000047, 0.04237529)
(0.000128, 0.042375302)	(0.000047, 0.04237529)
(0.000047, 0.04237529)	(0.000047, 0.04237529)

PFP, probability of false positive; PFN, probability of false negative. The table denotes that comparison of PFP and PFN in the uncorrected and PRT correction for the same number of positive results.

$p < 0.0003$, no corrected) (**Figure 4D**); weakened synchronous entropy connectivity from the left orbitofrontal cortex to the right primary visual cortex (BA 11L→ BA 17R) ($t = -3.718285$, $p < 0.05$, PRT corrected; $p < 0.0005$, no corrected) (**Figure 4E**). In addition, there was an atypical connectivity between the right angular gyrus and the left somatosensory association cortex, but it was not the prefrontal-posterior underconnectivity in this study (BA 39R→ BA 7L) ($t = -3.958346$, $p < 0.05$, PRT corrected; $p < 0.0002$, no corrected) (**Figure 4D**).

The Differences of Prefrontal-Posterior Connectivity Between ASDs and TDs Within the Female Group

In the female group (44 females with ASD vs. 48 females with TD), we only found the enhanced synchronous entropy connectivity from the left subcentral area to the right inferior prefrontal gyrus (BA 43L→ BA 47L) ($t = 3.672919$, $p < 0.05$, PRT corrected; $p < 0.008$, no corrected) (**Figure 4F**).

TABLE 4 | Demographic characteristics of participants.

Group	ASD ($n = 89$)	TD ($n = 94$)	Statistics (df)	*P*
Age(ys)	15.1 ± 5.1	16.0 ± 4.2	t = −1.331(181)	0.191
Male/Female	44/45	48/46	$X^2 = 0.048$	0.826
Handedness	R	R	–	–
ADOS(ASD)	–	–	–	–
ADOS_TOTAL	11.4 ± 4.1	N/A	N/A	–
ADOS_COMM	3.6 ± 1.7	N/A	N/A	–
ADOS_SOCIAL	7.8 ± 2.9	N/A	N/A	–
ADOS_STEREO_BEHA	1.9 ± 1.6	N/A	N/A	–
IQ(TD)	–	–	–	–
FIQ	N/A	109.2 ± 12.9	–	–
VIQ	N/A	110 ± 13.2	–	–
PIQ	N/A	105.9 ± 14.4	–	–

Data are in terms of mean ± standard deviation.
ASD, autism spectrum disorder; TD, normally developing child; ADOS_TOTAL, Classic Total ADOS Score (Communication subscore + Social Interaction subscore); ADOS_COMM, Communication Total Subscore of the Classic ADOS; ADOS_SOCIAL, Social Total Subscore of the Classic ADOS; ADOS_STEREO_BEHA, Stereotyped Behaviors and Restricted Interests Total Subscore of the Classic ADOS.

DISCUSSION

We used resting-state functional imaging of the ASD and TD groups to construct entropy connectivity and examined differences in functional connectivity between the prefrontal and posterior brain regions across both the ASD and TD groups. Specifically, in the present study, we found that ASDs presented weakened entropy connectivity between the prefrontal cortex (BAs 8R, 9L, 9R, 10L, 46R) and the primary somatosensory cortex (BAs 1L, 2L, 2R), somatosensory association cortex (BA 7R), middle temporal gyrus (BA 21L), angular gyrus (BA 39L), and supramarginal gyrus (BA 40L). In addition, we also discussed interactive prefrontal-posterior underconnectivity based on the predictive coding theory.

The Impaired Information Flow From the Prefrontal Cortex to the Posterior Brain Regions in ASD

To limit cognitive resources of maintaining context information (Braver and Cohen, 2001) and filter unnecessary information (Jun and Hoshi, 2008), as well as top-down attention (Buschman and Miller, 2007), the neural activities of the posterior brain areas are often accepted from the modulation of high-level brain regions, such as the prefrontal cortex. Some studies have reported that the absence of the active guidance mediated by the prefrontal cortex in individuals with ASD might have a negative impact on early sensory processing, execution function (Frith, 2008) and face recognition (Bird et al., 2006).

Abnormal functional connectivity between the prefrontal lobe (a higher-order brain region) and the primary somatosensory cortex (a lower-order brain region) has been found in some ASD studies. A previous study reported that children with

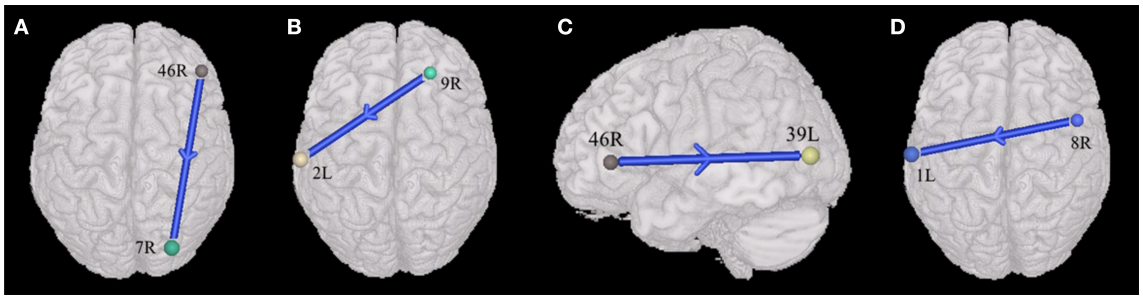

FIGURE 2 | Alterations of the entropy connectivity from the prefrontal cortex to posterior brain regions ($p < 0.05$, two-sided, PRT corrected; reproducible rate: 0.85; number of subjects: 70; PRT threshold: 1,000). **(A)** Synchronous entropy connectivity. **(B–D)** Asynchronous entropy connectivity. The blue arrows denote weakened interregional connections. Every colored sphere in the figure indicates the seed brain region of entropy connectivity, and the color of the sphere is randomly generated and has no special meaning. The number next to the sphere is the index of BA (see **Table 2** for details). The direction of the arrow indicates the direction of directional connectivity.

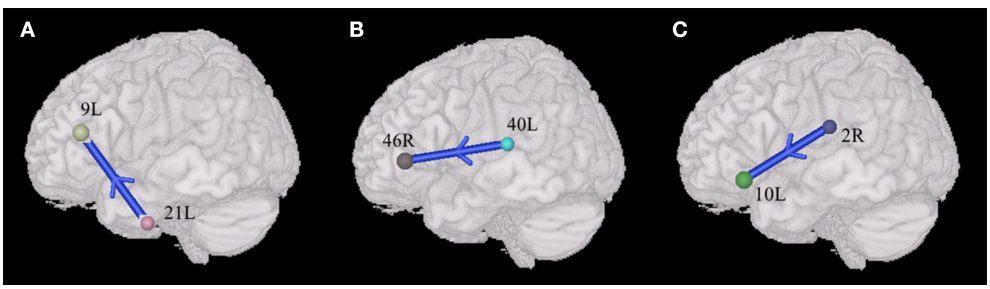

FIGURE 3 | Alterations of the entropy connectivity from the posterior brain regions to prefrontal cortex ($p < 0.05$, two-sided, PRT corrected; reproducible rate: 0.85; number of subjects: 70; PRT threshold: 1000). **(A,B)** Synchronous entropy connectivity. **(C)** Asynchronous entropy connectivity. The blue arrows denote weakened interregional connections. Every colored sphere in the figure indicates the seed brain region of entropy connectivity, and the color of the sphere is randomly generated and has no special meaning. The number next to the sphere is the index of BA (see **Table 2** for details). The direction of the arrow indicates the direction of directional connectivity.

ASD presented increased functional connectivity between the primary sensory and association regions (including the lateral frontal and parietal cortices) (Supekar et al., 2013). In the current study, we found decreased asynchronous entropy connectivity from the right dorsal prefrontal cortex (BAs 8R, 9R) to the left somatosensory cortex (BAs 1L, 2L), and the prefrontal cortex has been proven to play an important role in regulating information processing of the primary sensory cortex (Staines et al., 2002). A previous investigation found that patients with DLPFC injury displayed destroyed inhibitory regulation of inputs to the primary somatosensory cortex, and weakened inhibitory regulation often led to weakened processing for task-irrelevant sensory signals (Yamaguchi and Knight, 1990; Robert et al., 1999). Therefore, the weakened asynchronous entropy connectivity from the dorsal prefrontal to somatosensory area in ASD may indicate decreased selective collection of sensory information guided by the prefrontal cortex (restraining meaningless input and deciding relevant input) and further lead to decoupling between cognitive processes and sensory information from the environment (Miller and Cohen, 2001), which manifests as atypical somatosensory processing (Sapey-Triomphe et al., 2019).

Just and his colleagues (Koshino et al., 2005) found that the autistic patients with IQs in the normal range presented decreased functional connectivity between the right dorsolateral prefrontal lobe and the left inferior parietal lobule during an n-back visual working memory task, which was considered to be an important reason that ASDs need to rely more on the posterior brain region for information processing in visual tasks. In the current study based on resting-state fMRI, we also found that the right dorsolateral prefrontal lobe (BA 46R) in ASD displayed weakened synchronous output entropy connectivity to the ipsilateral parietal lobule (BA 7R) but asynchronous output entropy connectivity to the contralateral angular gyrus (BA 39L), and previous literature has reported that BA 7R is involved in the mental manipulation of information in working memory (Koenigs et al., 2009), the coordination of visual–tactile conflict (Ro et al., 2004) and visual-spatial processing (Ro et al., 2004). Additionally, BA 39L participates in multiple cognitive processes such as semantic processing, visual spatial processing, the manipulation of mental representations and memory retrieval

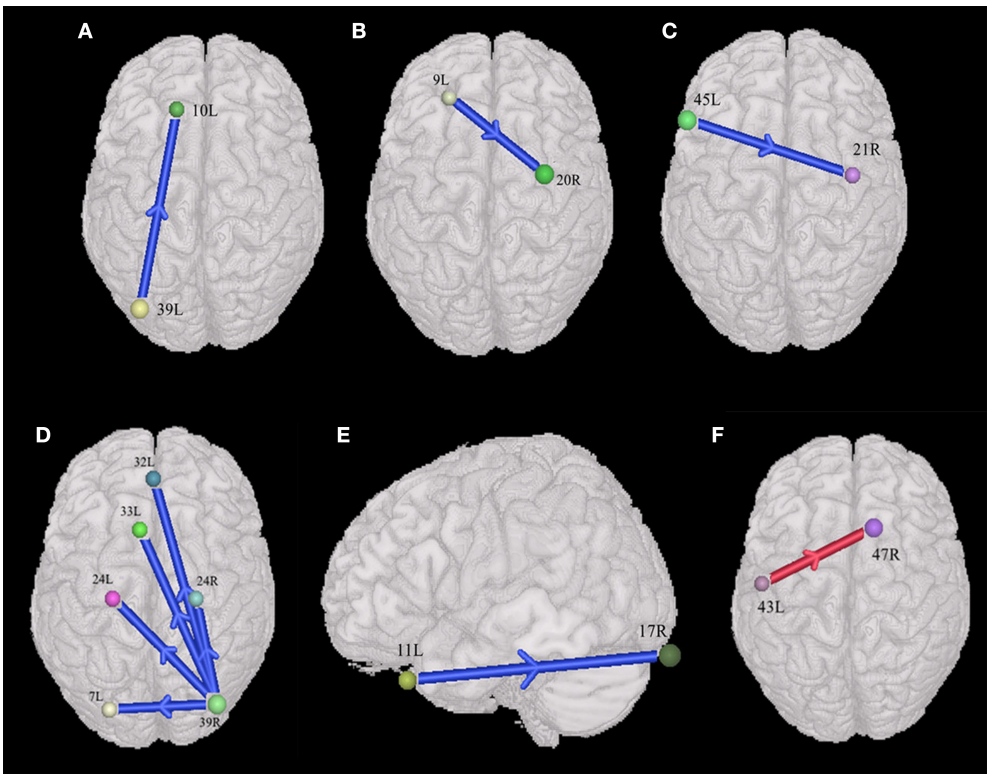

FIGURE 4 | Sex-specific differences of the entropy connectivity in ASD ($p < 0.05$, two-sided, PRT corrected; reproducible rate: 0.85; number of subjects: 30; PRT threshold: 1,000). **(A–E)** Synchronous entropy connectivity in the male group. **(F)** Synchronous entropy connectivity in the female group. The red arrow denotes enhanced interregional connection. The blue arrows denote weakened interregional connections. Every colored sphere in the figure indicates the seed brain region of entropy connectivity, and the color of the sphere is randomly generated and has no special meaning. The number next to the sphere is the index of BA (see **Table 2** for details). The direction of the arrow indicates the direction of directional connectivity.

(Seghier, 2013). Thus, these results might potentially affect the active maintenance of the prefrontal cortex for the information in the multimodal cortical architectures during "rest."

Autism Declined Capacity in Information Flow From the Posterior Brain Regions to the Prefrontal Cortex

The current study found weakened synchronous entropy connectivity from the middle temporal gyrus (BA 21L) to the dorsolateral prefrontal cortex (BA 9L), and the middle temporal gyrus plays an important role in multimodal visual information processing (Hidaka et al., 2015; Stock et al., 2017). In a study based on resting-state functional connectivity, an investigator found that insufficient functional correlation between the left anterior middle temporal gyrus and the right frontal polar cortex affected superior cognitive brain functions in children with ASD (Borras-Ferris et al., 2019).

Additionally, we also observed weakened synchronous entropy connectivity from the supramarginal gyrus (BA 40L) to the dorsolateral prefrontal cortex (BA 46R). BA 40 is a part of the inferior parietal lobe (IPL) (Caspers et al., 2006), and IPL is believed to integrate multisensory information and participate in various high-level cognitive activities, such as executive function and self-awareness (Torrey, 2007). It is believed that in executive function-related tasks, bandwidth limitations in the frontal and parietal lobes might lead to the disrupted information coordination between two regions, which is associated with cognitive deficits in ASD (Just et al., 2012). Furthermore, a study suggested that the lateral prefrontal cortex (LPFC) participates in integrating highly processed cognitive and motivational information from the posterior association cortices (e.g., IPL, middle temporal gyrus) and the orbitofrontal cortex, respectively, for adaptive goal-directed behavior (Watanabe and Sakagami, 2007). Therefore, we proposed that the weakened synchronous entropy connectivity mentioned above might affect the integrity of cognitive information input from the posterior brain regions to the prefrontal cortex.

Interestingly, we also found weakened asynchronous entropy connectivity between the primary somatosensory cortex (BA 2R) and the anterior prefrontal cortex (BA 10L). Peng et al. (2018) proposed that the anterior prefrontal cortex might participate in the integration and advanced processing of nociception and pain, and in this process, the BA 10 can integrate the sensory aspect of pain by the information flow from the sensorimotor network to the lateral BA 10. Some studies also presented that BA 10 contributes to the process of transforming tactile and somatosensory information into abstract representation

to maintain hapticospatial information during cognitive tasks (Kaas et al., 2007; Matsumoto et al., 2020). Thus, weakened entropy connectivity may potentially limit sensory information integration between brain areas in ASD.

The Interactive Prefrontal-Posterior Underconnectivity From the Perspective of Predictive Coding Theory

By using a method of directional connectivity, entropy connectivity, our results provide direct evidence that there is a functional interactive underconnectivity pattern between the prefrontal cortex and posterior brain areas. Notably, based on the previous discussion, we can summarize the pattern as the dysfunction of the interactive information processing between two brain regions, which consists of two weakened information pathways, i.e., the information flow from the prefrontal cortex to posterior brain regions and the information flow from the posterior brain regions to prefrontal cortex (**Figure 5**). Our suppositions are based on predictive coding theory, which constructs a framework that states that the brain is not a coded stimulus-response machine but a statistical organ that actively interprets the stimuli it encounters, tested on sensory evidence (Seth and Friston, 2016). Specifically, the brain builds prior knowledge to produce prediction signals, which are integrated with sensory signals to understand the external world, but if there is mismatching between two kinds of signals, the prediction error signals are uploaded to higher hierarchical structures to update prior knowledge to make the internal representation more compatible with the external environment or change the sensory information to make them more like predictions (Seth and Friston, 2016; Brodski-Guerniero et al., 2018; Coll et al., 2020). Notably, Smith et al. (2017) suggested that the prefrontal cortex possessing multimodal prior knowledge sends the downward high-precision estimates to other brain regions to promote goal-directed thought, and it accepts the modification to its prior knowledge from salient prediction error information broadcasted by other brain structures. According to the aforementioned theory, the weakened connectivity from the prefrontal cortex to posterior brain regions might imply the impairment of the cognitive control in ASDs. That is, the prefrontal cortex cannot flexibly allocate mental resources by adjusting the flow of information in the posterior brain areas to guide thoughts and actions in light of prior knowledge (Solomon et al., 2009). Inhibited connectivity from the posterior brain regions to prefrontal cortex might impair the process by which the prefrontal cortex (especially the DLPFC) recodes perception information from posterior cortical areas to an abstract form Wicker et al. (2008) to effectively store and control the information. On the other hand, the lack of some prediction error signals endowed with high precision to privilege access to the prefrontal cortex will make it difficult to update prior knowledge (Seth and Friston, 2016). Taken together, the interactive prefrontal-posterior underconnectivity may induce the deoptimization of the autistic brain networks due to potentially affecting the information processing pattern of the brain that integrating the information from the body and environment with prior knowledge (Friston, 2010; Sterling, 2012).

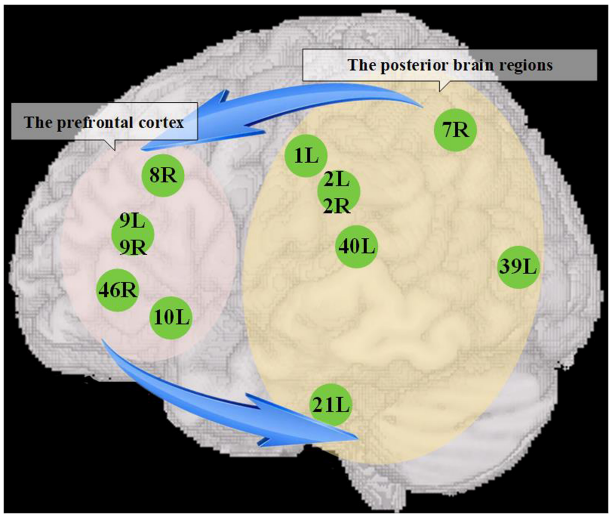

FIGURE 5 | The dysfunction of interactive information processing between the prefrontal cortex and posterior brain regions. The left elliptical shadow area covers the prefrontal cortex, and the right elliptical shadow area covers the posterior brain regions, and the figures in the shadow area show the seed brain regions with atypical entropy connectivity found in this study. The blue arrow represents that there might be a reduced mutual information transmission between them, which further contributed to accelerate the deoptimization of brain networks.

However, many studies agreed that patients with ASD have abnormal prediction signals and relatively complete and even enhanced prediction error signals (Maekawa et al., 2011; Brodski-Guerniero et al., 2018) from the perspective of predictive coding theory. We believe that one of the reasons for this conflict is a difference in approach: Maekawa et al. (2011) and Brodski-Guerniero et al. (2018) used EEG and MEG, respectively, to examine the changes in brain function in ASD. The directional functional connectivity we use is more visualized to reflect the direction of neurodynamics. An EEG study using the hierarchical frequency tagging task identified atypical integration of prediction and prediction error signals in ASD. Specifically, atypical precise weighted integration (IM component) of both prediction and prediction error signals was associated with lower ASD characteristics, while such changes were not found in patients with higher ASD characteristics (Coll et al., 2020). We proposed that the changes in interactive information processing in ASD would further worsen brain network optimization, and the symptoms of ASD might be more serious. This may explain the atypical interactive information processing found in the abovementioned study at lower rather than higher ASD characteristics.

In addition, we also found the enhanced asynchronous entropy connectivity between right dorsal frontal cortex (BA 8R) and left piriform cortex (BA 27L) ($t = 3.199877$, $p < 0.05$, PRT corrected; $p < 0.002$, no corrected). Many researchers have confirmed that the functional connectivity of the patients with ASD presented under- and over-connectivity simultaneously

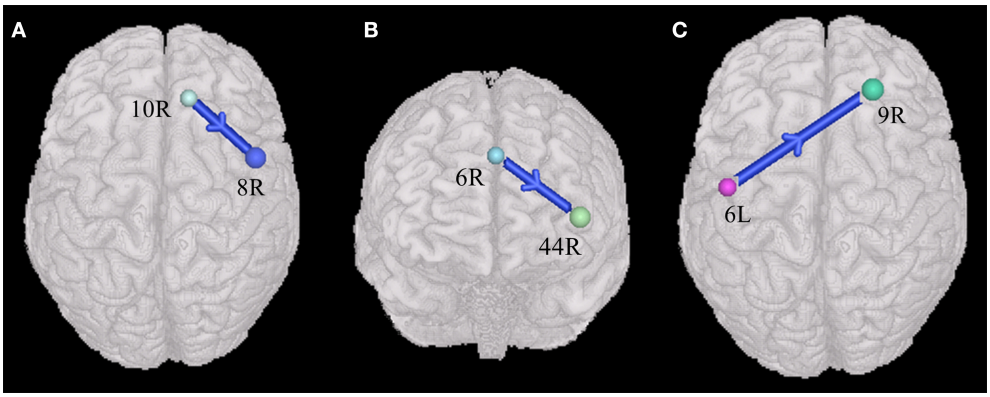

FIGURE 6 | Weakened synchronous entropy connectivity (BA 21L → BA 10L) ($p < 0.05$, two-sided, PRT corrected; reproducible rate: 0.85; number of subjects: 70; PRT threshold: 1,000). The blue arrow denotes weakened interregional connectivity. Every colored sphere in the figure indicates the seed brain region of entropy connectivity, and the color of the sphere is randomly generated and has no special meaning. The number next to the sphere is the index of BA (see **Table 2** for details). The direction of the arrow indicates the direction of directional connectivity.

(Hull et al., 2017). This result may indicate the heterogeneity of autistic functional connectivity. However, the reason why our study did not discuss this enhanced asynchronous connectivity was that the piriform cortex is the paleocortex rather than the posterior brain areas discussed in this study (De Curtis et al., 2019). Therefore, we could obtain a consistent conclusion: there is the interactive underconnectivity between the prefrontal cortex and posterior brain regions.

Comparison Between the Results Using ComBat and the Original

There was some consistent results between the prefrontal cortex and posterior brain regions in these two experiments with or without ComBat harmonization ($p < 0.05$, reproducible rate: 0.85, PRT corrected). Only those with significant entropy connectivity between the prefrontal cortex and posterior brain regions are described as follows. Weakened synchronous entropy connectivity from the right dorsolateral prefrontal cortex to the right somatosensory association cortex (BA 46R→ BA 7R) ($t = -3.360816$, $p < 0.05$, PRT corrected; $p < 0.002$, no corrected); weakened asynchronous connectivity from the right primary somatosensory cortex to the anterior prefrontal cortex (BA 2R→ BA 10L) (t = −3.57506, $p < 0.05$, PRT corrected; $p < 0.0005$, no corrected); weakened asynchronous entropy connectivity from the right dorsal frontal cortex to the left primary somatosensory cortex (BA 8R→ BA 1L) (t = −3.706519, $p < 0.05$, PRT corrected; $p < 0.0004$, no corrected); weakened asynchronous entropy connectivity from the right dorsolateral prefrontal cortex to the left angular gyrus (BA 46R→ BA 39L) (t = −3.214031, $p < 0.05$, PRT corrected; $p < 0.002$, no corrected). In addition, we also found weakened synchronous entropy connectivity from the left middle temporal gyrus to the left anterior prefrontal cortex (BA 21L→ BA 10L) (t = −3.827444, $p < 0.05$, PRT corrected; $p < 0.0002$, no corrected), which is a newly appearing result in the prefrontal cortex and the posterior brain region (**Figure 6**).

Although there are some differences between the ComBat result and the original, we obtain a consistent conclusion: there exists interactive underconnectivity between the prefrontal cortex and posterior brain regions on the basis of these two results.

Sex-Specific Differences of the Entropy Connectivity in ASD

Many previous studies provided much evidence of sex bias in autism from the perspective of gene, testosterone, immune system, and functional connectivity (Hu et al., 2015; Lai et al., 2017; Ferri et al., 2018; Cummings et al., 2020). However, it remains underaddressed whether there exist sex-specific differences in the directional connectivity network. In this present study, we investigated the differences of the entropy connectivity between ASDs and TDs within the female and male groups respectively. We found that the autistic males presented the interactive prefrontal-posterior underconnectivity compared with the males with TD (**Figures 4A–E**), but there existed an overconnectivity from the posterior cortex to prefrontal cortex between ASDs and TDs in the female (**Figure 4F**).

Alaerts et al. (2016) reported that the males with ASD generally presented underconnectivity but the females with ASD generally exhibited overconnectivity relative to sex-matched TDs in intrinsic functional connectivity, and they suggested the sex-specific differences of functional connectivity might be related to the disturbances in sex steroid levels (e.g., fetal testosterone). Thus, the aforementioned results indicated that the interactive prefrontal-posterior underconnectivity cannot necessarily be generalized to females with autism. We also suggested that future studies with larger samples of the males and females may help reveal the potential mechanism of the sex-specific differences of ASD in directional connectivity network.

CONCLUSIONS

In this article, we investigated alterations in entropy connectivity between the prefrontal cortex and posterior brain regions. Resting-state neural activity in individuals with ASD presented

interactive prefrontal-posterior underconnectivity. We suggested that interactive prefrontal-posterior underconnectivity might lead to less prior knowledge being used and updated from the perspective of predictive coding theory. Our proposals highlight that a combination of impaired interactive information flow between the prefrontal cortex and posterior brain regions accelerates the deoptimization of brain networks of ASD. Last, by analyzing the sex-specific differences of the entropy connectivity, we found this underconnectivity cannot necessarily be generalized to females with autism.

LIMITATIONS

There are several limitations of this study that warrant discussion. First, we limited the variability of handedness and the sex ratio of the data and removed unavailable data that were of poor imaging quality and were acquired from the patients with ASD using psychoactive drugs. We found no significant difference between the ASD and TD groups regarding age. To expand the data volume, we relatively loosened the treatment of the control about sites, resulting in variability of image data scanning parameters, which is consistent with the original intention of the database builders (Di Martino et al., 2014). Second, we did not investigate a dimensional associations with ASD symptoms due to the lack of clinical test scores (e.g., ADOS scores) in twenty-nine ASDs and eighty-four TDs. Moreover, some researchers proposed that the brain network of children with ASD under 12 years old showed overconnectivity compared with TD and the adolescents and adults with ASD appeared weakened functional connectivity (Uddin et al., 2013), which was speculated to be related to the abnormally accelerated growth of white matter in early children and subsequent loss of white matter in adolescence and adulthood (Waiter et al., 2005; Maximo et al., 2014). Therefore, we suggested that the finding of this study (i.e., the interactive prefrontal-posterior underconnectivity) only applicable to autistic adolescents and young males (15 ± 5 years) with IQs in the normal range. Developmental studies will be need to determine how these alterations in brain networks arise.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: http://fcon_1000.projects.nitrc.org/indi/abide/abide_I.html.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Ethics Committee of Shandong First Medical University. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements. Written informed consent was not obtained from the individual(s), nor the minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

RL and H-CZ conceived and designed the study. X-TC, RL, and G-YZ contributed to experimental design. H-CZ, RL, and G-YZ performed the experiments. H-CZ, QS, and RL wrote the first draft of the manuscript. X-TC, RL, H-CZ, L-MH, QS, and G-YZ discussed results. G-YZ, X-YB, X-YL, and C-YY revised the first draft of the manuscript. All authors contributed to the revision of the final version of the manuscript, read and approved the submitted version.

FUNDING

This research was supported by the Shandong Students' Platform for Innovation and Entrepreneurship Training Program (S202110439094), Shandong Provincial Natural Science Foundation, China (Grant Number: ZR2018MH033, ZR2015HL095, and ZR2014HM072), the High-Level Project Nurturing Program of Taishan Medical University (2018GCC13), the Shandong Province Higher Educational Science and Technology Program (No. J17KA262), and the Academic Promotion Program of Shandong First Medical University (2019LJ004).

ACKNOWLEDGMENTS

We thank the Center for Biomedical Research Excellence (COBRE) for sharing their data. We also thank all participants in this study.

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frontiers in Neuroscience

ORIGINAL RESEARCH
published: 07 February 2022
doi: 10.3389/fnins.2021.776860

Cortical Thickness and Clinical Findings in Prescholar Children With Autism Spectrum Disorder

*Simona Lucibello[1], Giovanna Bertè[2], Tommaso Verdolotti[3], Martina Lucignani[4], Antonio Napolitano[4], Rosa D'Abronzo[2], Maria G. Cicala[1], Elisa Pede[1], Daniela Chieffo[1], Paolo Mariotti[1], Cesare Colosimo[2,3], Eugenio Mercuri[1,5] and Roberta Battini[6,7]**

[1] Pediatric Neurology Unit, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy, [2] Dipartimento di Diagnostica per Immagini, Istituto di Radiologia, Università Cattolica del Sacro Cuore, Rome, Italy, [3] UOC Radiologia e Neuroradiologia, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy, [4] Medical Physics Unit, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy, [5] Centro Clinico Nemo, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy, [6] Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy, [7] Department of Developmental Neuroscience, IRCCS Fondazione Stella Maris, Pisa, Italy

The term autism spectrum disorder (ASD) includes a wide variability of clinical presentation, and this clinical heterogeneity seems to reflect a still unclear multifactorial etiopathogenesis, encompassing different genetic risk factors and susceptibility to environmental factors. Several studies and many theories recognize as mechanisms of autism a disruption of brain development and maturation time course, suggesting the existence of common neurobiological substrates, such as defective synaptic structure and aberrant brain connectivity. Magnetic resonance imaging (MRI) plays an important role in both assessment of region-specific structural changes and quantification of specific alterations in gray or white matter, which could lead to the identification of an MRI biomarker. In this study, we performed measurement of cortical thickness in a selected well-known group of preschool ASD subjects with the aim of finding correlation between cortical metrics and clinical scores to understand the underlying mechanism of symptoms and to support early clinical diagnosis. Our results confirm that recent brain MRI techniques combined with clinical data can provide some useful information in defining the cerebral regions involved in ASD although large sample studies with homogeneous analytical and multisite approaches are needed.

Keywords: autism spectrum disorder (ASD), cortical thickness, pre-scholar child, MRI, neuropsychological, local gyrification index

OPEN ACCESS

Edited by:
Francesco Craig,
University of Calabria, Italy

Reviewed by:
Uicheul Yoon,
Catholic University of Daegu, South Korea
Fabrizio Pizzagalli,
University of Turin, Italy

****Correspondence:***
Roberta Battini
rbattini@fsm.unipi.it

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: *14 September 2021*
Accepted: *16 December 2021*
Published: *07 February 2022*

Citation:
Lucibello S, Bertè G, Verdolotti T, Lucignani M, Napolitano A, D'Abronzo R, Cicala MG, Pede E, Chieffo D, Mariotti P, Colosimo C, Mercuri E and Battini R (2022) Cortical Thickness and Clinical Findings in Prescholar Children With Autism Spectrum Disorder. Front. Neurosci. 15:776860. doi: 10.3389/fnins.2021.776860

INTRODUCTION

According to the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5), autism spectrum disorder (ASD) is a neurodevelopmental disorder, characterized by the presence of impaired social communication and unusually repetitive behaviors or restricted interests (American Psychiatric Association, 2013).

However, the term ASD includes a wide variability of clinical presentation (Lord et al., 2000), which seems to reflect a multifactorial etiopathogenesis, still unclear, encompassing different genetic risk factors and susceptibility to environmental factors (Dietert et al., 2011; Tordjman et al., 2014).

Over the years, disruption of brain development and the maturation time course has been recognized as mechanism of autism (Amaral et al., 2008), suggesting the existence of common

neurobiological substrates, such as defective synaptic structure and aberrant brain connectivity (Ecker et al., 2016).

Therefore, several studies have assessed the utility of brain magnetic resonance imaging (MRI) in the evaluation of region-specific structural changes and quantification of specific alterations in gray (GM) or white matter (WM) [in terms of cortical thickness, gyrification index, GM surface and volume (Pagnozzi et al., 2018)].

The identification of an MRI biomarker could provide insight into the underlying mechanisms of symptoms and could additionally provide crucial support in early clinical diagnosis and dividing patients, sharing common features, into different groups to tailor specific interventions.

In literature, consensus exists on increased growth of total GM and WM volume (Courchesne et al., 2001; Sparks et al., 2002; Hardan et al., 2006; Hazlett et al., 2011; Nordahl et al., 2012; Mahajan and Mostofsky, 2015; Ismail et al., 2016; Libero et al., 2016; Lucibello et al., 2019), which involves specific brain regions, such as temporal lobes (Lai et al., 2013; Ecker et al., 2014; Riddle et al., 2017; Pappaianni et al., 2018), frontal lobes (Ecker et al., 2014; Zhou et al., 2014; Eilam-Stock et al., 2016; Patriquin et al., 2016), or both (Katuwal et al., 2015; Postema et al., 2019).

A comparable number of studies finds opposite results, reporting decreased volumes of the frontotemporal (Hardan et al., 2006; Ecker et al., 2010; Mak-Fan et al., 2012), and parietal cortex (Ecker et al., 2010), significantly smaller right (Haznedar et al., 1997), and bilateral (Ecker et al., 2010; Jiao et al., 2010), anterior cingulate gyrus, and decreased cortical volume in the orbitofrontal cortex bilaterally (Ecker et al., 2012).

Recently, many studies have focused their efforts on measurement of cortical thickness (Doyle-Thomas et al., 2013; Khundrakpam et al., 2017; Pereira et al., 2018; Prigge et al., 2018), and among them, the one with the largest ASD population (Van Rooij et al., 2018), showed promising results. Specifically, the authors found complex developmental trajectories involving different brain regions with significant differences in terms of increased cortical thickness in the frontal cortex and decreased thickness in the temporal cortex between ASD patients and controls during adolescence. Other authors focus their study on cortical gyrification, suggesting the involvement of both genetic and non-genetic factors in definition of cortical gyral and sulcal patterns (Lohmann et al., 1999; White et al., 2002; Kates et al., 2009; Kremen et al., 2010; Mata et al., 2010; Docherty et al., 2015; Kuhn et al., 2016; Bernardoni et al., 2018; Duan et al., 2020; Kruggel and Solodkin, 2020). Although analysis of gyrification has led to the identification of different cortical regions involved in ASD, there is high heterogeneity across studies.

However, no studies have investigated the correlation between clinical, genetic, and radiological findings in a well-selected pediatric population.

Aim of the Study

As part of an ongoing project collecting ASD preschooler data sets, we retrospectively selected MRI data sets and applied a semiautomatic brain segmentation methodology to investigate cortical thickness and gyrification. Correlations of cortical indexes and clinical features were subsequently performed.

MATERIALS AND METHODS

Study Design and Clinical Assessment

A retrospective study was designed. The study group included 39 preschool-age children regularly followed at the Child Neurology Unit of the Gemelli Hospital (Rome, Italy) with a diagnosis of ASD. As part of our routine assessment since March 2016, patients referred to our unit with a suspect clinical diagnosis of ASD undergo a detailed clinical assessment, including a neurological examination, Leiter or Wechsler scales according to age and cooperation, a comprehensive neuropsychiatric assessment using parent-reported questionnaires' (Child Behavior Checklist, CBCL), and autism-specific diagnostic tools, specifically Autism Diagnostic Interview Revised (ADI-R) and Autism Diagnostic Observation Schedule, second edition (ADOS2) (Lord et al., 1989, 1994). In addition, MRI is also routinely performed.

To have a relatively homogeneous cohort, the following exclusion criteria were used: (1) dysmorphic features with specific genetic syndrome identification, (2) presence of severe epilepsy, (3) presence of cerebral palsy or other major neurological signs, (4) malformations or other lesions at MRI.

The scores of all subscales of the ADI-R (social interaction, communication and language, restricted and repetitive behaviors) and ADOS2 (social affect score, restricted and repetitive behavior score, total score) were correlated with cerebral cortical thickness and cerebral cortical gyrification with the aim of finding a plausible neural substrate for the domains of deficit: impaired social communication and unusually repetitive behaviors or restricted interests.

Study Design and Neuroradiological Protocol

All participants underwent MRI with a 1.5T Philips Ingenia Scanner (Philips Healthcare, Eindhoven, Netherlands). The sequence used for postprocessing was a T1-weighted 3-D-TFE in a sagittal orientation (TR = 9.8 ms, TE = 4.6 ms with a delay time of 650 ms after a 180° prepulse, flip angle = 10°, FOV = 200 mm × 222 mm, 1.0 mm slice thickness with no gaps, total of 150 slices per slab, matrix size = 200 × 222, NSA = 2 with an in-plane resolution of 1.0 × 1.0 mm^2). This sequence is routinely acquired in our MRI protocol for children older than 2 years of age. ASD subjects were sedated with a general anesthesia with a halogenated agent while spontaneously breathing; contrast agent injection was never required. The written informed consent from a parent or guardian of children was obtained.

The quality of the structural MRI data was rated by an experienced neuroimaging researcher (TV) on a 3-point rating scale: 0 = no motion artifacts, excellent quality; 1 = few motion artifacts, fair quality; and 2 = moderate/severe motion artifacts, poor quality. Only data sets with scores of 0 were considered of adequate quality for research purposes. In case of imaging artifacts (ghosting, aliasing, chemical shift, and distortion), patients were excluded.

The raw 3-D T1 MRI data underwent automated processing for surface-based cortex reconstruction and volumetric segmentation using Freesurfer image analysis software (version 6.0.0), which is documented and freely available for download online[1], installed on an OSX El Capitan 10.11.6.

The technical details of these procedures are described in prior publications; briefly, the processing pipeline includes motion correction and averaging (Reuter et al., 2010) of volumetric T1 weighted images, removal of non-brain tissue (Ségonne et al., 2004), automated Talairach transformation, segmentation of the subcortical WM and deep GM volumetric structure (including hippocampus, amygdala, caudate, putamen, ventricles) (Fischl et al., 2002, 2004) intensity normalization (Sled et al., 1998), tessellation of the GM/WM boundary, automated topology correction (Fischl et al., 2001; Ségonne et al., 2007), and surface deformation following intensity gradients to optimally place the GM/WM and GM/CSF borders (Dale and Sereno, 1993; Dale et al., 1999; Fischl and Dale, 2000). Cortical thickness was quantified as the closest distance from the GM/WM boundary to the GM/CSF boundary at each vertex. Cortical parcelation and thickness estimations were based on the Desikan–Killiany Atlas (Desikan et al., 2006), resulting in average cortical thickness in 34 cortical parcels per hemisphere. Procedures for the measurement of cortical thickness have been validated against histological analysis (Rosas et al., 2002) and manual measurements (Kuperberg et al., 2003; Salat et al., 2004).

Local gyrification index (LGI) was computed using the approach proposed by Lyu et al. (2018)[2] that quantifies cortical gyrification within sulcal and gyral regions using a spatially varying kernel shape, able to adaptively encode cortical folding patterns. The proposed LGI is then computed within the adaptive kernel as a ratio of the cortical surface area and a fixed area on the outer hull ($\rho = 316\ mm^2$) (Lyu et al., 2018; Płonka et al., 2020).

The Ethics Committee of Policlinico Gemelli, Catholic University of Sacred Hearth, examined and approved the project study (February 2018).

Statistical Analysis

We investigated differences in cortical parameter distributions (i.e., cortical thickness, CT, and local gyrification, LGI) among subjects grouped on the basis of ADOS2 and ADI-R domains scores. To this purpose, we first mapped vertex-wise CT and LGI values on a common spherical coordinate system using spherical transformation, and then we assessed differences among groups using permutation tests (1,000 permutations for all tests) based on the *t*-statistics, performed with the Permutation Analysis of Linear Models (PALM) FSL package ([3]version 6.0). In particular, we used group age as a covariate to produce threshold-free cluster enhancement (TFCE) statistical maps, where the initial raw statistical images were enhanced using both the intensity of the data point and information from neighboring voxels (Mensen and Khatami, 2013). We detected group differences on both family-wise error (FWE) corrected and uncorrected *p*-value maps. Moreover, correlation analyses were evaluated vertex-wise between cortical parameters (CT and LGI) and several clinical variables, including social interaction (SI), communication (COM), and total scores (TOT, defined as SI + COM), together with their autism diagnostic interview (ADI) counterparts, i.e., ADI_SI, ADI_COM, repetition ADI (ADI_rep) and ADI_TOT as sum of the previously mentioned scores. Correlation analyses were performed testing Pearson correlation with PALM permutation test (1000 permutations).

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

[2]https://github.com/ilwoolyu/LocalGyrificationIndex

[3]https://fsl.fmrib.ox.ac.uk/fsl/fslwiki

RESULTS

After image quality check, five patients were excluded. Data from 34 patients, 4 females (11.8%) and 30 males (88.2%), were eventually included in the analysis. Their age range was between 3.4 and 6 years. All subjects of the sample selected had normal MRI findings and normal FMR1 and CGH array analysis.

Seven patient (20.6%) had a normal intellectual quotient, 12 (35.3%) a mild intellectual disability, and three (8.8%) a moderate intellectual disability.

About ADOS valuation, 5/34 ASD patients (14.7%) had a low level of severity, 20/34 (58.8%) had a medium level of severity, and 5/34 (14.7%) had a high level of severity (**Table 1**).

Correlations

Autism Diagnostic Interview – Social Interaction Domain

Statistical analysis showed a correlation between the subtest of ADI that investigates social interaction aims and thickness of different left-brain regions: fusiform ($p = 0.046$), lingual (0.049), posterior cingulate (0.048), pre-cuneus ($p = 0.046$), superior parietal ($p = 0.033$), inferior parietal ($p = 0.033$), superior temporal ($p = 0.043$), inferior temporal ($p = 0.041$), middle temporal (0.037), temporal pole ($p = 0.041$), and lateral occipital (0.041).

A correlation was found also between the subtest of ADI that investigates social interaction aims and gyrification of different cortical regions: in left hemisphere insula ($p = 0.01$), rostral anterior cingulate (0.008), pars orbitalis ($p = 0.009$), superior frontal ($p = 0.007$), medial orbito-frontal ($p = 0.006$), lateral orbito-frontal ($p = 0.008$), precentral ($p = 0.008$), postcentral ($p = 0.004$), supramarginal ($p = 0.007$), transverse temporal ($p = 0.009$); in right hemisphere, superior frontal ($p = 0.008$), precentral ($p = 0.007$), paracentral ($p = 0.009$) (**Figure 1**).

TABLE 1 | Neuropsychological variables of the sample enrolled (34 patients).

	Mean	SD
Intellectual quotient	70.07	12.286
ADOS social affect score	10.81	3.894
ADOS repetitive behavior score	4.76	2.700
ADOS total score	15.41	4.790
ADI social interaction score	13.04	7.190
ADI communication score	8.08	3.049
ADI repetitive behavior score	6.08	3.006

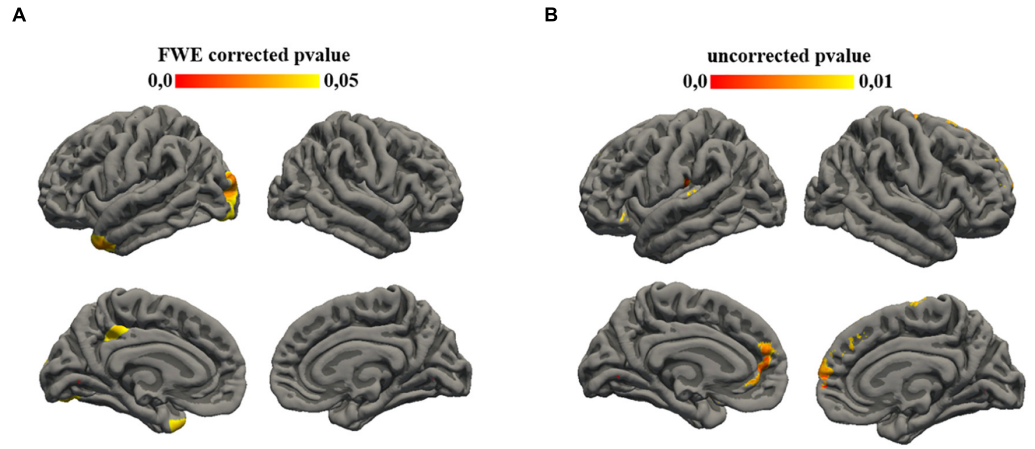

FIGURE 1 | Statistical results mapped on the surface template. **(A)** Family-wise error (FWE) corrected *p*-value maps for cortical thickness ADI-social interaction correlation. Significance was set at 0.05. **(B)** Uncorrected *p*-value maps for gyrification ADI-social interaction correlation. Significance was set at 0.01.

Autism Diagnostic Interview – Communication Domain

Statistical analysis showed a correlation between the subtest of ADI that investigates communication aims and gyrification of different left cortical regions: caudal anterior cingulate ($p = 0.007$), posterior cingulate ($p = 0.008$) (**Figure 2**).

Autism Diagnostic Interview – Repetitive Behavior Domain

Statistical analysis showed a correlation between the subtest of ADI that investigates social interaction aims and gyrification of different left regions: fusiform ($p = 0.008$), lingual ($p = 0.008$), para-hippocampal ($p = 0.008$), middle temporal ($p = 0.006$), inferior temporal ($p = 0.005$), lateral occipital ($p = 0.008$), but also with different right region: superior frontal ($p = 0.006$), frontal pole ($p = 0.008$), rostral middle frontal ($p = 0.004$), caudal middle frontal ($p = 0.007$), and precentral ($p = 0.006$) (**Figure 3**).

Autism Diagnostic Observation Schedule – Social Affect Score

Statistical analysis showed a correlation between the subtest of ADOS that investigates restricted and repetitive behavior aims and thickness of different left cortical regions: pre-cuneus ($p = 0.007$), posterior cingulate ($p = 0.006$), lateral orbito-frontal ($p = 0.006$), medial orbito-frontal ($p = 0.005$), precentral ($p = 0.007$), paracentral ($p = 0.009$), postcentral ($p = 0.007$), and superior parietal ($p = 0.007$).

A correlation was found also between the subtest of ADOS that investigates restricted and repetitive behavior aims and

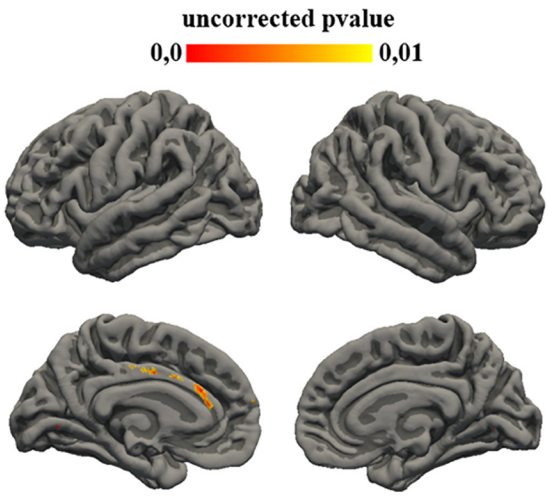

FIGURE 2 | Uncorrected *p*-value maps for gyrification ADI-communication correlation. Significance was set at 0.01.

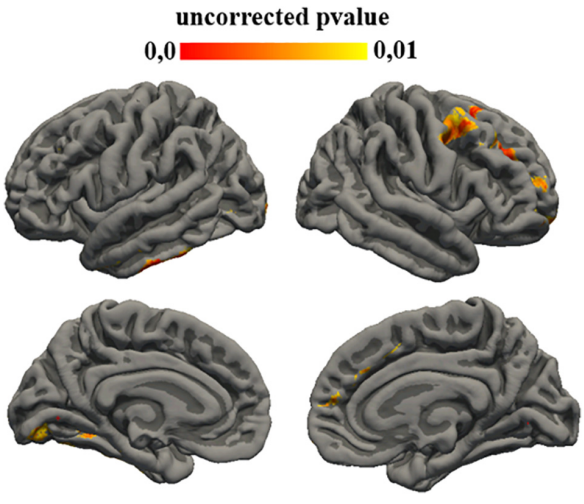

FIGURE 3 | Uncorrected *p*-value maps for gyrification ADI-repetitive behavior correlation. Significance was set at 0.01.

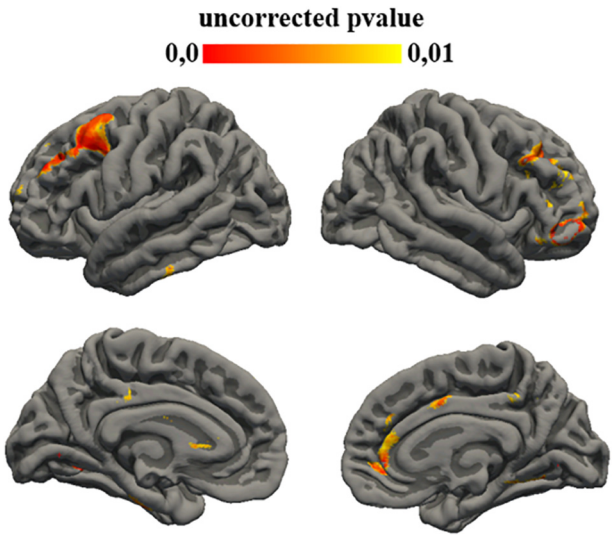

FIGURE 4 | Uncorrected *p*-value maps for gyrification ADOS-restricted and repetitive behavior score correlation. Significance was set at 0.01.

gyrification of left cortical regions: middle temporal (p = 0.008) and superior temporal (p = 0.008).

Autism Diagnostic Observation Schedule – Restricted and Repetitive Behaviors Score

Statistical analysis showed a correlation between the subtest of ADOS that investigates social affect aims and gyrification of different left cortical regions: fusiform (p = 0.008), lingual (p = 0.005), entorhinal (p = 0.008), para-hippocampal (p = 0.008), posterior cingulate (p = 0.009), superior frontal (p = 0.009), caudal middle frontal (p = 0.004), rostral middle frontal (p = 0.004), inferior temporal (p = 0.008) and also with different right regions: lingual (p = 0.008), para-hippocampal (p = 0.009), pars triangularis (p = 0.007), pre-cuneus (p = 0.009), posterior cingulate (p = 0.008), rostral anterior cingulate (p = 0.006), caudal anterior cingulate (p = 0.009), pars orbitalis (p = 0.004), superior frontal (p = 0.009), rostral middle frontal (p = 0.006), medial orbito-frontal (p = 0.006), and caudal middle frontal (p = 0.008) (**Figure 4**).

Autism Diagnostic Observation Schedule – Total Score

Statistical analysis showed a correlation between the total score of ADOS and gyrification of left cortical regions: middle temporal (p = 0.009) and inferior temporal (p = 0.007) (**Figure 5**).

DISCUSSION

Autism is a complex and heterogeneous neurodevelopmental disorder with widely varied clinical features and a multitude of possible etiological factors.

Emerging evidence supports that ASD undergoes an atypical trajectory of brain maturation (Doyle-Thomas et al., 2013; Ecker et al., 2014) or abnormal lateralization of brain networks (Conti et al., 2015). Findings of atypical brain morphology in ASD, however, are highly heterogeneous and there are still not clear neuroanatomical markers to accurately identify individuals with ASD.

Whereas most neuroradiological studies on autism have focused on finding a specific pattern of brain involvement, only a few studies aimed to establish a correlation between clinical severity and cortical thickness (Doyle-Thomas et al., 2013). Additionally, many of these studies exclusively investigate well-known cortical areas involved in specific function, whereas we have tried to assess the correlation of several cortical areas to verify some correspondence with already known brain circuits. Whereas some results have reinforced well-known

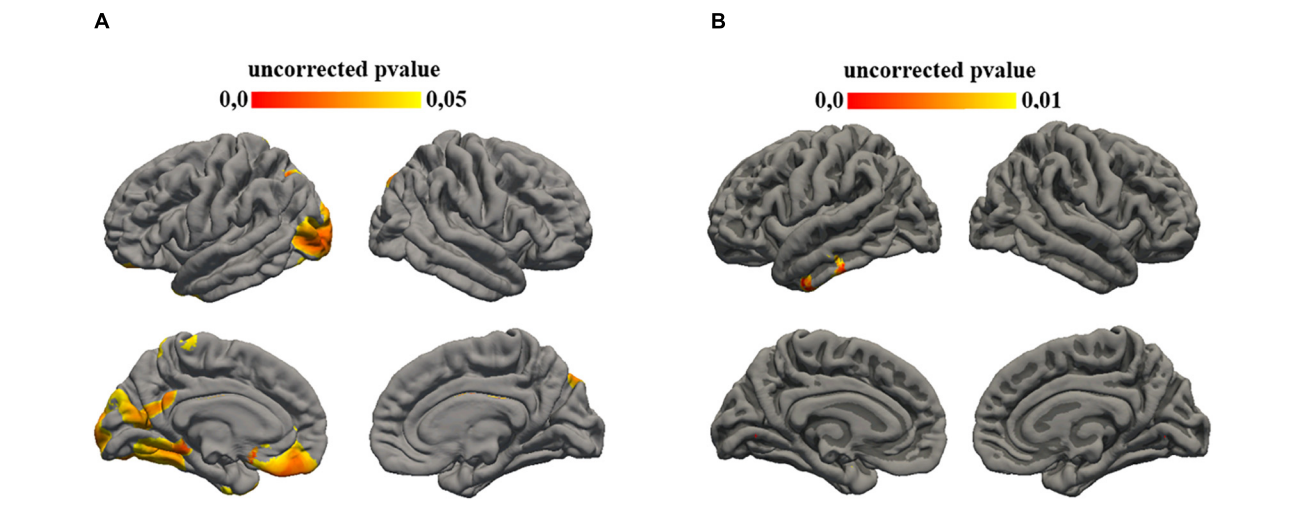

FIGURE 5 | Statistical results mapped on the surface template. **(A)** Uncorrected *p*-value maps for cortical thickness-ADOS_total score correlation. Significance was set at 0.05. **(B)** Uncorrected *p*-value maps for gyrification-ADOS-total score correlation. Significance was set at 0.01.

concepts already widely described in literature, others are quite difficult to frame.

The correlation between cingulate and frontal regions, particularly the left medial orbito-frontal area, with a social affect score of both ADOS and ADI-R scales is not surprising as the involvement of this area in social-affective processing is already described as part of the ventral social affective processing system (Belger et al., 2011). These results are consistent with what is already known about cortical gyrification morphology in neurodevelopmental disorders (Kohli et al., 2019).

Less clear is the correlation with other areas, such as the left lateral-occipital area, even if other authors have already shown this result in general in ASD patients (Kohli et al., 2019).

Among the results obtained, it is useful to underline a partial congruence between the results of the ADOS2 and ADI-R subtests investigating social affective behaviors and cortical gyrification index. This highlights how the two diagnostic tests support each other and how it is essential to always try to perform a complete patient assessment.

In the same way, it is easy to understand the correlation found between the restricted and repetitive behavior indices of both scales with gyrification of para-hippocampal, temporal, and middle frontal area.

Many human neuroimaging studies have indeed shown increased activation in ventral striatum and ventromedial frontal cortex in response to unexpected negative feedback that implies a change of behavior strategy (Glascher et al., 2009; D'Cruz et al., 2016; Sharda et al., 2017).

Conversely, it is unclear how the cingulate cortex could be involved in communication tasks although some neuroimaging studies suggest the association with this region and indirect language and social inferential capacity, such as the comprehension of non-literal language or construction-based pragmatic information (D'Cruz et al., 2011).

In general, the assessment of cortical areas based on the ADOS and ADI scores cannot be univocal. These scores, indeed, assess other skills, such as language or different non-verbal communication ability that could be underpinned by more cortical areas. Certainly, many of these areas, such as temporal and frontal or middle temporal areas, are already described as involved in patients with ASD.

This study confirms and expands previous results while reducing the variability of cohort patients as, in other studies a systematic selection of ASD subjects was not performed, and combining imaging with more detailed clinical data. The well-selected population of our study allowed us to reduce the variability observed between different ages.

Exclusion criteria, such as dysmorphic features or epilepsy or other major neurological signs, have led to less variability related to secondary factors, and focusing on only preschool-age patients has reduced the bias linked to the noted changes in the thickness of the cerebral cortex related to age (Shibata et al., 2010).

Neuroimaging studies indeed report numerous region-specific alterations in cortical thickness in patients with ASD. However, there are many inconsistent findings, and this is probably due to atypical CT developmental trajectories in ASD, characterized by decreased cortical thinning during early adolescence and increased thinning at later stages, involving mostly frontal and parietal areas (Mensen and Khatami, 2013; Ecker et al., 2014).

Limitations of our study include a small sample size, which reduces the power of statistical analysis.

Though the study has investigated the brain structure–function correlation with the aim of catching a possible clinical-related sensitivity marker at this age, the lack of controls constitutes a limitation of the study. The need for sedation during MRI scans, indeed, limits the opportunity to collect age-matched typical subjects.

Moreover, because pediatric brains are different in size and shape from adult brains commonly used as frameworks for spatial normalization (e.g., Talairach space), specific cortical areas could show correlation of deformation with age. In this context, several studies assessed the influence of age on various spatial normalization parameters (Wilke et al., 2002; Fonov et al., 2011), and consequently, our results should be taken with caution.

In conclusion, our results confirm that recent brain MRI techniques integrated to clinical data can provide some useful information in defining the cerebral regions involved in ASD although large sample studies characterized by homogeneous analytical and multisite approaches 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 author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Fondazione Policlinico Gemelli Hospital – Catholic University, Rome, Italy. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

RB, SL, and EM contributed to conception and design of the study. SL, TV, and GB organized the database. EP, MC, DC, and RD'A contributed to investigation. AN and ML performed the statistical analysis. SL wrote the first draft of the manuscript. TV and AN wrote sections of the manuscript. PM, CC, and EM contributed to the supervision of manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was partially supported by the Current Research (RC) of Italian Ministry of Health, IRCCS Stella Maris Foundation (FSM), entitled "Early functional and neurobiological markers of diseases associated to neurodevelopmental disorders" and 5 for thousands of IRCCS FSM (RB).

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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.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 09 February 2022
doi: 10.3389/fnins.2022.721987

Effect of Transcranial Direct Current Stimulation on the Mismatch Negativity Features of Deviated Stimuli in Children With Autism Spectrum Disorder

Changcheng Sun[1,2], Zhuoyue Zhao[2], Longlong Cheng[3], Rong Tian[2], Wenchang Zhao[2], Jingang Du[2], Ying Zhang[2] and Chunfang Wang[2]**

[1] Academy of Medical Engineering and Translational Medicine, Tianjin University, Tianjin, China, [2] Department of Rehabilitation Medical, Tianjin Union Medical Centre, Tianjin, China, [3] China Electronics Cloud Brain (Tianjin) Technology Co., Ltd., Tianjin, China

Autism spectrum disorder (ASD) is a devastating mental disorder in children. Currently, there is no effective treatment for ASD. Transcranial direct current stimulation (tDCS), which is a non-invasive brain stimulation neuromodulation technology, is a promising method for the treatment of ASD. However, the manner in which tDCS changes the electrophysiological process in the brain is still unclear. In this study, we used tDCS to stimulate the dorsolateral prefrontal cortex area of children with ASD (one group received anode tDCS, and the other received sham tDCS) and investigated the changes in evoked EEG signals and behavioral abilities before and after anode and sham stimulations. In addition to tDCS, all patients received conventional rehabilitation treatment. Results show that although conventional treatment can effectively improve the behavioral ability of children with ASD, the use of anode tDCS with conventional rehabilitation can boost this improvement, thus leading to increased treatment efficacy. By analyzing the electroencephalography pre- and post-treatment, we noticed a decrease in the mismatch negativity (MMN) latency and an increase in the MMN amplitude in both groups, these features are considered similar to MMN features from healthy children. However, no statistical difference between the two groups was observed after 4 weeks of treatment. In addition, the MMN features correlate well with the aberrant behavior checklist (ABC) scale, particularly the amplitude of MMN, thus suggesting the feasibility of using MMN features to assess the behavioral ability of children with ASD.

Keywords: autism spectrum disorder (ASD), transcranial direct current stimulation (tDCS), EEG, mismatch negativity (MMN), dorsolateral prefrontal cortex (DLPFC)

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Edited by:
Eugenia Conti, Stella Maris Foundation, Scientific Institute for Research, Hospitalization and Healthcare (IRCCS), Italy

Reviewed by:
Ayman Sabry El-Baz, University of Louisville, United States
Verner Knott, University of Ottawa, Canada

****Correspondence:***
Ying Zhang
15510982989@163.com
Chunfang Wang
chfwang@tju.edu.cn

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: *08 June 2021*
Accepted: *19 January 2022*
Published: *09 February 2022*

Citation:
Sun C, Zhao Z, Cheng L, Tian R, Zhao W, Du J, Zhang Y and Wang C (2022) Effect of Transcranial Direct Current Stimulation on the Mismatch Negativity Features of Deviated Stimuli in Children With Autism Spectrum Disorder. Front. Neurosci. 16:721987. doi: 10.3389/fnins.2022.721987

INTRODUCTION

Autism spectrum disorder (ASD) is a biologically based neurodevelopmental disorder (developmental disability) that is defined by the following diagnostic criteria: deficits in social communication and interaction and presence of restricted, repetitive patterns of behavior, interests, or activities that can persist throughout life (Battle, 2013; Principi and Esposito, 2020). The

population of children with ASD is generally large; approximately 1 in 54 children has been identified with ASD in the United States in 2016 (Maenner et al., 2020). There are no exact statistics on the number of patients with ASD in China. Some scholars believe that the actual number may reach 2.6–8 million. Worryingly, the prevalence of ASD is increasing year by year, with the United States experiencing an increase of 150% from 2000 to 2014. This situation is more severe in China, which has a rate of increase of 200,000 ASD cases per year. Therefore, the increasing incidence of ASD is a problem that needs the attention of the global community.

Different factors could increase the susceptibility of a child to develop ASD, including environmental, biological, genetic, pregnancy-related, and behavioral factors (Hallmayer et al., 2011; Anagnostou et al., 2014; Gesundheit and Rosenzweig, 2017). The factor with the highest probability is genetic deficits, but susceptibility to ASD is also correlated with the living environment. The exact cause of ASD remains unclear. Several interventions have been investigated and developed for ASD, including behavioral intervention therapy, drug therapy, psychological analysis, sensory integration therapy, and assistive technology. Most of these therapies originate from the perspectives of psychology and behavior and achieve treatment and relief by correcting the abnormal behavior of children with ASD or by changing their psychological state. Currently, no treatment has been shown to cure ASD or eliminate its core symptoms.

Most researchers agree that ASD is a cognitive developmental disorder caused by impaired brain function (Miyahara, 2013; Jutla et al., 2021). Dickinson et al. (2016) and Lee et al. (2017) showed the imbalances between excitation and inhibition in synaptic transmission and neural circuits in patients with ASD Anatomically, patients with ASD have more cortical mini-columns and smaller volumes than healthy individuals, particularly in the dorsolateral prefrontal cortex (DLPFC) area (Casanova, 2006; Casanova et al., 2006). Physical stimulation regulates the balance between neuronal excitation and inhibition in the nerve conduction circuit and may play a positive role in the treatment of ASD. At present, researchers have investigated various detection and repair technologies for the impairment of cognitive function in the central nervous system. Transcranial direct current stimulation (tDCS) is a non-invasive brain stimulation technique that uses weak direct current to induce changes in intracortical excitability. The direction of these modulations depends on stimulation polarity: Anodal stimulation increases excitability, whereas cathodal stimulation diminishes excitability (Vaseghi et al., 2015). The main advantages of tDCS are safety, stability, non-invasiveness, and ease of clinical application. tDCS is widely used in neurological and psychiatric diseases, such as stroke, depression, and Parkinson's disease. It has also been studied in attention-deficit hyperactivity disorder (ADHD), cerebral palsy, and language disorders, and no serious adverse reactions have been reported.

Electroencephalography (EEG) is a non-invasive method for recording the electrical activity of the brain along the scalp and is a powerful tool for studying complex neuropsychiatric disorders (Kang et al., 2018). In addition to EEG, functional magnetic resonance imaging (fMRI), which is an important neuroimaging detection method, has also been applied in many studies. However, considering that the subjects of the current study were children with ASD, it is difficult to complete long-term data collection in a small space with nuclear magnetic equipment. Therefore, the changes in evoked EEG signals and behavioral abilities of each child were investigated in the current study. Event-related potential (ERP) is an evoked potential with phase-locked time-specific events, which can reflect the neurophysiological changes of the brain during cognitive processes. When the nervous system receives a specific stimulus, relevant EEG waveforms can be detected in the corresponding regions of the brain, such as P1, N1, P2, P3, and mismatch negativity (MMN). The MMN is a change-specific component of auditory ERP, which is evoked by a discriminable change in any physical feature of a frequently presented stimulus (Ćeponiene et al., 1998; Näätänen et al., 2004). MMN is generally considered an endogenous ERP component that represents an automatic neuronal change-detection process. In addition, MMN can be measured in an unconscious situation and does not require the patient to take the initiative to participate. It is more suitable for the evaluation of children with ASD who cannot concentrate. Therefore, it is now widely used in clinical applications for the diagnosis of certain neurological deficits. Relevant studies have shown that compared with healthy children, patients with ASD have robust MMN deficits, thus suggesting an altered central ability in auditory discrimination (Vlaskamp et al., 2017; Chen et al., 2020; Di Lorenzo et al., 2020). In the current study, a single-blind, sham-controlled experiment was designed for subjects with ASD. We placed the anode of tDCS over the left DLPFC and analyzed the changes in behavior and MMN features of children with ASD after 4 weeks of treatment to verify the clinical efficacy of tDCS.

MATERIALS AND METHODS

Patient Demographics and Recruitment

Forty children with ASD (age range: 4–12 years; 33 males and 7 females) were recruited in this study from December 2018 to January 2020. The participants were all diagnosed with ASD by using the fifth edition of the *Diagnostic and Statistical Manual of Mental Disorders* (Battle, 2013), and the diagnoses were confirmed by clinical experts.

Participants were randomly assigned to the anodal tDCS stimulation group (atDCS group) or sham stimulation group (stDCS group), with 20 participants in each group. One participant dropped out of the study because of personal reasons, and two participants were excluded from the analysis because of excessive noise in the EEG recording. Therefore, we analyzed the data from 37 participants. At the end of this study, 19 participants (15 males and 4 females; mean ± SD age: 8.0 ± 1.9 years) received anodal tDCS stimulation, and 18 participants (15 males and 3 females; mean ± SD age: 7.6 ± 2.1 years) received sham stimulation. Most of the enrolled patients were accompanied by various degrees of comorbidities, including anxiety disorder,

ADHD, attenuated psychosis syndrome (APS). As shown in **Table 1**, one child may have multiple comorbidities. All patients received conventional treatment methods (details shown in section "Conventional Rehabilitation Treatment"), in addition to tDCS intervention during the enrollment period, and were drug free for at least 2 weeks before the study began. There was no statistical difference between the two groups of participants in terms of gender, age, handiness, and comorbidities. This study was approved by the ethics committee of Tianjin Union Medical Center, Tianjin, China. All participants and their guardians provided written informed consent before participation.

Transcranial Direct Current Stimulation Procedures

The tDCS device used in this study was an IS200 intelligent electrical stimulator produced by Sichuan Intelligent Electronic Industry Co., Ltd. The current was delivered through a pair of 35 cm^2 sponge electrodes (i.e., the anode and cathode) that were soaked in sterile saline before the experiment to ensure good electrical conductivity. The anodal electrode was placed over the left DLPFC (F3 in the 10/20 system), and the cathode was placed over the right supraorbital area for all participants. For the anodal tDCS, stimulation current began with an 8 s ramp-up to 1.5 mA, lasted for 20 min at 1.5 mA, and ended with an 8 s ramp-down to 0 mA. For sham stimulation, the current increased at the same rate as the anodal stimulation at the beginning; the difference is that the stimulation current of 1.5 mA only lasted for 30 s, and no current was given after the first 30 s. Both the atDCS and stDCS groups had the same intervention duration (21 min). The 30 s fade-in and fade-out times were set because the procedure ensured that the participants felt a tingling sensation at the beginning of the stimulation. Transient EEG artifacts were observed only during the fade-in and fade-out phases of tDCS stimulation. A similar protocol was used in comparable studies (Kunze et al., 2016; Kang et al., 2018). The tDCS intervention was a single-blind experiment, and the patients did not know what kind of treatment they received. tDCS stimulation was performed for a total of 4 weeks for three times a week, and the two treatment sessions were separated by at least 1 day.

Conventional Rehabilitation Treatment

There are many different manifestations in children with ASD, including social communication and behavioral and cognitive abilities. Therefore, treatment plans are usually multidisciplinary. We chose the rehabilitation treatment according to the child's individual needs, including behavioral intervention strategies (Tiura et al., 2017), sensory integration therapy (Case-Smith et al., 2015), and speech therapy methods (Li et al., 2018). **Table 2** shows the statistical results of the two groups of patients receiving different rehabilitation treatment items, and the combination of the types of treatment received by the patients was used as the evaluation index. The chi-square test was used for statistical analysis, and the results showed that there was no statistical difference between the two groups of patients. The treatment plan was the same as that for tDCS, which was also performed three times a week, and each treatment session lasted approximately 40 min.

Behavioral Measures and Evaluation

In addition to EEG acquisition, we used the aberrant behavior checklist (ABC) to evaluate the behavior of children with ASD (Kat et al., 2020). The ABC scale covers almost all aspects of the symptoms of patients with ASD. It assesses five problem aspects, including communication, feeling, body movement, language, and self-care ability, and contains 57 sub-items. The assessment requires the cooperation of the participant's guardians, who understand the child's current situation. We mainly focused on children's performance in the previous week.

To avoid bias caused by human subjective factors on the experiment results, the evaluation process of the scale adopted a double-blind method, i.e., participants and their guardians did not know whether they were in the atDCS stimulation group or the stDCS stimulation group. Furthermore, the therapist who evaluated the subject also did not know which group the participant was placed. At the same time, to ensure the accuracy of the assessment, all scale scores were completed under the guidance of an experienced therapist.

Electroencephalography Recording and Processing

The EEG signals were recorded using an extended international 10/20 system with a Neuroscan 64-electrode EEG cap. Before starting the recording, the skin resistances of 64 channels were measured and maintained below 10 kΩ. All channels were measured against a common reference (Cz) with a sampling rate of 1,000 Hz. Eye movements were monitored with two pairs of electrodes: one pair above and below the left eye and another pair at the outer corner of the left and right eyes.

In this study, the MMN was induced using an auditory oddball task, and a series of standard auditory stimuli were interspersed with deviation tones. MMN was the negative EEG wave induced by the deviation stimulus minus the standard stimulus, which generally appears 100–250 ms after stimulus onset. The oddball task contained 4 types of auditory stimuli for a total of 1,000 times. The standard stimulus frequency was 1,000 Hz, the duration was 50 ms, the sound intensity was 60 db, and the probability of occurrence was 70%. There were three types of deviation stimuli: frequency deviation stimulation (FDS; 1,500 Hz, 50 ms, 60 dB, 10%), time deviation stimulation (TDS; 1,000 Hz, 100 ms, 60 dB, 10%), and time-frequency

TABLE 1 | Number of comorbidities in the two groups.

Groups	Anxiety	ADHD	Learning disorder	Obsessive–compulsive	Oppositional defiant	Eating disorder	APS
atDCS	4	8	5	4	2	1	1
stDCS	3	9	5	4	2	0	1

TABLE 2 | Statistical analysis of the treatment items received by the two groups.

Groups	b + s1	b + s2	b + s1 + s2
atDCS (n = 19)	11	2	6
stDCS (n = 18)	9	2	7
χ^2		0.25	
p		0.882	

b, behavioral intervention strategies; s1, sensory integration therapy; s2, speech therapy.

TABLE 3 | Evaluation results of the ABC scale pre- and post-tDCS in the two groups.

Groups	Pre-tDCS	Post-tDCS	p
atDCS	80.21 ± 22.04	59.32 ± 17.30	**<0.001**
stDCS	84.00 ± 20.69	71.39 ± 17.91	**<0.001**
p	−2.086	**0.044**	

Statistical significance is shown in bold font style.

deviation stimulation (TFDS; 1,500 Hz, 100 ms, 60 dB, 10%). All auditory stimuli were standard sine waves with an intensity of 75 db. Stimuli were produced and delivered using the NeuroStim equipment. The auditory stimuli were presented through a loudspeaker, which was placed 1 m in front of the subjects. Each participant received 1,000 stimuli.

The EEG signals were re-referenced offline to bilateral mastoid electrodes (M1 and M2) at a 200 Hz sampling rate, after which the eye movement interference and baseline drift were removed. In all channels, except the four channels located on the eyes, if the standard deviation of EEG data in any 200 ms epoch was greater than 30 uV, it was considered that this segment of data might be affected by interference and caused excessive fluctuations. The epochs containing such segments were deleted in the following analysis. EEG epochs of −100 to 400 ms were extracted and averaged, and 0 ms denotes the start time when the stimulus is presented.

Before and after 4 weeks of treatment, behavioral ability assessment and EEG data collection were performed on all subjects.

Statistical Analysis

Data are presented as the mean ± standard error of the mean ($\bar{x} \pm s$). The categorical variables (gender, handiness, and comorbidity) in the basic information of the two groups were compared using the chi-square test, and type I errors were corrected using the Bonferroni method. The discrete variables (age) were compared using one-way analysis of variance. To quantitatively evaluate changes in metrics across the pre- and post-tDCS intervals, the normality of data was first investigated using the Shapiro–Wilk test ($p > 0.05$). An independent sample t-test was used to compare the data changes before and after tDCS between the sham and anode groups, and a paired sample t-test was used to analyze the changes in the same group of subjects. Pearson's correlation coefficients were used to examine the relationships between the changes in the scale evaluation results and the MMN component features.

RESULTS

Behavioral Evaluations

The ABC scores post-tDCS to pre-tDCS were compared for the two groups. The t-test returned normality at $p = 0.05$. As shown in **Table 3**, there was no significant difference in the ABC scores between the two groups before tDCS treatment ($p > 0.05$). After treatment, the behavioral abilities of both groups improved and were statistically significant compared with those before treatment ($p < 0.05$). The comparison between the two groups of patients showed that the atDCS group performed significantly better than the stDCS group, thus indicating that the anode tDCS has a positive effect on the treatment of ASD.

Mismatch Negativity Features

The responses were averaged separately for each stimulus type for each subject. To quantify the MMNs, the evoked response to the standard tone was subtracted from the corresponding deviant stimulus response, and the amplitude and latency at peak were measured for all electrodes.

Figure 1 shows the MMN waveforms from different deviant tones in the Fz channel of two typical subjects (one from the atDCS group and the other from the stDCS group). It can be seen from the figure that the MMNs from FDS have lower amplitudes and shorter latencies than with TDS and TFDS. After 4 weeks of treatment, the participants' MMNs showed an increase in amplitude and decrease in latency, and the average changes in the atDCS group were greater than those in the stDCS group. **Figure 2** shows the grand averages of Fz for each group. Similar to **Figure 1**, the MMN amplitude of the two groups of subjects increased after treatment, but the difference between the groups did not seem to be significant.

Figure 3 shows the TD-evoked MMN waveforms of all electrodes pre- and post-treatment for a subject in the atDCS group and the amplitude brain topographies at the peak moments of most electrodes. It can be seen from the MMN waveforms that most of the amplitudes increased after treatment and that the latencies decreased. **Figures 3C,D** show that the blue area of the brain topographic map increased after treatment, thus indicating that the activation range was enlarged.

Table 4 shows the statistical analysis focused on MMN latency and amplitude in the atDCS and stDCS groups at the Fz electrode measured under the three deviant conditions. There was no statistical difference in the amplitude and latency of all participants' MMNs when participating in this study. After 4 weeks of treatment, the average MMN amplitude and latency, respectively, increased and decreased for both groups under the three types of deviation stimuli. The improvement in the atDCS group was more significant than that in the stDCS group. The p-value in bold font style represents statistical difference ($p < 0.05$). In the atDCS group, all evaluated items were statistically significant, except for the latency of the FDS. The changes in the amplitude of TDS and TFDS and the latency of TDS in

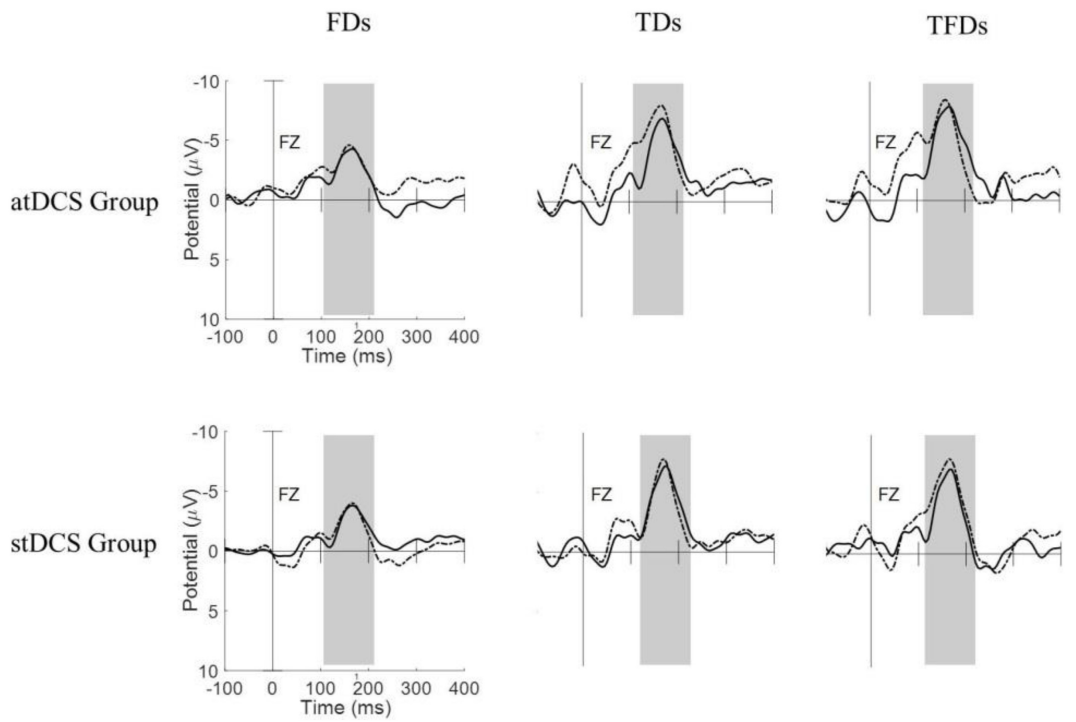

FIGURE 1 | Responses of the three types of deviation stimuli (FDS, TDS, and TFDS) from pre- and post-tDCS states in the two groups of subjects (atDCS group and stDCS group). Dashed line = pre-tDCS, solid line = post-tDCS.

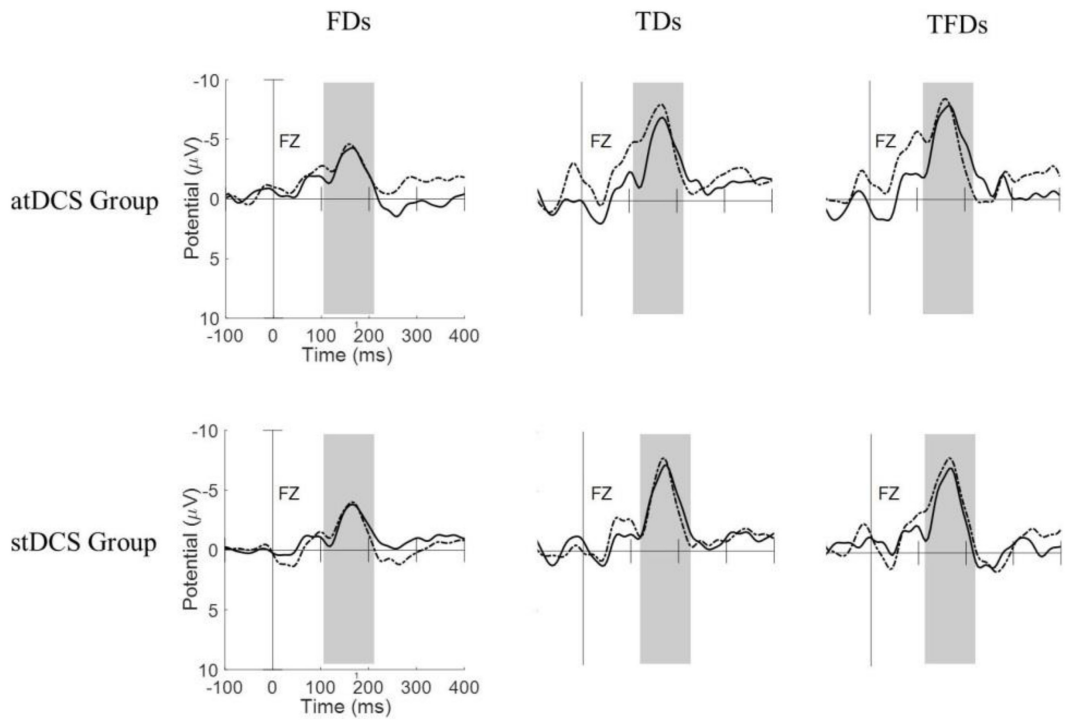

FIGURE 2 | Grand averages of MMNs for all subjects of each group under the three types of deviation stimuli. Dashed line = pre-tDCS, solid line = post-tDCS.

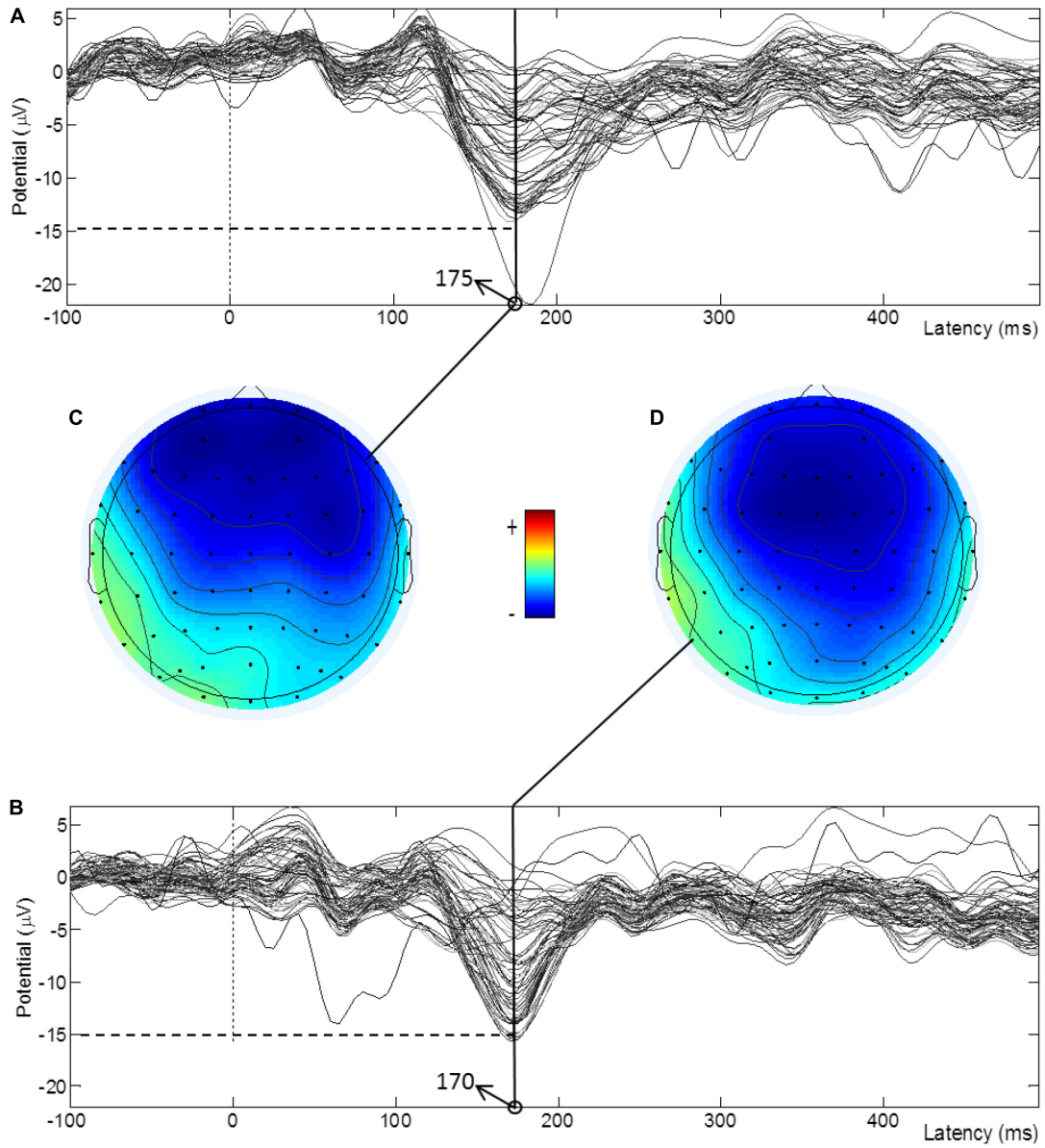

FIGURE 3 | TDS-evoked MMN waveforms of all electrodes pre- and post-treatment for a subject in the atDCS group and the amplitude brain topographies at the peak moments. **(A)** MMN waveform before treatment. **(B)** MMN waveform after treatment. **(C)** Peak amplitude topographic map before treatment. **(D)** Peak amplitude topographic map after treatment.

the stDCS group were statistically significant. Although the differences in MMN amplitude and latency were not statistically significant, the average MMN latency and amplitude were, respectively, shorter and higher in the atDCS group than in the stDCS group.

In addition to Fz, we analyzed the changes in the MMN features of all other leads. **Table 5** shows the electrode names with statistical differences pre- and post-tDCS intervention, which can be seen mainly in the prefrontal, temporal, and central areas. The atDCS group had more electrodes with significant differences than the stDCS group. Among the three types of deviated stimuli, TDS have the largest number of differential electrodes, followed by TFDS, and FDS had the lowest number of differential electrodes.

Correlation Between Aberrant Behavior Checklist and Mismatch Negativity

The features of ERP components are generally affected by individual differences. This study adopted a special normalization process to accurately reflect the improvement of patients. The ratio of post-tDCS to pre-tDCS was used as an index to evaluate the degree of MMN improvement. The difference between the post-tDCS and pre-tDCS scale scores was used as an indicator

TABLE 4 | Mean amplitudes and latencies of MMN in two groups of children at the Fz electrode.

Deviant type	MMN wave	atDCS group (n = 19)	stDCS group (n = 18)	p
FDS	Pre-tDCS latency (ms)	163.68 ± 5.74	164.44 ± 5.66	0.688
	Post-tDCS latency (ms)	161.05 ± 4.59	162.78 ± 4.28	0.698
	p	0.056	0.083	
	Pre-tDCS amplitude (uV)	4.40 ± 0.92	4.19 ± 1.04	0.525
	Post-tDCS amplitude (uV)	4.97 ± 0.78	4.38 ± 1.00	0.051
	p	**0.015**	0.454	
TDS	pre-tDCS latency (ms)	171.84 ± 6.91	172.78 ± 5.75	0.658
	post-tDCS latency (ms)	167.63 ± 5.86	169.17 ± 4.62	0.384
	p	**0.002**	**0.019**	
	Pre-tDCS amplitude (uV)	8.07 ± 2.09	7.92 ± 1.99	0.826
	Post-tDCS amplitude (uV)	8.86 ± 1.93	8.54 ± 1.81	0.608
	p	**0.002**	**0.004**	
TFDS	Pre-tDCS latency (ms)	167.11 ± 6.08	167.22 ± 5.48	0.951
	Post-tDCS latency (ms)	162.37 ± 5.62	165.28 ± 6.06	0.139
	p	**0.02**	0.261	
	Pre-tDCS amplitude (uV)	8.41 ± 2.18	8.14 ± 1.92	0.688
	Post-tDCS amplitude (uV)	9.32 ± 2.16	8.83 ± 1.54	0.436
	p	**0.011**	**0.031**	

Statistical significance is shown in bold.

TABLE 5 | List of electrodes with significant differences ($p < 0.05$) in the amplitude and latency of the three deviation stimuli between the two groups.

Deviant type	MMN features	Electrodes	
		atDCS group	stDCS group
FDS			
	Latency	FP1, F3, F1, FC1, C5, C3, T8	AF3, F5, F6, F8, C5, CP5
	Amplitude	F5, Fz, FC1, FCz, CP1, P3, PO3	F1, FT7, T7, TP7, CP4
TDS			
	Latency	F1, Fz, F2, FC1, FCz, Cz, C2, CPz, CP6, P6, PO8	Fz, FC3, FC1, C3, P5, PO5, O1
	Amplitude	FP1, AF3, F5, F1, Fz, F4, FC1, FCz, CPz, P4, P6, O2	FPz, FP2, F5, Fz, F2, FC2, CP2, POz, O2
TFDS			
	Latency	FP1, FPz, AF4, Fz, F2, FCz, FC2 C2, CP2, CP4	F3, F1, F2, C3, C1, P5, P2, POz
	Amplitude	FP1, F7, F5, F1, Fz, FC1, FC4, CP3, P4, O2	F3, Fz, F2, FC2, C2, CP3

of behavioral changes. **Figure 4** shows the correlation between the changes in MMN components at the Fz electrode and the changes in behavioral abilities for all subjects under the three types of deviation stimuli. Only results with $p < 0.05$ are shown in **Figure 4**. The amplitude and latency of the FDS demonstrated a significant linear correlation with the improvement in behavioral ability pre- and post-tDCS ($p < 0.05$). For TDS and TFDS, only the latency of MMN had a significant linear correlation with the improvement in behavioral ability ($p < 0.05$), and there was no statistical relationship between behavioral ability and amplitude ($p > 0.05$).

DISCUSSION

tDCS is a typical representative of non-invasive brain stimulation (NIBS) that can use a weak current to adjust the activity of cerebral cortex neurons and change the potential of transmembrane neurons, thereby affecting the level of excitability and regulating the firing rate of neurons (Vines et al., 2008). In the current study, the anode tDCS was used to intervene with the DLPFC area of children with ASD, and the effects of behavioral abilities and evoked EEG characteristics were investigated in a controlled study. The results show that 4 weeks of rehabilitation treatment can effectively increase the amplitude of auditory-induced MMN, decrease the latency, and improve behavioral ability. After the treatment, the improvements in participants with anode stimulation were generally greater than those with sham stimulation.

In this study, the anode electrode was placed in the left DLPFC. Abnormalities in the prefrontal area are closely related to symptoms such as attention distribution disorder, weak executive ability, and organization disorder in patients with ASD (Zhou et al., 2020). Dickinson et al. (2016) showed that there is an imbalance between excitation and inhibition in the neural circuits of patients with ASD, particularly in the DLPFC. The results of the current study showed that the behavioral abilities of the two groups of patients significantly improved after treatment. The atDCS group improved more than the stDCS group, thus indicating that the effect of anode tDCS in the DLPFC area can effectively regulate the relationship between neuronal excitation and nerve conduction circuit inhibition. The results of our study reveal that tDCS can improve the coordination ability of the brain and nervous system and have a positive effect on the treatment of ASD.

The MMN reflects the processing of sound differences by the human auditory system. The latency represents the functional state of the auditory sensory pathway, and the amplitude is closely related to the state of the cortex (Näätänen et al., 2007). Relevant studies have shown that the MMN of children with ASD is significantly weaker than that of healthy children (lower amplitude and longer latency), thus indicating that children with ASD may have a weaker response to abnormal environmental stimuli at the auditory level (Vlaskamp et al., 2017; Chien et al., 2018). Impey et al. (2016) used MMN to evaluate the effect of tDCS on the auditory perception of healthy participants, and the results showed that tDCS can enhance the MMN component (increased amplitude and shortened latency). In the current study, the evoked EEG data of the three types of deviated stimuli were collected. As shown in **Figure 1**, the average amplitude of the FDS was lower than that of the other two deviated

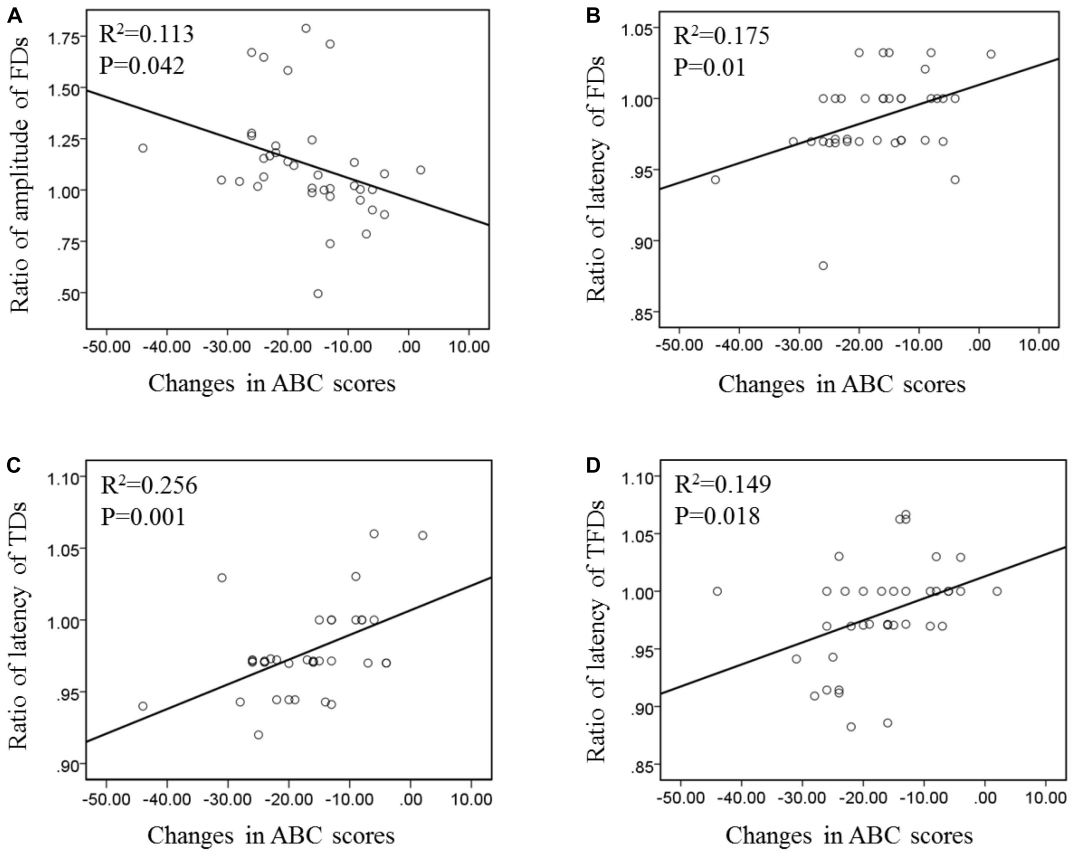

FIGURE 4 | Correlation of the changes between MMN and behavior ability under the three types of deviation stimuli pre- and post-tDCS. **(A)** Correlation between the amplitude of FDS and ABC. **(B)** Correlation between the latency of FDS and ABC. **(C)** Correlation between the amplitude of TDS and ABC. **(D)** Correlation between the amplitude of TFDS and ABC.

stimuli, and this finding might be caused by the difficulty in distinguishing the difference between 1,000 and 1,500 Hz sound stimuli. The latency of the FDS was the shortest among the MMN components of the three types of deviation stimuli. We believe that the time deviation is a process that requires accumulation over time, whereas the frequency deviation can be distinguished instantaneously. Therefore, the MMN evoked by FDS appears to have less latency. The increase in amplitude and decrease in latency suggest functional improvements in neurological pathways and are correlated with ABC score improvements. In the atDCS group, except for the change in latency from FDS, pre-tDCS did not show statistical significance compared with post-tDCS, but the other features improved significantly. The latency amplitude of TDS and TFDS and the latency of TDS in the stDCS group improved significantly after treatment. Overall, the improvement in the atDCS group was higher than that in the stDCS group, but there was no statistical difference in MMN features between the two groups after treatment. Considering that the syndromes of ASD and the neurological deficits that lead to ASD might be complicated, a 4 week treatment might not be enough to cure ASD completely. Therefore, the long-term efficacy of tDCS treatment on ASD should be investigated with precise control of other stimulation parameters in the future.

Another important information given in **Figure 3** is that after the treatment of anode tDCS, the activation area of the brain increased, thus indicating that tDCS has a positive significance in the neuroregulation of children with ASD; this result also verifies the results of previous studies (Amatachaya et al., 2015; Palm et al., 2016).

Liu et al. (2017) showed that subjects with lower cognitive function scores had a longer ERP latency and a lower amplitude. In the current study, the correlations of the changes between MMN and behavioral ability under these three types of deviation stimuli were analyzed in ASD participants pre- and post-tDCS. The results show that the changes in the amplitudes of the proposed three types of deviant stimuli are linearly related to the changes in the ABC scale scores, thus indicating that MMN features can reflect the behavioral ability of children with ASD and can be used as an objective and quantitative evaluation method. This finding is consistent with the results of Wang et al. (2018), who showed that tDCS can improve the MMN amplitude of patients with impaired consciousness and concluded that MMN is expected to serve as an auxiliary evaluation tool for the treatment effect of tDCS. Only the FD stimulus induced the latency features of MMN and correlated with changes in behavioral ability, thus showing that the response speed of MMN

evoked by FDS can better reflect the behavioral ability level in children with ASD.

The current study has some limitations. First, rehabilitation treatment for ASD is generally a long process. Four weeks of tDCS intervention might not be long enough to demonstrate its unique advantages in improving the MMN features of some participants. Second, the pre- and post-tDCS clinical data of subjects may not be sufficient. Follow-up studies with a more comprehensive evaluation of patients, including fMRI and functional near-infrared spectroscopy, are needed to further investigate the effectiveness of tDCS in children with ASD.

CONCLUSION

In this study, we utilized tDCS to stimulate the DLPFC area among children with ASD and analyzed the changes in evoked EEG signals and behavioral abilities in the anode and sham stimulation groups. The results showed that the latency of MMN in children with ASD tended to decrease and that the average amplitude tended to increase after stimulation; these features are similar to the MMN features from healthy children. The ABC scale evaluation results show that both tDCS treatment and traditional rehabilitation treatment can effectively improve the behavioral ability of children with ASD, and the improvement is more significant after the use of anode tDCS. The results of the correlation analysis between EEG and the scale show that MMN features have a good correlation with the behavioral ability of children with ASD, particularly the latency features of MMN. Despite the limitations of this study, it is evident that tDCS has positive effects on children with ASD, and the features of MMN can be considered in the assessment of children with autistic behavior ability.

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 Ethics Committee of Tianjin Union Medical Centre, Tianjin, China. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CS, YZ, and CW contributed to study design and patient recruitment. ZZ, RT, and WZ contributed to data collection. CS and LC contributed to data analysis, data interpretation, and manuscript preparation. JD and YZ contributed to data interpretation and critically revised. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Natural Science Foundation of China (Grant No. 81871469) and the Tianjin Health Science and Technology Project (Grant No. QN20029).

ACKNOWLEDGMENTS

We wish to thank Hongli Yu for her help in data analysis and Xin Zhang for his assistance in document preparation. Also, we would like to thank Editage (www.editage.com) for English language editing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2022.721987/full#supplementary-material

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Conflict of Interest: LC was employed by China Electronics Cloud Brain (Tianjin) Technology Co., Ltd., Tianjin, China.

The remaining 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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frontiers in Neuroscience

ORIGINAL RESEARCH
published: 18 February 2022
doi: 10.3389/fnins.2022.806990

The Synaptic Gene Study: Design and Methodology to Identify Neurocognitive Markers in Phelan-McDermid Syndrome and *NRXN1* Deletions

Jennifer Cooke[1†], Ciara J. Molloy[2†], Antonia San José Cáceres[1,3,4], Thomas Dinneen[2], Thomas Bourgeron[5], Declan Murphy[1], Louise Gallagher[2‡] and Eva Loth[1*‡]*

[1] Forensic and Neurodevelopmental Sciences, Institute of Psychiatry, Psychology & Neuroscience, King's College London, London, United Kingdom, [2] Department of Psychiatry, School of Medicine, Trinity College Dublin, Dublin, Ireland, [3] Fundación para la Investigación Biomédica del Hospital General Universitario Gregorio Marañón, Madrid, Spain, [4] Biomedical Research Networking Center for Mental Health Network (CIBERSAM), Madrid, Spain, [5] Department of Neuroscience, Institut Pasteur, Paris, France

OPEN ACCESS

Edited by:
Francesco Craig, University of Calabria, Italy

Reviewed by:
A. Gulhan Ercan-Sencicek, Masonic Medical Research Institute (MMRI), United States
Elena Bacchelli, University of Bologna, Italy

***Correspondence:**
Jennifer Cooke
jennifer.cooke@kcl.ac.uk
Eva Loth
eva.loth@kcl.ac.uk

†These authors have contributed equally to this work and share first authorship

‡These authors have contributed equally to this work and share last authorship

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: *01 November 2021*
Accepted: *26 January 2022*
Published: *18 February 2022*

Citation:
Cooke J, Molloy CJ, Cáceres ASJ, Dinneen T, Bourgeron T, Murphy D, Gallagher L and Loth E (2022) The Synaptic Gene Study: Design and Methodology to Identify Neurocognitive Markers in Phelan-McDermid Syndrome and NRXN1 Deletions. Front. Neurosci. 16:806990. doi: 10.3389/fnins.2022.806990

Synaptic gene conditions, i.e., "synaptopathies," involve disruption to genes expressed at the synapse and account for between 0.5 and 2% of autism cases. They provide a unique entry point to understanding the molecular and biological mechanisms underpinning autism-related phenotypes. Phelan-McDermid Syndrome (PMS, also known as 22q13 deletion syndrome) and *NRXN1* deletions (NRXN1ds) are two synaptopathies associated with autism and related neurodevelopmental disorders (NDDs). PMS often incorporates disruption to the *SHANK3* gene, implicated in excitatory postsynaptic scaffolding, whereas the *NRXN1* gene encodes neurexin-1, a presynaptic cell adhesion protein; both are implicated in *trans*-synaptic signaling in the brain. Around 70% of individuals with PMS and 43–70% of those with NRXN1ds receive a diagnosis of autism, suggesting that alterations in synaptic development may play a crucial role in explaining the aetiology of autism. However, a substantial amount of heterogeneity exists between conditions. Most individuals with PMS have moderate to profound intellectual disability (ID), while those with NRXN1ds have no ID to severe ID. Speech abnormalities are common to both, although appear more severe in PMS. Very little is currently known about the neurocognitive underpinnings of phenotypic presentations in PMS and NRXN1ds. The Synaptic Gene (SynaG) study adopts a gene-first approach and comprehensively assesses these two syndromic forms of autism. The study compliments preclinical efforts within AIMS-2-TRIALS focused on *SHANK3* and *NRXN1*. The aims of the study are to (1) establish the frequency of autism diagnosis and features in individuals with PMS and NRXN1ds, (2) to compare the clinical profile of PMS, NRXN1ds, and individuals with 'idiopathic' autism (iASD), (3) to identify mechanistic biomarkers that may account for autistic features and/or heterogeneity in clinical profiles, and (4) investigate the impact of second or multiple genetic hits on heterogeneity in clinical profiles. In the current paper we describe our methodology for phenotyping the

sample and our planned comparisons, with information on the necessary adaptations made during the global COVID-19 pandemic. We also describe the demographics of the data collected thus far, including 25 PMS, 36 NRXN1ds, 33 iASD, and 52 NTD participants, and present an interim analysis of autistic features and adaptive functioning.

Keywords: synaptopathies, autism, rare genetic variants, biomarker, Phelan-McDermid syndrome, *NRXN1* deletion

INTRODUCTION

Autism Spectrum Disorder (ASD)[1] is characterized by difficulties in communication and social interaction, as well as the presence of restricted and repetitive behaviors and sensory anomalies (American Psychiatric Association [APA], 2013). Currently, around 1 in 54 children are identified as autistic (Maenner et al., 2020), yet still little is known about the links between genomics and clinical outcomes. A significant amount of clinical heterogeneity is observed in autism, with around 70% of people also experiencing other conditions, such as ADHD, anxiety, or epilepsy (Simonoff et al., 2008; Hofvander et al., 2009; Ung et al., 2013; Besag, 2018; Boxhoorn et al., 2018; Lukmanji et al., 2019; Hossain et al., 2020). Despite significant individual variability, autism is a highly heritable condition with strong evidence for the contribution of genetic factors including both inherited and rare *de novo* mutations (Sebat et al., 2007; Buxbaum, 2009; Sanders et al., 2015; Sandin et al., 2017; Bai et al., 2019; Yoon et al., 2021). Although many genes are implicated, these are known to converge on key biological processes, particularly synaptic development and plasticity (Voineagu et al., 2011; De Rubeis et al., 2014; Leblond et al., 2021). Thus, genetic profiles may go some way to explaining the clinical heterogeneity observed in autism and provide key insights into phenotypes linked with specific aetiologies.

Synaptic gene conditions, i.e., "synaptopathies," involve disruption to genes expressed at the synapse. As some synaptopathies have high penetrance for autism, they provide a unique entry point to better understand the molecular and biological mechanisms underpinning autism-related phenotypes. Such insights are needed to identify new therapeutic targets. They have added to the interest in 'gene-first' approaches, which focus on stratifying based on genetic diagnosis, as they open the possibility that new mechanistic treatments, identified in rare synaptopathies, may be applicable for wider sub-groups of individuals with so-called 'idiopathic' autism (i.e., without any known genetic rare variants). Hence, the present study was designed to compare two synaptic gene conditions with high penetrance for autism; Phelan-McDermid Syndrome (PMS, also known as 22q13 deletion syndrome, typically including haploinsufficiency of the *SHANK3* gene) and *NRXN1* deletions (NRXN1ds). It is estimated that around 70% of individuals with PMS and 43–70% of individuals with NRXN1ds receive a diagnosis of autism (Soorya et al., 2013; Castronovo et al., 2020). However, despite the reported prevalence of autism within PMS and NRXN1ds, not all individuals receive a diagnosis. In fact, mounting evidence suggests that there may be greater clinical heterogeneity within syndromic or 'monogenic' conditions than previously thought (Oberman et al., 2015). For example, both conditions also frequently involve other neurodevelopmental or psychiatric symptoms, including ADHD, psychosis, schizophrenia, bipolar disorder and others (Guilmatre et al., 2014; Kirov et al., 2014; Kolevzon et al., 2019; Cosemans et al., 2020; Vogels et al., 2021). Whilst co-occurring intellectual disability (ID) is common in both synaptopathies, it may vary in severity. Individuals with PMS typically present with moderate to profound ID and delayed or absent speech (Phelan and McDermid, 2012; Soorya et al., 2013; Kolevzon et al., 2014; Oberman et al., 2015). Meanwhile, those with NRXN1ds can present with a more variable cognitive abilities ranging from none to severe ID (Al Shehhi et al., 2019; Cosemans et al., 2020). Heterogeneity in the wider clinical phenotypes of these two conditions may in part be explained by underlying genetic heterogeneity, such that in NRXN1ds, clinical outcome is thought to be attributed to deletion location and possibly size within the *NRXN1* gene (Schaaf et al., 2012; Castronovo et al., 2020; Cosemans et al., 2020). This heterogeneity even within particular monogenic synaptopathies raises both the challenge and opportunity to trace mechanisms by which atypical synaptic transmission, at different developmental levels, brain cells and circuits, may lead to convergent or divergent biological, cognitive and clinical features.

Both *NRXN1* and *SHANK3* genes have an important role in the development of synaptic efficiency, including the maintenance of excitatory and inhibitory *trans*-synaptic signaling. *NRXN1* encodes a presynaptic cell adhesion protein that is implicated in *trans*-synaptic signaling at both excitatory and inhibitory (i.e., glutamatergic and GABA-ergic) synapses within the brain (Reissner et al., 2008, 2013; Anderson et al., 2015; Tong et al., 2017). Meanwhile, *SHANK3* encodes a glutamatergic postsynaptic scaffolding protein that affects the morphology of dendritic spines and synaptic transmission (Gouder et al., 2019; Huang et al., 2019). Initiatives such as the Synaptopathies Consortium, led by Dr. Deepak Srivastava at King's College London, investigate synaptic biology with a specific focus on glutamatergic synapses, and have shown that stem-cells derived from autistic individuals with *SHANK3* mutations can be linked to early neuronal morphogenesis, suggesting that synaptic development plays a vital role in the pathogenesis of autism (Kathuria et al., 2018). Synaptopathies are thought to affect a fundamental property of brain function, namely the coordinated excitatory and inhibitory activity both at the level of individual neurons (by maintaining appropriate ratios of excitatory vs. inhibitory synaptic inputs), and at the level of global firing

[1]Henceforth referred to as autism in accordance with the express wishes of the autistic community.

patterns within or across networks (Gao and Penzes, 2015). An "imbalance" in excitatory and inhibitory (E/I) transmission has emerged as an influential, albeit somewhat unspecified, theory of a putative pathophysiological mechanism underpinning phenotypic features of autism, such as sensory hypersensitivity, and related neurodevelopmental/neuropsychiatric disorders. At present, animal models exploring *NRXN1* and *SHANK3* expression support E/I imbalance as an index for autistic neural processing (Etherton et al., 2009; Lee et al., 2015, 2017; Sudhof, 2017). E/I imbalance is also suggested from iPSC lines derived from autistic individuals with NRXN1ds, such that derived neurons show upregulated voltage-gated calcium channels and increased Calcium activity (Avazzadeh et al., 2019), as well as increased neuronal excitability (Avazzadeh et al., 2021). This altered neuronal excitability observed in electrophysiological studies brings us closer to identifying neurocognitive markers linked to autistic phenotypes.

It is difficult to directly measure E/I at any level in the living human brain. Although there are now several ligands available to measure glutamate or GABA receptor densities using Positron Emission Tomography (Finnema et al., 2015), the technique is invasive and unsuited for children and/or vulnerable populations. Magnetic Resonance Spectroscopy (MRS) measures, for example glutamate or GABA concentrations in particular brain regions, with the caveat that so-called voxels can span an entire brain structure, thus providing very coarse resolution. Recently, several markers derived from electroencephalography (EEG) have been suggested as 'proxy' measures of E/I balance with the potential advantage that EEG is a widely available, relatively inexpensive methodology. Such putative proxy measures include oscillations in the high-frequency gamma and beta ranges, which are thought to reflect, respectively, synchronized fluctuations of the membrane potential of excitatory and inhibitory neurons and involvement of inhibitory interneurons, gated by GABA(A). In addition, neural dynamics, such as the 1/f power law function has been proposed as an index of global network function, while kappa computed dynamically throughout EEG recordings may index critical state change in the brain phases between asynchrony and synchrony (Shew et al., 2009).

Large scale multi-modal natural history studies of rare genetic syndromes are still limited. Although, in the United States, the US Developmental Synaptopathies Consortium[2], compares people with PMS, Tuberous Sclerosis Complex (TSC1/2), and PTEN Hamartoma Tumor Syndrome.

The Synaptic Gene Study (SynaG) is the first European study that uses a multi-modal approach to address four specific objectives associated with explaining heterogeneity in autism: (1) investigating the frequency of autistic and other neurodevelopmental/neuropsychiatric features within clinically ascertained synaptic gene conditions (PMS and NRXN1ds), (2) determining the extent of shared versus unique autistic features between synaptopathies and those with idiopathic autism, (3) identifying neurocognitive stratification biomarkers, such as E/I imbalance, linked to genotype, and (4) examining the impact of deletion size and additional common or rare genetic factors, such as common genetic burden or additional rare copy number variants (CNV) or single nucleotide variants (SNV), on heterogeneity in the clinical profile of synaptopathies.

SynaG was originally set up as part of EU-AIMS (Loth et al., 2014) and was designed to comprehensively phenotype synaptopathies linked to autism, with a specific focus on PMS. This natural history study aimed to complement pre-clinical work streams using *SHANK3* and *NRNX1* animal models, and human cellular assays focused on synaptopathies that were conducted under the auspices of EU-AIMS to understand the pathophysiological mechanisms underpinning autism-related phenotypes and features (Etherton et al., 2009; Kathuria et al., 2018; Avazzadeh et al., 2021). From this, potential targets for treatment may be highlighted, leading to more personalized approaches to specific neurobiological mechanisms involved in autism.

It is important to clarify at this point that SynaG promotes representation within research and does not seek to pathologise the lived experience of those with synaptopathies or reduce the value of individual variability within autism. The emphasis on biomarker discovery within SynaG serves only to promote treatment choices for those who feel such an approach may improve a person's quality of life. It is in no way to imply that drug treatment in autism is viewed by authors as required or necessary.

The NRXN1ds research began as a separate study with a different protocol and was added to SynaG more recently as part of AIMS-2-TRIALS[3]. The study protocols have been aligned across two research sites to allow for comparisons across cohorts within SynaG (see **Supplementary Table 1** for SynaG Protocol). Historically, the two studies included each separate comparison groups of, respectively, mental and chronological age-matched participants with typical development. However, SynaG is now embedded within the clinical research portfolio of AIMS-2-TRIALS longitudinal, multidisciplinary research studies. This includes a longitudinal infant sibling studies of 300 babies at high familial likelihood of developing autism or ADHD by virtue of having a first degree family member with either condition, the Preschool Brain Imaging and Behavior Study (PIP), which follows 500 children with autism, ADHD, ID, epilepsy from the age of 3–6 years, and the Longitudinal European Autism Project (LEAP), which follows 730 individuals with autism, mild ID or typical development aged 6–30 years across three time points over 8 years (Loth et al., 2017). Aligning the SynaG protocol with PIP and LEAP, depending on the participant's age or ability level, allows us to make use of a large number of comparison participants with typical development, ID or ASD. In particular, it enables us to adopt a "normative modeling" approach across experimental measures (i.e., cognition, eye-tracking, EEG, and MRI indices) which is akin to normative growth charts frequently used by pediatricians to assess a child's height or weight (Marquand et al., 2019). This approach first involves deriving "standardized" scores of different age groups in terms of say, regional cortical thickness, and then ascertains how far scores of a particular individual (with PMS and NRXN1ds) differ from expectations based on the participant's age, mental

[2]https://www1.rarediseasesnetwork.org/cms/dsc

[3]https://www.aims-2-trials.eu/

age, sex and/or other variables (see **Figure 1**). This approach is well-suited to make predictions about an *individual* (as opposed to group-level inferences), which is critical for the clinical use of biomarkers.

In addition, some aspects of the SynaG protocol are also aligned with the Developmental Synaptopathies Consortiums natural history study of PMS protocol (led by Alexander Kolevzon at Icahn School of Medicine at Mount Sinai[4]) for data pooling and replication, which is critical when working with rare disorders.

The current paper sets out the design and methodology for the SynaG study. Given the prevalence of mild to profound ID within our genetic populations of interest, we also include strategies used to facilitate engagement, in addition to the necessary adaptations made during the global COVID-19 pandemic. As data acquisition is ongoing, we present sample demographics and task completion numbers for each modality at the time of writing (August 2021).

MATERIALS AND METHODS

Design

The study adopts a cross-syndrome design including two genetically defined cohorts: PMS, NRXN1ds, and two comparison groups of idiopathic autism (iASD) (with ID) and neurotypical development (NTD). The individual and both biological parents are invited to take part in the study so that the heritability of certain genetic features and clinical characteristics associated with autism can be assessed.

Sample

The objective is to assess 50 PMS, 50 NRXN1ds, 30 iASD with ID, 100 typically developing (NTD) matched controls (N = 230). Sample sizes for each participant group were determined with consideration of data collection from the existing EU-AIMS LEAP study and with the feasibility of data collection in mind, given that both PMS and NRXN1ds are rare conditions. Participants are recruited and tested at two different sites, King's College London (KCL) and Trinity College Dublin (TCD). Written consent or assent is obtained from participants, and from a parent/legal guardian where the participant is below the age of 16 (United Kingdom) or 18 years (Ireland) and where participants lack mental capacity to consent for themselves, for example in cases of profound ID. Where necessary, mental capacity is assessed by trained members of the research team in conjunction with parent/legal guardian appraisal. Ethical approval was obtained from St. James' Hospital/The Adelaide and Meath National Children's Hospital (REC ref: 2015/03/01) and the St. James' Hospital/Tallaght University Hospital Joint Research Ethics Committee [REC ref: 2019-09 List 35 (10)] at TCD, and the UK Health Research Authority via the Queens Square Research Ethics Committee (REC ref: 15/LO/0305) and the South London and Maudsley NHS Foundation Trust (SLaM ref: CSA/17/001) at KCL.

Mental age-matched NTD and iASD comparison groups have been recruited to account for the impact of severe to profound ID on phenotypic presentation in PMS. As severe

[4]https://www1.rarediseasesnetwork.org/cms/dsc/Get-Involved/Studies/7902

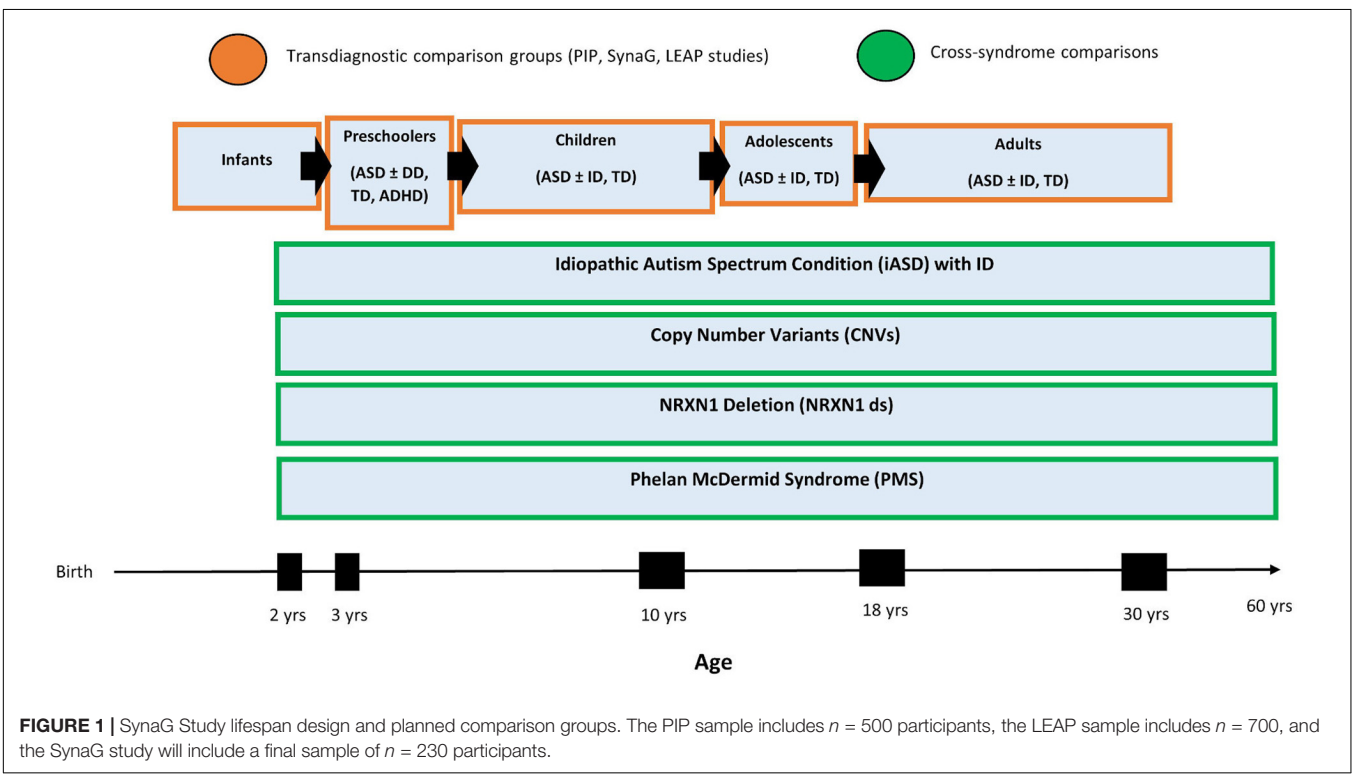

FIGURE 1 | SynaG Study lifespan design and planned comparison groups. The PIP sample includes n = 500 participants, the LEAP sample includes n = 700, and the SynaG study will include a final sample of n = 230 participants.

ID is less prevalent within NRXN1ds, chronological age and gender-matched NTD participants have also been recruited to serve as an adequate comparison of age and ability level for this group. As a result, the current NTD group includes a wide range of ages (18 months – 54 years) and developmental levels (i.e., toddlerhood – adulthood). To ensure a fair comparison of ability level between our affected groups (PMS, NRXN1ds, and iASD) and typical development we have categorized our current NTD group as follows for this paper: NTD-PMS, referring to those younger NTD participants more closely representing the ability level of PMS and iASD participants with severe to profound ID, and NTD-NRXN, who are age and gender-matched to NRXN1ds participants and more closely represent the ability level of this genetic group. Current sample demographics, including chronological age (CA) and non-verbal mental age (NVMA) are presented in **Table 1**.

Crucially, our current iASD comparison group includes only individuals with co-occurring ID to control for severe to profound ID in our PMS group. Although, as outlined previously, greatly enriched comparison groups will ultimately be generated by combining samples from several studies within the AIMS-2-TRIALS research portfolio.

Inclusion and Exclusion Criteria

Participants with PMS or NRXN1ds are included if they have clinically confirmed CNVs via existing clinical genetics reports. For PMS, we allow both individuals with and without a *SHANK3* deletion. CNVs are confirmed using aCGH or SNP array from accredited clinical genetics services in the United Kingdom or Ireland, or through SNP array by our AIMS-2-TRIALS consortium partners at Institut Pasteur in Paris, France. iASD is defined in our study as first diagnosis autism in the absence of any known genetic syndrome, at the time of study enrollment, which is then confirmed by consortium partners at Institut Pasteur. iASD participants are in receipt of a formal diagnosis of Autism Spectrum Disorder (ASD) according to either the Diagnostic and Statistical Manual for Mental Disorders (DSM) or the International Classifications of Diseases (ICD) criteria (American Psychiatric Association [APA], 2000, 2013; World Health Organization [WHO], 2013).

For PMS, NRXN1ds, and iASD cohorts, additional co-occurring neurodevelopmental or psychiatric conditions are allowed (except for psychosis in the iASD group), given the prevalence of comorbidity and because ascertaining neurodevelopmental/neuropsychiatric features is a key objective of the study. Participants on stable medication (minimum of 8 weeks at entrance point) are also eligible for the study (in all groups, including NTD). Exclusion criteria for the NTD group is a formal diagnosis of any neurodevelopmental or neuropsychiatric condition and/or the presence of any professional concerns or investigations around development, i.e., from teachers or health professionals.

Noteworthy, as NRXN1ds can be inherited, any biological parent subsequently identified as a carrier of a NRXN1ds following their child's genetic diagnosis are also included in the study as participants, but often have no known diagnosis of a neurodevelopmental condition.

Study Visit Structure

Participating families are invited to attend 2-day visits at the research institute. Day 1 consists of clinical and cognitive phenotyping assessments, parent interviews, eye-tracking tasks, EEG recording, and biosampling. Day 2 typically involves the MRI scanning protocol and the completion of outstanding tasks. The visit structure broadly follows that of the EU-AIMS LEAP and is described in more depth in a previous publication (Loth et al., 2017).

Participants are allocated to testing schedules based on their estimated NVMA as ascertained during screening, and therefore complete measures suitable to their level of non-verbal ability. Consequently, certain tasks/measures are not completed in all groups. All tasks in the study protocol are considered optional, thus, families can consult with the research team and choose to opt-out of certain tasks, depending on a participant's individual

TABLE 1 | Current sample demographics including both KCL and TCD sites.

		PMS	NRXN1ds	iASD	NTD-PMS	NTD-NRXN
Total *N*		25	36	33	30	22
Gender (f:m)		12:13	17:19	4:29	13:17	9:13
Autism dx (%)		32	33	100	0	0
Non-verbal mental age (NVMA) in years*	***n***	**21**	**20**	**28**	**28**	**21**
	Mdn	1.54	32.51	3.33	4.59	35.23
	IQR	10.02	27.80	2.31	2.38	25.05
	Min–max	0.50–47.32	7.75–51.68	0.75*–10.44	1.46–7.19	9.49–55.70
Chronological age (CA) in years	***n***	**25**	**36**	**33**	**30**	**22**
	Mdn	6.56	20.56	6.80	4.24	33.70
	IQR	5.66	25.49	6.04	2.84	26.80
	Min–max	2.00–47.32	2.25–52.20	2.86–19.42	1.57–6.92	10.20–53.62

**NVMA was estimated as the average age equivalent for the visual reception and fine motor domains for the MSEL, and as (chronological age x perceptual reasoning index)/100 for the WASI-II. The accuracy of these estimates may vary in cases of severe to profound ID where the precision of behavioral assessment decreases.*

needs and ability level. Support to engage with all aspects of the study protocol is provided by the research team and tailored to each family's individual needs (more information is provided in **Supplementary Box 1**). For a full list of SynaG testing schedules and measures described in the methods section please see **Supplementary Table 1**.

Frequency of Autism and Neurodevelopmental/Neuropsychiatric Features

To address SynaG study objectives 1 and 2, a range of clinical and cognitive assessments, and self or parent-report questionnaires are used to comprehensively phenotype our clinically ascertained synaptopathies and comparison groups.

Characterization of Autism

Autistic features are assessed in our genetic cohorts and confirmed for our iASD group using the Autism Diagnostic Observation Schedule 2 (ADOS-2) and the Autism Diagnostic Interview-Revised (ADI-R) (Lord et al., 2012; Rutter et al., 2012). Autism diagnostic assessments are not completed with NTD participants. However, parents of all children are asked to complete the Social Communication Questionnaire - Lifetime (SCQ-Lifetime) and the Children's Routines Inventory - Revised (CRI-R) to dimensionally assess level of social communication symptoms and restricted and repetitive behaviors, across all groups, including NTD. Autism diagnostic assessments are also not collected in parent NRXN1ds cases; instead, the Autism Quotient, Social Responsiveness Scale, Adulthood Routines Inventory- Revised (ARI-R) and SCQ-L are collected to measure autism features.

Further Neurodevelopmental and Neuropsychiatric Characterization

Temperament, behavioral disturbances, and motor coordination are assessed using a range of validated parent or self-report questionnaires depending on the age and developmental level of the individual. Attention Deficit Hyperactivity Disorder (ADHD) and sensory dysregulation are also assessed using standardized parent or self-report measures (McIntosh et al., 1999). For a full list of named questionnaires see **Supplementary Table 1**.

Cognitive and Adaptive Functioning

Both verbal and non-verbal mental ages are established for all participants using an assessment suitable to the individual's ability and level of language, which is estimated by researchers through parent-report and initial observations of the child at the visit. Assessment measures include the Mullens Scales of Early Learning (MSEL), the British Picture Vocabulary Scale: Third Edition (BPVS) and Raven's Colored Progressive Matrices (CPM), the British Ability Scales (BAS), and the Wechsler Abbreviated Scale of Intelligence - Second Edition (WASI–II) (see **Supplementary Figure 1** for how assessments are assigned in each schedule). Daily adaptive functioning, including social adaptive functioning, is assessed for all participants using the Vineland Adaptive Behavior Scales second edition (Vineland-II) parent interview format (Sparrow et al., 2005). Global functioning and coordination disorders are additionally screened.

Medical and Family Background

In order to establish a comprehensive phenotypic profile for each participant, a full medical and psychiatric history is recorded. Early environmental factors, such as maternal depression and drug, alcohol or medication use during pregnancy, are also recorded as they have been linked to neurodevelopmental outcomes in previous work (Kim et al., 2019). Sleep disorders are commonly reported in neurodevelopmental conditions (Devnani and Hegde, 2015); therefore, we additionally measure sleep habits for all participants.

Parent Phenotyping

Parents are screened for autistic features, ADHD, anxiety, and depression to better understand the potential role of family background on clinical heterogeneity. These measures are collected in both biological parents where possible and biological samples (bloods or saliva, and hair) are also collected to further assess genetic heritability in our populations of interest.

Social Cognition

To assess social cognition in younger and non-verbal participants, two play-based tasks probe for social attention and Theory of Mind (ToM), namely the Social Orienting task and the Penny Hiding Game (Dawson et al., 2004; San Jose Caceres et al., 2014). Additionally, an unstructured observation of parent-child play is recorded to capture a naturalistic interaction in the absence of any task demands. Previous research has linked reduced social attention and limited spontaneous initiation of social interaction with a caregiver to early development to autism (Devnani and Hegde, 2015).

Identification of Neurocognitive Stratification Biomarkers

To address objective 3 of the SynaG study we leverage eye-tracking, EEG and MRI metrics to uncover neurocognitive markers of autism linked to specific genotypes. Of particular interest are proxy markers of synaptic transmission, such as E/I imbalance, which may represent an aetiological marker for autism in these groups.

Eye-Tracking

Eye-tracking is used as an objective measure of visual attention, including social attention, through assessment of eye-movements and gaze patterns. Reduced motivation for social engagement and differences in attention to social content has been associated with autism (Chevallier et al., 2012). We will assess if eye-tracking measures of visual attention can be leveraged as a potential neurocognitive marker of autism associated synaptopathies. A battery of tasks were chosen based on their suitability across a range of ages and ability levels, including young children and those with ID, which only require a participant to look at and explore stimuli displayed on the screen. The battery includes a set of static and dynamic social stimuli, which are used to measure spontaneous social attention. A biological motion

task using point light display stimuli to measure preference for biological motion or non-biological (scrambled) motion (Annaz et al., 2012). A third task, the implicit false belief task, is included as a measure theory of mind, which is the ability of a person to attribute mental states to oneself and another person. In this task we assess gaze patterns during a short video to determine if participants can represent the person in the video's false belief. Finally, the gap overlap task (Landry and Bryson, 2004; Elsabbagh et al., 2013) is a gaze contingent paradigm that measures visual attention disengagement from a central to a peripheral stimulus, which is thought to be related to top-down attentional control. Attentional disengagement has been shown to be a good measure of the development and maturation of visual attention. Differences in the time taken to disengage and shift attention to another stimulus has also been suggested to reflect differences in processing of local and global information, often reported as a feature of early visual attentional processing in autism.

Electroencephalography

Electroencephalography provides an index of neural responsiveness and synchronization that reflects neurochemical changes at the level of the synapse. Altered synaptic development and functionality are pre-existing features of the synaptopathies within SynaG. Atypical attentional processing and variation in network level neural firing patterns, such as E/I imbalance, can be tracked using EEG and have been linked to autistic brain processing in the literature as previously described (Dickinson et al., 2016; Bruining et al., 2020). Therefore, we utilize EEG methodology for biomarker detection in SynaG as a way to identify neural signatures that can be linked to the genotype of our synaptopathies of interest (PMS and NRXNds).

Two EEG metrics, namely event-related potentials (ERPs) and event-related oscillations (EROs) are implemented to stratify groups. To assess group and individual differences we firstly explore pre-attentive ERP responses associated with change detection (mismatch negativity, MMN) and novelty detection (P3a), thought to reflect the organization of attention-related processes within the brain (Kujala et al., 2013; Fitzgerald and Todd, 2020). Secondly, we use frequency-based analyses of beta, theta and alpha bands to explore neural synchronization during the passive viewing of social and non-social videos. Functional connectivity analysis will also be used to examine short and long-range synchronization (i.e., connectivity) within and between different brain networks. Lastly, we use an auditory gamma task to probe for power in the gamma frequency, phase-locking factor, and power in the stimulation frequency (Rojas and Wilson, 2014). Power within the gamma frequency across all tasks will be examined as a proxy marker for E/I imbalance given the specific relevance to autism and synaptopathies (Rojas and Wilson, 2014; Kolesnik et al., 2019).

Magnetic Resonance Imaging

Magnetic resonance imaging is used to characterize brain structure and connectivity across individuals and groups within SynaG. We aim to identify system-level biomarkers relating to genotype that may explain variability observed at the phenotypic-level. High resolution anatomical T1-weighted scans are acquired to investigate structural biomarkers of brain volume, cortical thickness and surface area. Whilst, diffusion tensor imaging (DTI) is used to analyze white matter structural architecture. E/I imbalance is probed using magnetic resonance spectroscopy (MRS), whereby we measure absolute metabolite concentrations, specifically Glutamate and GABA, in the anterior cingulate cortex, concordant with recent findings in autism (Horder et al., 2018; Ajram et al., 2019), and the known genetic basis of our two synaptopathies of interest (PMS and NRXNds).

Several procedures have been put in place to facilitate MRI scanning at KCL, particularly with young children and individuals with severe or profound ID. At KCL, a space narrative has been introduced, which includes a space-themed tent that can be erected to partially cover the scanner, and 'space friends,' which are MRI-safe soft characters that can be attached to the space tent using Velcro (see **Supplementary Box 1** and **Supplementary Figure 2**). KCL have also recently introduced a sleep scanning protocol whereby participants can attend the scan session in the evening for an unlimited time with their parents or carers. Furthermore, participants may also receive melatonin as an aid to sleep prior to entering the scanner, where all relevant medical checks have been made. The procedure was introduced after consultation with a number of participating families who felt their children would not be able to remain still in the MRI scanner whilst awake.

Biological Samples

To address objective 4, blood and saliva samples are collected to examine how genetic variation may relate to the heterogeneity in clinical phenotype, and how this may potentially relate to altered underlying function at the cellular level. Blood, saliva, hair and skin biopsy samples are collected from study participants, their parents, and where appropriate, a sibling.

Genomics

DNA is extracted from blood and saliva samples. Saliva samples are used for a SNP array using Illumina NovaSeq 6000 platform. Blood samples are used for whole-genome sequencing which allows for CNV breakpoint mapping and also the identification of common and rare variation elsewhere in the genome. Following sequencing, quality control and variant calling/validation will be performed following methods outlined by AIMS-2-TRIALS LEAP WGS. We also confirm *NRXN1* and *SHANK3* deletion size and the CNV's breakpoints to better understand their influence on severity of clinical characteristics, as well as behavior and cognition. Next, we test whether a participant carries one or more additional CNVs associated with neurodevelopmental conditions at the same locus (a double hit), or at another locus as defined by Kendall et al. (2019). Finally, we examine the burden of other hits, i.e., additional, putatively loss-of-function, deleterious genetic variants, or primary hits, i.e., clinically validated genetic variants associated with neurodevelopmental disorders. It is hoped that understanding the contribution of this

other hit burden to the observed clinical, behavioral, neurological and physical phenotypes may improve our understanding of the heterogeneity in clinical and cognitive outcomes in NRXN1ds and PMS.

Development of Induced Pluripotent Stem Cells

Hair samples (keratinocytes) are used to generate induced pluripotent stem cells (iPSCs) of selected volunteers with a particular phenotypic (e.g., ASD vs. no-ASD) and/or genomic profile (e.g., *SHANK3* plus double-hit, or a particular genetic background) following a protocol developed by Professor Jack Price and colleagues at KCL (Warre-Cornish et al., 2020). Skin biopsy samples collected from autistic NRXN1ds participants have previously been used to derive iPSC lines to examine neuronal function (Avazzadeh et al., 2019, 2021). Human iPSC studies derived from individuals with *SHANK3* and *NRXN1* deletions may reveal important information about differences in neuronal activity and function, which may benefit our understanding of clinical phenotype, as well as the development and testing of targeted treatments.

Planned Analyses

To address aim 1 of the SynaG study, we will conduct traditional mean-group comparisons to establish whether our synaptopathies differ on average from each other with respect to the frequency of autistic and associated neurodevelopmental/neuropsychiatric features.

To address aim 2, we will assess whether mean-group differences exist between our synaptopathies and an idiopathic autistic group (i.e., autism without a known genetic association) and also a typically developing group (i.e., those without diagnosis or suspicions of neurodevelopmental conditions).

However, mean group differences are not well suited to understand individual variability within group (Dumas et al., 2021). Therefore, broadly following the approach used in standardized assessment tools, we aim to standardize scores on each experimental measure by creating 'norms' based on the larger data sets available via the AIMS-2-TRIALS PIP and LEAP cohorts. The approach enables us to derive 'scaled' scores relative to each person's chronological age and/or mental age (developmental level). This is a particularly poignant approach for research into rare genetic conditions because it directly addresses the common issue of small sample bias in group-level comparisons and enables us to make predictions about individuals. That is, given the inherent rarity of such genetic conditions it can be difficult to generalize group level findings to an entire affected population. With a normative modeling approach (also outlined previously), we will be able to compare individual performance of those with synaptopathies against an extensive comparison population that spans several stages of human development from toddlerhood to adulthood, including those with neurodevelopmental conditions.

Assessing individual performance in this way affords us the opportunity to identify variations in the developmental trajectories of those with synaptopathies and make predictions about possible future outcomes, which is key to improving our understanding of the natural history of neurodevelopmental conditions, but also important for managing ongoing support or treatments for these individuals. Additionally, the introduction of such an extensive comparison group will also be used to enrich our between-group comparisons (particularly where our current groups may not be matched on sex, age or developmental level).

Aim 3 will be addressed using the data modeling and comparison approach previously outlined, so that we may identify neurocognitive, functional, and/or structural biomarkers specific to our synaptopathies of interest.

Aim 4 will involve observing genotype-phenotype correlations to investigate in what ways phenotypic presentation may be related to genetic profile in our synaptopathies of interest and idiopathic autistic groups. Genetic profiling in SynaG will include location and size of deletion, and examination of common genetic burden or additional rare CNVs or SNVs and their impact on clinical phenotype.

Facilitating Study Engagement

Several strategies are implemented to increase chances that participants with PMS and NRXN1ds (i.e., mild to profound ID, and complex needs) are able to complete the various experimental measures (see **Supplementary Box 1** for description of strategies). Researchers work closely with parents to understand the specific needs of each child, which may include limited attention span, difficulties sitting still for long periods of time, or sensory difficulties that could impact toleration of the EEG cap or the noise of the MRI scans. The approaches used in SynaG are a combination of expertise within the research team, consultation with participating families, and ongoing discussions and ideas drawn from research groups and publications in the area (Webb et al., 2015; DiStefano et al., 2019). Specific credit should go to the MIND Institute, Sacramento, CA, United States, for their advice in conducting MRI sleep scans (Nordahl et al., 2008, 2016; Amaral et al., 2017).

COVID-19 Adaptations – Remote Testing Procedures

In March 2020 all face-to-face testing for the study was suspended due to COVID-19 restrictions. In response to these restrictions, and due to uncertainties regarding the resumption of in-person testing, both research groups adapted the study protocol to continue collection of some assessments remotely. Specifically, our remote testing procedure includes scheduling ADI-R, Vineland-II and family history interviews as online video call interviews with parents instead of during visits to our neurocognition lab. All parents and self-reporting participants are also now sent a link through email and asked to complete questionnaires online at home. Finally, saliva sample kits are sent by post to family homes and samples are collected from participants and their parents. These samples are then returned by post. ADOS-2, ET, EEG, and MRI data collection were all suspended as of March 2020. The research labs are now reopening for face-to-face testing allowing us to continue with our protocol as of September 2021. Dates and timelines for all testing have been recorded to account for any delay between remote and face-to-face assessment.

RESULTS

Interim Analyses – Sample Demographics

25 PMS, 36 NRXN1ds (including previous and SynaG studies), 33 iASD, 30 NTD-PMS, and 22 NTD-NRXN participants have been enrolled into the SynaG study so far (N = 146) (see **Table 1**). Median chronological age (CA) was 6.56 years for PMS (2.00–47.32 years), 20.56 years for NRXN1ds (2.25–52.20 years), 6.80 years for iASD (2.86–19.42 years), 4.24 years for NTD-PMS (1.57–6.92 years), and 33.70 years for NTD-NRXN (10.20–53.62 years) (see **Table 1**). A Kruskal–Wallis test confirmed that CA was different across groups [$H(146,4) = 70.305$, $p < 0.001$]. *Post hoc* (Dunn's) pairwise comparisons with Bonferroni correction confirmed that our current PMS ($p < 0.01$, $p < 0.001$), iASD ($ps < 0.001$), and NTD-PMS ($ps < 0.001$) groups are younger on average than the NRXN1ds and NTD-NRXN groups respectively, in accordance with the comparison sample matching procedures outlined under Section "Sample." Furthermore, NTD-PMS participants are younger on average than both PMS and iASD participants ($ps < 0.05$). NRXN1ds and NTD-NRXN groups are currently matched on CA as predicted ($p = 1$). PMS and iASD groups are also currently matched on CA ($p = 1$).

A higher proportion of males to females (29:4) was observed only in the iASD group, consistent with existing literature on autism (see **Table 1**) (Werling and Geschwind, 2013; Loomes et al., 2017). Prior to entering the study, 32% of PMS and 33% of NRXN1ds were in receipt of a formal diagnosis of autism (see **Table 1**).

Differences in non-verbal mental age (NVMA) were present across groups as anticipated [$H(118,4) = 80.446$, $p < 0.001$]. *Post hoc* pairwise comparisons with correction revealed that on average, NRXN1ds (mdn = 32.51) and NTD-NRXN (mdn = 35.23) participants had a higher NVMA than PMS (mdn = 1.54, $ps < 0.001$), iASD (mdn = 3.33, $ps < 0.001$), and NTD-PMS participants (mdn = 4.59; $ps < 0.001$) (see **Table 1**). PMS, iASD, and NTD-PMS groups are currently matched on NVMA ($ps > 0.1$), in accordance with comparison sample matching procedures. NRXN1ds and NTD-NRXN groups are also currently matched on NVMA ($p = 1$).

Discrepancy between CA and NVMA as a general indication of developmental delay was assessed in each group using paired samples Wilcoxon signed rank tests (for contributing group n's please see n's for NVMA in **Table 1**). On average, developmental delay (i.e., NVMA significantly lower that CA) was present for PMS ($Z = 3.920$, $p < 0.001$), iASD ($Z = 4.509$, $p < 0.001$), and NRXN1ds groups ($Z = 3.061$, $p < 0.01$), with no difference for NTD-PMS ($Z = -0.775$, $p = 0.439$) and NTD-NRXN ($Z = -1.269$, $p = 0.204$) comparison groups (see **Figure 2**).

Interim Analyses – Task Completion Numbers

Supplementary Table 2 provides a full breakdown of task completion numbers by group. Autistic features have been assessed in between 84–100% of PMS, 19–28% of NRXN1ds, and 85–91% of iASD participants thus far. Cognitive functioning has so far been assessed in 88% of PMS, 61% of NRXN1ds, 91% of iASD, 100% of NTD-PMS, and 95% of NTD-NRXN participants. Additionally, 58% of NRXN1ds and 95% of NTD-NRXN participants have been further assessed using the CANTAB. Adaptive functioning has been assessed in 96% of PMS, 22% of NRXN1ds, 88% of iASD, and 93% of NTD-PMS participants.

At the KCL site, between 72–80% of PMS, 76–91% of iASD, and 97–100% of NTD-PMS participants have completed behavioral measures of social cognition, specifically the Social Orienting task and Parent-Child Interaction. Only 28% of PMS participants have completed the Penny-Hiding game task as the majority lacked the fine motor skill necessary to complete all aspects of the game for assessment when attempted. At TCD, 69% of NRXN1ds participants have completed interviews/questionnaires probing for comorbidities (i.e., the DAWBA).

Neurocognitive measures such as ET and EEG have thus far been completed by between 72–84% of PMS, 6–8% of NRXN1ds, 48–70% of iASD, 73–100% of NTD-PMS, and 50–100% of NTD-NRXN participants. MRI completion is currently at 16% in PMS, 47% in NRXN1ds, 18% in iASD, 20% in NTD-PMS, and 77% in NTD-NRXN. Biosamples including bloods, saliva, hair, and skin biopsy (only at TCD), have thus far been completed by between 60–84% of PMS, 6–31% of NRXN1ds, 9–79% of iASD, and 3–77% of NTD-PMS participants.

Please be aware that due to COVID-19 restrictions, several NRXN1ds participants and a smaller number of PMS participants, are still awaiting a face-to-face research visit. Therefore, the acquisition of measures such as ET, EEG, MRI scanning, and biosample collection have been severely impacted. Despite this, parent interviews and online questionnaires have been completed remotely wherever possible. All aspects of the SynaG research protocol are now to be resumed (as of September 2021).

Also of note is the optional nature of the SynaG protocol, whereby parents can select for their child to opt-out of aspects of the study protocol they feel will be too challenging for their child. The types of assessment frequently abandoned and why are to be explored within a subsequent publication. As outlined in more detail elsewhere (**Supplementary Box 1**), the research team work closely with all families to facilitate participation where possible and support the best interests of the child.

DISCUSSION

The SynaG study adopts a gene-first approach to autism that aims to understand the impact of specific genetic susceptibility factors on neurocognitive and behavioral development. Two specific synaptic gene conditions ("synaptopathies") with a high penetrance for autism, namely PMS and NRXN1ds, are considered. The study is an important step toward explaining heterogeneity within autism in a biologically meaningful way. To our knowledge, this is the first European study that compares these two specific CNVs on a comprehensive multi-disciplinary protocol.

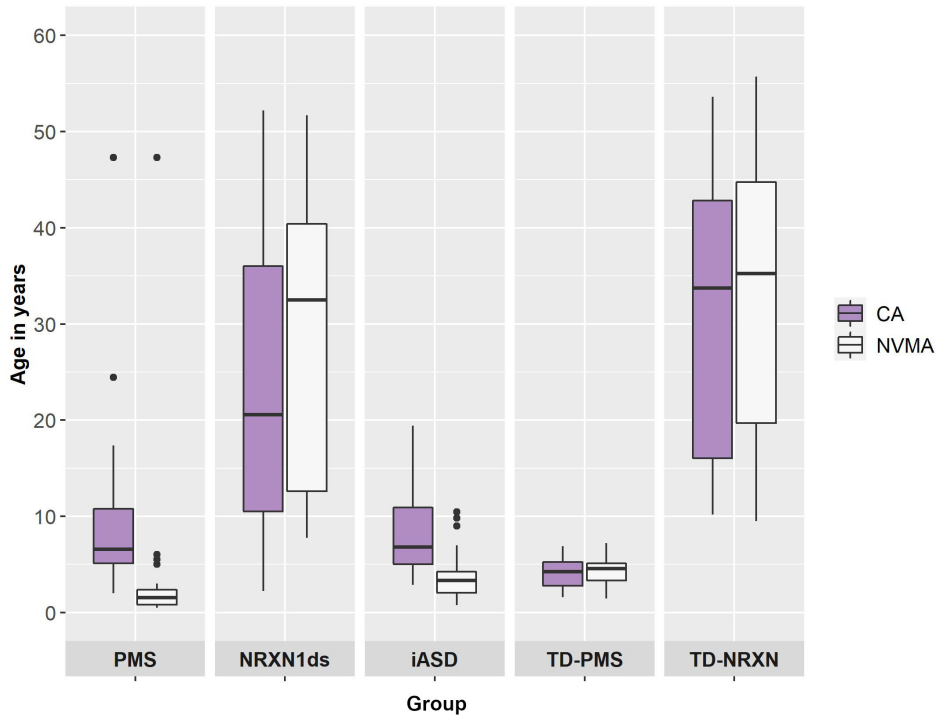

FIGURE 2 | Chronological age (CA) and non-verbal mental age (NVMA) comparisons by diagnostic group.

In order to comprehensively phenotype individuals, a variety of clinical and cognitive measures are collected, ranging from parent questionnaires and behavioral assessments to participant EEG recordings and MRI scans. Study recruitment is ongoing and so the current paper serves to showcase what progress has been made from the inception of the SynaG study in 2014 until the present day, as well as highlight that in spite of many challenges (e.g., individuals with significant intellectual disability, and the current COVID 19 pandemic) it is possible to deeply phenotype rare and complex populations.

Current Sample

The current study sample, including comparison groups, consists of a broad range of ages and ability levels. Around one third of our PMS and NRXN1ds groups (32 and 33%, respectively) were in receipt of a formal diagnosis of autism spectrum disorder prior to entering the study. Crucially, half of our current PMS cohort are under the age of 6 years, which may explain why we observe a lower number of autism cases than previously reported at this point (Soorya et al., 2013). That is to say, that autistic features may have yet to manifest in a way that warrants further assessment for these individuals. Moreover, it is possible that medical concerns/conditions take priority for assessment and treatment in the early stages of development. As individuals with PMS commonly present with multiple and profound disabilities, autistic features become part of a complex clinical presentation that requires skilled formulation and assessment. There is some evidence to suggest that general developmental delay may inflate the likelihood of autism diagnosis within PMS (Oberman et al., 2015). Furthermore, it has been found that the autistic phenotype may be influenced by the contribution of both deleted and preserved genes within the *SHANK3* region (Oberman et al., 2015). In order to address some of these challenges, Soorya et al. (2018) have published a framework for the assessment of individuals with rare genetic disorders, as a guide for professionals.

For our NRXN1ds group, it should be noted that parent NRXN1ds carriers ascertained thus far do not frequently have pre-existing diagnoses of autism and so may contribute to the current levels of autism we observe in NRXN1ds being on the lower percentage of reported in the literature. Ascertainment and inclusion criteria may contribute to the variability in frequency of reported autism diagnosis across studies.

Study Progress

Task completion numbers are promising given the extensive nature of the study protocol and the complex needs of our sample. The encouraging level of engagement from families may be due to the carefully designed and accessible protocol, as well as training and experience of the researchers in working with populations with ID, and attention to environmental modifications. The majority of participants are engaging well with behavioral assessments of autism and cognitive functioning. Parents/guardians are equally engaging well with interviews about their child's early development and adaptive functioning. Acquisition of blood samples has been a particular challenge in the iASD group due to a high prevalence of tactile sensitivity. For our more recently added NRXN1ds cohort, task completion

numbers are currently low due to face-to-face assessments being suspended during the COVID-19 pandemic. Furthermore, several PMS and iASD participants are currently awaiting MRI scanning under our melatonin protocol as a result of COVID-19 related suspension. With the resumption of face-to-face research visits as of October 2021, specific focus will be given to improving task completion numbers for MRI and biosampling measures. In order to achieve this, the research team will continue to work closely with families to implement supportive behavioral strategies, as well as offering our sleep scanning MRI protocol with melatonin.

Until now, the SynaG study has cataloged a sample of individuals with synaptic conditions, along with iASD and NTD comparison groups, but the research is ongoing. Data collection in rare genetic syndromes is challenging given the inherent rarity of cases, with the prevalence of co-occurring ID and complex needs also acting as a barrier to accessing extensive research protocols, such as that within SynaG. However, with the upscaling of remote testing during the global COVID-19 pandemic, the introduction of MRI scanning with melatonin, and the continued utilization of supportive behavioral strategies, we are confident that we will reach our proposed recruitment targets.

Future Study Plans

Much of what is currently known about the links between synaptopathies and autism comes from pre-clinical studies that are far removed from the clinical outcomes experienced by autistic individuals. SynaG sets a precedent for translational neuroscience by combining with the prior knowledge-base of preclinical studies to put individuals with synaptic gene conditions at the heart of the research. What is more, current clinical trials within the wider AIMS-2-TRIALS consortium are focused on targeting GABA receptors to address E/I imbalance with respect to improving social adaptive functioning in autistic children and adolescents. By partnering with initiatives such as the Synaptopathies Consortium and the wider AIMS-2-TRIALS consortium, we hope to uncover neural markers linked to synaptic functioning that may serve as potential treatment targets in future. Biomarker discovery may also inform the effectiveness of non-drug-based interventions that could improve quality of life.

Future plans for the SynaG study involve expansion to a longitudinal design whereby developmental trajectories and outcomes can be mapped in relation to iASD. One substantial benefit of being part of the AIMS-2-TRIALS consortium is the possibility of comparing our rare samples against the largest longitudinal cohort of autistic individuals ever recorded in LEAP (Loth et al., 2017). In this way, we can identify whether individuals with synaptic gene conditions follow similar developmental trajectories to those with iASD, as well as how and where they may deviate. The approach may be extremely informative in relation to explaining heterogeneity as the inherent rarity of these conditions translates to small research samples and an inability to make accurate population level inferences. What is more, the ability to associate unique developmental trajectories with specific genotypes takes us one step further in understanding and predicting clinical outcomes in autism. Collaboration between research teams is crucial for generating large samples in rare disorders that allow for population level inference to be drawn accurately. SynaG will continue to actively collaborate with the Developmental Synaptopathies Consortium and remains open to collaboration with other research teams around the world working in the area of synaptic gene conditions.

Conclusion

The SynaG study takes a gene-first approach to explaining heterogeneity in autism, focusing on PMS and NRXN1ds. The study compliments existing pre-clinical work looking at the downstream effects of alterations to *SHANK3* and *NRXN1* genes. Biomarker discovery in synaptopathies, such as PMS and NRXN1ds, is a promising area of research for the identification of potential treatment targets and more personalized treatment approaches.

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.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by St. James' Hospital/The Adelaide and Meath National Children's Hospital (REC ref: 2015/03/01) and the St. James' Hospital/Tallaght University Hospital Joint Research Ethics Committee [REC ref: 2019-09 List 35 (10)] at Trinity College Dublin, and the UK Health Research Authority via the Queens Square Research Ethics Committee (REC ref: 15/LO/0305) and the South London and Maudsley NHS Foundation Trust (SLaM ref: CSA/17/001) at King's College London. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

EL, TB, and DM were involved in the conception of the original study design. EL and ASJC were responsible for initiating the study at the KCL site. LG, EL, CM, and JC were involved in expanding the study at the TCD site. JC and CM compiled and analyzed the data and wrote the article in full. ASJC, TD, EL, and LG contributed to the composition and drafting of the article. JC, ASJC, and CM have made significant contributions to data collection for this study. All authors approved the final version to be published.

FUNDING

This work was supported by the Innovative Medicines Initiative 2 Joint Undertaking under grant agreement No. 777394 for

the project AIMS-2-TRIALS. This Joint Undertaking receives support from the European Union's Horizon 2020 research and innovation programme and EFPIA and AUTISM SPEAKS, Autistica, SFARI. This work was also supported by the Science Foundation Ireland grant 13/IA/1787.

ACKNOWLEDGMENTS

We would specifically like to thank the Phelan-McDermid Syndrome Foundation UK for their ongoing support and cooperation with our research. Thanks also must go to our AIMS-2-TRIALS consortium colleagues without whom this study would not exist. Our express thanks goes to Daisy Crawley, Hannah Hayward, Bethany Oakley, Chiara Terzo, Jacqueline Fitzgerald, Richard O'Conaill, Matthew O'Sullivan, Niamh McDevitt, Charlotte Blackmore, and Hannah Meyer-Lindenberg who all contributed to data collection and quality control thus far. And a thanks to our collaborators Maryam Al-Shehhi, Richard Reilly, Sally-Ann Lynch, and Sanbing Shen. Lastly but most importantly, we would like to sincerely thank all participants and their families for taking part in the study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2022.806990/full#supplementary-material

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Conflict of Interest: ASJC has worked as a consultant in several different projects for Servier and Roche.

The remaining 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.

The reviewer EB declared a past co-authorship with the authors TB and LG to the handling editor.

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frontiers in Neuroscience

REVIEW
published: 02 March 2022
doi: 10.3389/fnins.2022.785993

Looking for "fNIRS Signature" in Autism Spectrum: A Systematic Review Starting From Preschoolers

Eugenia Conti[1†], Elena Scaffei[1,2†], Chiara Bosetti[1], Viviana Marchi[1], Valeria Costanzo[1], Valerio Dell'Oste[3], Raffaele Mazziotti[1], Liliana Dell'Osso[3], Claudia Carmassi[3], Filippo Muratori[1,3], Laura Baroncelli[1,4], Sara Calderoni[1,3] and Roberta Battini[1,3]*

[1] Department of Developmental Neuroscience, IRCCS Stella Maris Foundation, Pisa, Italy, [2] Department of Neuroscience, Psychology, Drug Research and Child Health NEUROFARBA, University of Florence, Florence, Italy, [3] Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy, [4] Institute of Neuroscience, National Research Council, Pisa, Italy

OPEN ACCESS

Edited by:
Marco Turi, Fondazione Stella Maris Mediterraneo Onlus, Italy

Reviewed by:
Jennifer L. Bruno, Stanford University, United States
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***Correspondence:**
Elena Scaffei
elena.scaffei@fsm.unipi.it

[†] These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: *29 September 2021*
Accepted: *08 February 2022*
Published: *02 March 2022*

Citation:
Conti E, Scaffei E, Bosetti C, Marchi V, Costanzo V, Dell'Oste V, Mazziotti R, Dell'Osso L, Carmassi C, Muratori F, Baroncelli L, Calderoni S and Battini R (2022) Looking for "fNIRS Signature" in Autism Spectrum: A Systematic Review Starting From Preschoolers. Front. Neurosci. 16:785993. doi: 10.3389/fnins.2022.785993

Accumulating evidence suggests that functional Near-Infrared Spectroscopy (fNIRS) can provide an essential bridge between our current understanding of neural circuit organization and cortical activity in the developing brain. Indeed, fNIRS allows studying brain functions through the measurement of neurovascular coupling that links neural activity to subsequent changes in cerebral blood flow and hemoglobin oxygenation levels. While the literature offers a multitude of fNIRS applications to typical development, only recently this tool has been extended to the study of neurodevelopmental disorders (NDDs). The exponential rise of scientific publications on this topic during the last years reflects the interest to identify a "fNIRS signature" as a biomarker of high translational value to support both early clinical diagnosis and treatment outcome. The purpose of this systematic review is to describe the updating clinical applications of fNIRS in NDDs, with a specific focus on preschool population. Starting from this rationale, a systematic search was conducted for relevant studies in different scientific databases (Pubmed, Scopus, and Web of Science) resulting in 13 published articles. In these studies, fNIRS was applied in individuals with Autism Spectrum Disorder (ASD) or infants at high risk of developing ASD. Both functional connectivity in resting-state conditions and task-evoked brain activation using multiple experimental paradigms were used in the selected investigations, suggesting that fNIRS might be considered a promising method for identifying early quantitative biomarkers in the autism field.

Keywords: fNIRS, near-infrared spectroscopy, functional neuroimaging, preschooler, neurodevelopmental disorders, autism spectrum disorder, high-risk infant

INTRODUCTION

Functional Near-Infrared Spectroscopy (fNIRS) allows performing *in vivo* a continuous and non-invasive monitoring of oxygenation levels of chromophores like hemoglobin (Van de Rijt et al., 2018), thanks to the transparency of biological tissues -including the human brain- to the light in the near-infrared spectrum. Indeed, fNIRS quantifies regional changes in the concentration of oxygenated, deoxygenated, and total hemoglobin (OxyHb, DeoxyHb, and TotHb).

The blood-oxygen-level-dependent (BOLD) signal detected by fNIRS provides a direct measure of modifications in the brain blood flow, which are coupled to neuronal activity by the complex process known as "neurovascular coupling" (Villringer and Chance, 1997; Logothetis and Wandell, 2004). Typically, an increase of neural activity is paralleled by a peak of OxyHb and TotHb, with a drop of DeoxyHb. fNIRS devices consist of red light-sources paired to specific detectors, which can be placed into a textile EEG-like cap to form an array of multi-distant channels. For data acquisition, the system executes the parallel reading of optical sensors. fNIRS systems give the opportunity to perform recording with different wavelengths of the near-infrared spectrum (700–1000 nm). However, for functional activation studies in humans the devices typically focus on paired wavelengths of approximately 690 and 830 nm to optimize the accuracy of OxyHb and DeoxyHb measurements (Lloyd-Fox et al., 2010). Indeed, separability between the chromophore signals seems to be optimal if one wavelength is below 720 nm and the other is above 730 nm (Uludag et al., 2004).

Although several neuroimaging methods can directly detect the electrical activity of brain circuits (EEG and MEG) or record the related hemodynamic response (fMRI), most of them have limiting factors restricting their use in the developmental time window (for instance, sensitivity to motion artifacts, requiring high grade of subject compliance). Indeed, fMRI is the gold standard for *in vivo* imaging of the human brain, but fNIRS stands out for its high portability, robustness to noise, relatively low costs and small size, bringing functional imaging into much more realistic environments (Cui et al., 2011; Duan et al., 2012; Scarapicchia et al., 2017). In particular, strength to motion artifacts make this technique an ideal candidate for research in preschool children (until the age of 6), even in infancy and toddlerhood (Lloyd-Fox et al., 2010). Despite lower spatial resolution and imaging depth, fNIRS offers a higher temporal resolution than fMRI. On the other hand, EEG and fNIRS share high portability, low-cost features and recording depth, but they measure different perspectives of brain activity: indeed, EEG signal reflects the bioelectrical activation of cortical neurons, while fNIRS assesses hemodynamic responses (Chiarelli et al., 2017; Berger et al., 2019). In order to obtain a more detailed picture of brain activity, fNIRS can be combined with other neuroimaging and electrophysiological methods (e.g., fMRI and EEG), without causing any measurement interference (Chen et al., 2015) and potentially gaining a multimodal data set (Yuan et al., 2010; Chiarelli et al., 2017). Notably, EEG and fNIRS use similar experimental settings (they are both scalp-located procedures), but show different temporal resolutions that might allow dissecting the electrical from the hemodynamic contribution to the recorded signal.

Although introduced into clinical care almost 40 years ago, only recently, fNIRS gained much popularity in the study of brain development and neurodevelopmental disorders (NDDs) (Greisen, 2006; Vanderwert and Nelson, 2014). FNIRS has been widely used to investigate the typical maturation of speech perception and language development, sensory and motor functions, social communication and interaction, object processing, human action processing in both toddlers and children (Lloyd-Fox et al., 2010). In contrast, the application of fNIRS in the field of NDDs is a growing research area and studies looking for a "fNIRS signature" as a brain biomarker useful for the diagnosis and the treatment outcome are still underrepresented (Gervain et al., 2011). NDDs are a heterogeneous group of complex disorders resulting from the interaction of genetic, epigenetic, neurobiological and environmental factors (from alterations *in utero* to postnatal environment), and characterized by early disruption in cognition, emotion, and behavior. NDDs include attention-deficit/hyperactivity disorder (ADHD), autism spectrum disorder (ASD), communication and specific learning disorders, intellectual disability (ID) and motor disorders (American Psychiatric Association, 2013). NDDs often present overlapping clinical features or may occur simultaneously in the same patient, making the differential diagnosis potentially challenging (Hansen et al., 2018). Moreover, NDDs show fairly broad behavioral phenotypes, reflecting also etiological heterogeneity of these disorders that can occur as "idiopathic" or "secondary" conditions (e.g., well documented causes of secondary ASD include monogenic disorders such as tuberous sclerosis and Fragile-X syndrome) (Muhle et al., 2004). Thus, reliable functional biomarkers would be very helpful to support early clinical diagnosis and to monitor developmental trajectories through *in vivo* longitudinal studies, also focused on the potential outcome of tailored intervention. Moreover functional biomarkers might help clinicians to separate behavioral symptoms of NDDs into more stable and objective phenotypes, including gender-related endophenotypes.

Clinically useful neuroimaging markers for NDDs are sorely missing yet (Vasung et al., 2019; Azhari et al., 2020), but emerging evidence suggests that fNIRS might be exploited to generate unbiased and reliable measures to explore brain functions, thus possibly driving clinicians toward a faster and more precise diagnosis (Zhang and Roeyers, 2019).

Moreover, fNIRS offers the opportunity to study cortical networks focusing not only on neuronal activation, but also on the neuromodulatory action of non-neuronal cells (such as glial cells or astrocytes that contribute to neurovascular and metabolic homeostasis of the brain). Thus, fNIRS is emerging as a fundamental tool, alone or in combination with other techniques, to dissect the key players in brain physiology and pathophysiology (Lines et al., 2020).

The main purpose of this systematic review has been to investigate the recent literature covering the use of fNIRS in the field of NDDs. We decided to focus on the preschool population in order to highlight the promising value of fNIRS as a reliable and non-invasive tool to detect brain biomarkers in clinical settings, even in populations commonly showing reduced compliance to experimental environments. This specific inclusion criterium led to narrow the focus of this review to children with idiopathic ASD or high-risk (HR) infants.

MATERIALS AND METHODS

A systematic search was conducted for relevant studies in three databases (PubMed, Scopus, and Web of Science), using the

following search terms that were refined from previous reviews (Liu et al., 2019; Zhang and Roeyers, 2019) and according to PICOS framework: ("fNIRS" OR "functional nirs" OR "near-infrared spectroscopy") AND ("neurodevelopmental disorder" OR "ASD" OR "ADHD") AND ("children" OR "toddler" OR "preschooler").

Additionally, manual searches were conducted among the reference sections of the retrieved studies and reviews. Publication year was not restricted, and the latest database search was performed in March 2021. Reviews were not included in the selection, but were only used to collect original studies. After removing duplicate records, articles emerged from the search were required to meet pre-established criteria. Inclusion and exclusion criteria, established prior to the literature search, are outlined below.

Inclusion Criteria

(i) Studies applying fNIRS;
(ii) Studies including a clinical population with a confirmed diagnosis (according to DSM-5) or a well-established condition of risk for NDDs;
(iii) Studies including preschool children (sample mean age < 7 years).

Exclusion Criteria

(i) Articles published in languages other than English;
(ii) Meta-analyses or literature reviews;
(iii) Other reasons referring to report characteristics, as not published clinical studies (e.g., conference paper, thesis, book chapter) or studies that do not involve human participants (e.g., focused on methodological issues).

RESULTS

Study Selection

One hundred and sixty-three potentially relevant studies were identified from PubMed, 136 from Scopus, 127 from Web of Science; in addition, four articles that met the selection criteria were retrieved from screened review articles. After omitting duplicates, 224 articles were examined. On the basis of the title, a total of 52 studies that did not meet the inclusion criteria were disregarded. Finally, within the172 articles screened for abstract or full-text evaluation, a total of 13 studies were selected for this review. An in-depth detail of paper selection is shown in the PRISMA diagram (**Figure 1**).

Crucial information was extrapolated from included papers. **Tables 1**, **2** summarize the mean age of participants, the sample size and gender ratio, the experimental procedures (technical aspects of recording sessions and experimental design) used during fNIRS measurement.

Although the initial aim was to include different type of NDDs (see keywords used for the search), only studies focused on children with idiopathic ASD or high-risk (HR) infants (infants at high familial risk of autism) satisfied the inclusion and exclusion criteria.

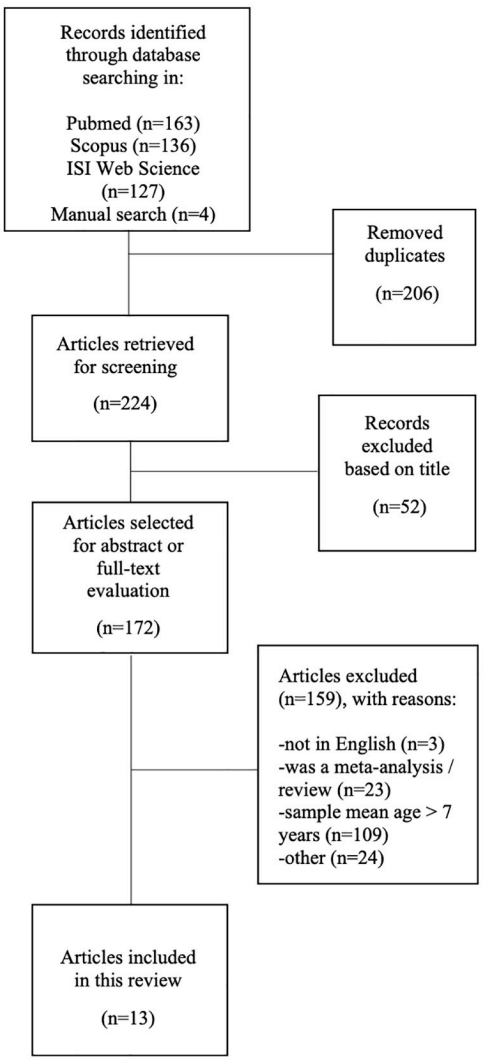

FIGURE 1 | Flow diagram of the literature search and subsequent screening performed in this review.

Regarding other NDDs, studies on ADHD included children older than 7 years, due to the pharmacological aim of their experimental design: at the time of our last search, indeed, 55 studies focused on fNIRS and ADHD, but none of them was performed on the preschool population. Moreover, within the growing bulk of literature that uses fNIRS for evaluating the emerging language ability in infants, only two studies (Chang, 2014; Hosseini et al., 2018) focused on children with Language Disorders; however, both of them, did not meet the inclusion criteria (mean age of participants older than 7 years) and were therefore excluded from this review. fNIRS was rarely used to explore neuropsychological features in learning disorders such as Dyslexia (Zhu et al., 2012; Pecyna and Pokorski, 2013; Song et al., 2013; Sela et al., 2014; Cutini et al., 2016), but this field obviously includes only school-aged children. Application of fNIRS to Intellectual Disability (ID) is only anecdotal yet

TABLE 1 | Overview of the reviewed studies using Resting State (R-S) fNIRS recordings: references, technical issues, characteristics of participants as well as main findings are shown.

References	**NIRS technique**			**Experimental paradigm**	**Characteristics of participants**		**Main findings**
First Author, year	**System**	**Channels (N)**	**Probe Geometry Source: Detector (cortical areas covered)**	**Experimental setting "trick" for Resting-State recording**	**Clinical population N (M:F) Mean age**	**Comparison group N (M:F) Mean age**	
Kikuchi et al., 2013	Foire 3000, (Shimadzu Kyoto, Japan)	2	2:1 optodes probe (covering anterior prefrontal cortex, bilaterally)	R-S fNIRS measurement while watching picture-card show	ASD 15 (13:2) 45–82 months^	TD 15 (13:2) 47–86 months^	Higher anterior prefrontal cortex (PFC) connectivity for 0.02-Hz fluctuations in children with ASD compared to TD group. Inter-hemispheric connectivity in ASD group positively correlated with the severity of social deficit.
Li and Yu, 2016	LABNIRS (Shimadzu Corporation, Kyoto, Japan)	44	16:16 optodes probe (covering prefrontal, temporal and occipital areas, bilaterally)	R-S fNIRS measurement while watching cartoon	ASD 12 (9:3) 6.1 years	TD 12 (9:3) 6.1 years	Weak functional network efficiency in young children with ASD compared to TD group. In particular, weak lobe-level inter – region connectivity in right prefrontal cortex (including its linkages with left prefrontal cortex and bilateral temporal cortex) was found.
Li and Yu, 2018	LABNIRS (Shimadzu Corporation, Kyoto, Japan)	44	16:16 optodes probe (covering prefrontal, temporal and occipital areas, bilaterally)	R-S fNIRS measurement while watching cartoon	ASD 46 (36:10) 5 years	n.a.	Significant associations between age and network efficiency, especially evident in the deoxy- and total-Hb-based-networks, indicating the network efficiency decreases with age in young ASD children. Significant associations between levels of autistic behaviors and network efficiency in the oxy-Hb-based-network, indicating decreased functional global and local network efficiency in ASDs with a relatively higher level of autistic behaviors.
Li et al., 2018	LABNIRS (Shimadzu Corporation, Kyoto, Japan)	44	16:16 optodes probe (covering prefrontal, temporal and occipital areas, bilaterally)	R-S fNIRS measurement while watching cartoon	ASD 29 (23:9) 6 years	TD 29 (20:9) 6.5 years	Spatial complexity of functional connectivity (SCFC) differs between ASD and control group. Global SCFC was significantly higher in ASDs, along with considerably higher intraregion SCFCs in the prefrontal and temporal lobes. Moreover, higher interregion SCFCs between right PFC and the other brain regions were found in young children with ASD compared to TD children.
Jia et al., 2018	LABNIRS (Shimadzu Corporation, Kyoto, Japan)	44	16:16 optodes probe (covering prefrontal, temporal and occipital areas, bilaterally)	R-S fNIRS measurement while watching cartoon	ASD 35 (23:12) 5.9 years	TD 31 (20:11) 6.6 years	The long-range temporal correlations (LRTCs) of hemoglobin concentration signals were attenuated in young children with ASD over left temporal region (for oxy-Hb signal) and bilateral temporo-occipital regions (for deoxy-Hb signal). Detrended fluctuation analysis (DFA), used to quantify LRTCs, of oxy-Hb in left temporal region were negatively correlated with autistic symptom severity. Relationship between age and LRTCs of Hb concentration signals differs between two groups, correlating with autistic symptom severity.

^ *Sample mean age not available: age range of participants is shown.*
N.A. means not applicable: no comparison group is included in this study.

TABLE 2 | Overview of the reviewed studies using Task-Related (T-R) fNIRS recordings: references, technical issues, characteristics of participants as well as main findings are shown.

References	NIRS technique			fNIRS paradigm	Demographics		Main findings
First Author, year	**System**	**Channels (N)**	**Probe Geometry Source: Detector (cortical areas covered)**	**Type of stimuli proposed in Task-Related (T-R) protocol**	**Clinical population N (M:F) Mean age**	**Comparison group N (M:F) Mean age**	
Fox et al., 2013	Hitachi ETG-4000 system	24	Two arrays of optodes probe: -Frontal areas (OFC), bilaterally (Ch 1-12) -Temporal lateral areas, only in right hemisphere (Ch 13-24)	Face processing task	HR 10 (4:6) 7 months	LR 10 (4:6) 6.9 months	Greater OxyHb responses in lateral regions for LR as compared to HR, and significantly greater DeoxyHb responses in frontal channels for HR as compared to LR during face processing task. Greater DeoxyHb response to mother's face as opposed to stranger face within the HR group across both frontal and lateral regions. Greater OxyHb response to smiling versus neutral conditions only for the LR.
Lloyd-Fox et al., 2013	UCL-NIRS topography system	26	10:10 optodes probe (covering temporal areas, as IFG, pSTS-TPJ and aMTG-STG, bilaterally)	Social perception task	HR 18 (8:10) * 5 months	LR 16 (10:6) * 5 months	Visual social stimuli produced a diminished response in HR infants relative to LR infants, with difference mostly evident in the left STS region of the cortex. Regarding auditory social stimuli, no vocal-specialized areas in HR group was found, compared to greater vocal selectivity in the right mid-posterior STS region in LR group.
Lloyd-Fox et al., 2018	UCL-NIRS topography system	26	10:10 optodes probe (covering temporal areas, as IFG, pSTS-TPJ and aMTG-STG, bilaterally)	Social perception task	HR˜ 20 (10:10) * 5 months	LR 16 (10:6) * 5 months	Early fNIRS measurements (4–6 months) in response to social perception task correlate with later clinical outcome and symptoms level at 36 months. Reduced activation to visual social stimuli in those infants with later diagnosis of ASD. Reduced activation to vocal relative to non-vocal sounds in those infants with later diagnosis of ASD compared to LR and HR-without later diagnosis confirmation.
Braukmann et al., 2018	UCL-NIRS topography system	26	10:10 optodes probe (covering temporal areas, as IFG, pSTS-TPJ and aMTG-STG, bilaterally)	Social perception task	HR 16 (7:9) 5 months	LR 13 (9:4) 5 months	LR showed a socially selective cortical response in the right posterior temporal cortex in response to visual social stimuli, whereas this response was not significant in the HR.
Bhat et al., 2019	Hitachi ETG-4000 system	24	10:8 optodes probe (covering temporal areas, bilaterally)	Naturalistic social interactions	HR 9 (7:2) 7 months	LR 6 (1:5) 7.5 months	HR infants showed reduced right and left-hemispheric activation compared to LR infants based on OxyHb and DeoxyHb signal trends. Indeed, HR infants had greater functional connectivity than LR infants during the pre- and post-social periods and showed a drop in connectivity during the social period.
Keehn et al., 2013	Hitachi ETG-4000 system	24	10:8 optode probe (covering anterior and posterior temporal areas, bilaterally)	Language-related task	HR 27°	LR 37°	In a prospective longitudinal view, LR infants showed a pattern of increasing functional connectivity from 3- to 12-months in selected ROIs, while HR exhibited a pattern of decreasing connectivity with age. At 12-months, HR have reduced intra-hemispheric connectivity for the left hemisphere compared to LR infants.
Edwards et al., 2017	Hitachi ETG-4000 system	24	10:8 optode probe (covering anterior and posterior temporal areas, bilaterally)	Language-related task	HR 21 (13:8) 3.6 months	LR 17 (10:7) 3.6 months	During an auditory stimuli containing syllable repetitions (ABB vs. ABC syllables pattern), HR females exhibited significantly higher OxyHb responses than HR males, LR females or LR males in both left and right anterior regions. HR females exhibited different temporal neural response patterns than LR females across both ABB and ABC stimuli pattern (no habituation response to repetition in speech in HR females group).
Pecukonis et al., 2021	Hitachi ETG-4000 system	24	10:8 optode probe (covering anterior and posterior temporal areas, bilaterally)	Language-related task	HR˜ 14 (7:7) 7 months	LR 18 (9:9) 7 months	LR infants exhibited strongest activation in bilateral anterior ROIs, while HR exhibited similar activation across all brain regions in study. Compared to LRs, HR-ASD infants had reduced brain response in the bilateral anterior ROIs, while HR-noASD had increased brain response in the right posterior ROI. This atypical brain response was not predictive of 24-month language abilities in HR infants.

**Note that 32 of the 36 infants (16 low risk and 16 high risk) contributed data to a previous publication (Lloyd-Fox et al., 2013).*

°Longitudinal Study: 3-, 6-, 9-, and 12- months visit; variable gender ratio according to timepoint.

˜Longitudinal data outcome available: within HR cohort of Lloyd-Fox et al. (2018) at 36-months visit 15 HR-noASD and 5 HR-ASD; within HR cohort of Pecukonis et al. (2021) at 36-months visit 9 HR-noASD; 5 HR-ASD.

(only one scientific report found in literature database consulted) (Bembich et al., 2021).

Characteristics of Participants

As mentioned above, all studies included in this review applied fNIRS in preschool children with ASD, a clinical condition characterized by socio-communicative impairment associated with restricted interests and repetitive behaviors, or HR infants (younger siblings of children with ASD). Only four studies included more than 40 participants (Keehn et al., 2013; Jia et al., 2018; Li and Yu, 2018; Li et al., 2018); in most cases subjects were equally distributed between the clinical population and the control group. Interestingly, the cohorts included both males and females in all studies, but the two populations were not homogeneously distributed in terms of sample size, except for two investigations (Lloyd-Fox et al., 2018; Pecukonis et al., 2021).

Five studies included in this review (Kikuchi et al., 2013; Li and Yu, 2016, 2018; Jia et al., 2018; Li et al., 2018) comprised ASD children with a mean age was between 3 and 7 years and compared the ASD group with age- and gender-matched typically developing (TD) peers in resting state condition (see **Table 1**). It is to note that all selected studies included children with an average IQ score and no comorbidity with ADHD.

The remaining eight studies (Fox et al., 2013; Keehn et al., 2013; Lloyd-Fox et al., 2013, 2018; Edwards et al., 2017; Braukmann et al., 2018; Bhat et al., 2019; Pecukonis et al., 2021) focused on infants/toddlers (children younger than 3 years old), and compared fNIRS data between HR group and in Low-Risk groups (LR; condition defined as subjects with no family history of ASD) in task-related study designs (see **Table 2**). Of these studies, only two (Lloyd-Fox et al., 2018; Pecukonis et al., 2021) analyzed the correlation between the fNIRS brain responses of infants during the first year of life and their diagnostic outcome at 24 or 36 months.

fNIRS Measurement: Technical Issues and Experimental Design

A total of four different fNIRS recording systems were employed in the reviewed studies (Foire 3000, LABNIRS, Hitachi ETG-4000 system, UCL-NIRS topography system). The number of channels used ranged from a minimum of 2 (Kikuchi et al., 2013) to a maximum of 44 (Li and Yu, 2016, 2018; Jia et al., 2018; Li et al., 2018). The remaining investigations used an intermediate number of channels (24–26 pairs of optodes), but the density of montages may be considered comparable among almost all studies, due to the younger age of the cohort investigated and the relatively smaller dimension of the probe. Despite the variability of probe geometry, in all cases optodes placement followed the international 10–20 EEG system.

Within the 13 articles included, eight studies explored the brain activation evoked by specific tasks (task-related paradigm or T-R; an overview of experimental procedures is reported in **Table 2**) and the remaining five investigated functional brain organization in resting-state (R-S) condition (an overview of experimental procedures is reported in **Table 1**). Considering the significant difficulties to remain still under stimulation-free conditions for young children, all resting-state data were recorded while participants watched a cartoon or, only in one case, a picture-card show (Kikuchi et al., 2013) in order to optimize subject compliance. Papers exploring functional cortical activation in task-related condition can be sub-grouped into two categories that reflect the nature of the task proposed: (i) social stimuli, including social perception (Lloyd-Fox et al., 2013, 2018; Braukmann et al., 2018), face processing (Fox et al., 2013) and naturalistic social interactions (Bhat et al., 2019); (ii) language-related stimuli (Keehn et al., 2013; Edwards et al., 2017; Pecukonis et al., 2021).

DISCUSSION

Papers systematically selected for this review allow discussing some research questions that might pave the way toward the fNIRS application in the field of NDDs. The final selection, mainly based on age criteria, focused our attention on ASD and HR populations. This field is characterized by huge heterogeneity both regarding the phenotypic and the neurobiological level, and a great amount of literature is devoted to uncover biomarkers dissecting sub-phenotypes and supporting the clinical assessment (Loth et al., 2016; Lombardo et al., 2019). Indeed, while an early detection of ASD is highly recommended to receive the services and supports the children need to reach their full potential (Dawson et al., 2010; Hyman et al., 2020), a final diagnosis is still obtained approximately during the third year of life (Salomone et al., 2016). However, neuroimaging and electrophysiological evidence consistently suggested that ASD-related atypical brain pattern in cortical regions crucial for socio-communicative skills (such as fronto-temporal areas) could be detected before the full-blown expression of symptoms (Conti et al., 2015; Ha et al., 2015; O'Reilly et al., 2017; Pagnozzi et al., 2018).

Is "fNIRS Signature" Useful as a Brain Biomarker for Autism Spectrum Disorder?

Resting-state fNIRS measurements have been proposed as auxiliary indexes for the objective assessment of ASD in both young (Xu et al., 2021) and adult population (Yanagisawa et al., 2016).

Regarding preschoolers, Kikuchi et al. (2013) first demonstrated an aberrant functional connectivity between the right and the left anterior prefrontal cortex (aPFC) in young children with ASD under resting state conditions. Notably, they found a significantly higher inter-hemispheric connectivity in the ASD compared to the TD group, reinforcing previous data about the aberrant cortical organization at structural level in ASD children (McCaffery and Deutsch, 2005; Courchesne et al., 2011). The higher inter-hemispheric connectivity in ASD reported by Kikuchi et al. (2013) was positively correlated with the severity of social deficit, as scored with the Autism Diagnostic Observation Schedule (Lord et al., 2012). It is to note that the anterior prefrontal cortex has already been implicated in ASD pathophysiology in previous structural and functional studies, as

being involved in social perception and processing abilities that are altered in the ASD condition (Uddin et al., 2013; Pagnozzi et al., 2018).

In contrast, studies from Li and Yu (2016, 2018) and Li et al. (2018) showed weaker functional network efficiency in young children with ASD compared to TD peers, mainly in short- and long-range connectivity of the right prefrontal cortex (Li and Yu, 2016). Moreover, a subsequent study of the same research group (Li et al., 2018) showed an inverse association between network efficiency, age (especially evident in the Deoxy- and TotHb-based networks) and severity of autistic behaviors (especially evident in the OxyHb-based-network) in high-functioning ASD subjects. Statistically significant findings were also obtained when comparing ASD and TD groups through spatial complexity of functional connectivity (SCFC) analysis (Li et al., 2018).

Other approaches to analyze resting state recordings also confirmed that fNIRS might be a candidate tool to investigate the pathophysiological mechanism of ASD, looking at the temporal dynamics of neuronal oscillations (Jia et al., 2018). Specifically, the long-range temporal correlations (LRTCs) of hemoglobin concentration signals were studied by quantifying a detrended fluctuation analysis (DFA) exponent. Comparing data between ASD and TD group, significant differences were found over the left temporal region for OxyHb signal, and over bilateral temporo-occipital regions for DeoxyHb signals, suggesting a shift-to-randomness of brain oscillations in children with ASD. Moreover, the relationship between age and DFA exponents revealed that this association could be modulated by autism. Furthermore, the DFA exponents of OxyHb in the left temporal region were negatively correlated with autistic symptom severity. Between-group differences remained significant also looking at correlation coefficients between age and DFA.

Altogether, these results confirm that fNIRS can detect a different pattern of functional connectivity in the brain of ASD children. Accordingly, the considerable bulk of prospective studies focused on neurostructural and neurofunctional measures of brain development validated the concept of ASD as characterized by early altered connectivity patterns (Bosl et al., 2011; Wolff et al., 2012; Solso et al., 2016). In particular, early social brain network alterations were previously reported in structural (Shen et al., 2018) and functional MRI (Emerson et al., 2017) as well in observational studies (Elsabbagh and Johnson, 2016). However, no resting-state fNIRS data are yet available in cohorts younger than 3 years, thus restricting the predictive value of resting state analyses so far. Moreover, the mathematical algorithms used for the interpretation of resting-state data are very complex to be applied in the clinical setting and the limited coherence among models still prevents from drawing conclusive interpretations.

Is "fNIRS Signature" Useful as an Early Predictor in "At Risk" Population?

Younger siblings of children with ASD are considered as HR infants because around 20% of them receive a diagnosis of ASD at the age of 3 years (Ozonoff et al., 2011) and a further 20–30% exhibit other neurodevelopmental problems (Messinger et al., 2013). In addition, a high rate of behavioral traits overlapping with those observed in children with an ASD diagnosis (referred as the Broader Autism Phenotype) has been documented in the sibling population (Charman et al., 2017). Thus, the prospective study of the HR population allows detecting behavioral risk signs or biomarkers of NDDs at a very early age, prior to the full clinical manifestation (Zwaigenbaum et al., 2015).

Studies on younger siblings of children with ASD compared HR infants with infants without familial history of ASD or NDDs (LR infants), regardless their developmental outcome or differentiating HR infants with or without a later diagnosis of ASD. In this review, we included both types of studies as we are interested in understanding how fNIRS signal can be used as a potential biomarker of atypical development, and not only as an ASD related outcome.

In the past decade, the number of studies exploring the applicability of fNIRS technique in HR infants has rapidly grown, focusing especially on core ASD neurocognitive domains. Accordingly, our study selection includes five papers testing social perception and three others assessing possible alterations of language development in the HR group.

With regard to social processing, starting from previous data in typically developing infants (Lloyd-Fox et al., 2012), Lloyd-Fox et al. (2013) examined whether the temporal lobe specialization for processing visual and auditory social stimuli during the first months of life differed between HR and LR infants. They found significantly diminished evoked hemodynamic responses in HR compared to LR infants in the superior temporal sulcus (STS), both in visual and auditory trials, suggesting a lack of cortical specialization to social stimuli, already within the first 6 months of life. Notably, a more recent prospective study in the same cohort of HR infants (increased by only 2 more subjects) demonstrated that the reduced activation to visual social and vocal stimuli across cortical regions of interest was specific for infants with a later diagnosis of ASD (Lloyd-Fox et al., 2018).

Moreover, Braukmann et al. (2018) provided further evidence for a social processing difference in infants at risk of autism, highlighting a reduced hemodynamic response evoked by social visual stimuli in the right posterior temporal cortex. Similarly, a reduced bilateral cortical activation in HR children compared to LR infants was reported in the social perception within the infant-mother dyad (Bhat et al., 2019). Of note, this is the only study using a fNIRS experimental protocol with live stimuli (face-to-face naturalistic social interaction between infant and mother).

Social behavior was also explored by Fox et al. (2013), recording fNIRS hemodynamic responses to a face perception task (facial identity and emotion perception on video clips performed by infant's mothers) during the first year of life. This study suggested differences in patterns of functional connectivity in the frontal lobe (short- and long-range connections) between the HR and LR groups. HR infants showed significantly greater DeoxyHb responses in frontal channels and lower OxyHb responses in right temporal regions during face processing task; a significant difference between groups was detectable in response to mother's face as to a stranger face (greater DeoxyHb response within the HR group across both frontal and lateral regions). For this cohort, no longitudinal data were available for discriminating

infants with or without later ASD diagnosis and establishing the predictive value of early fNIRS recordings.

To summarize, these results suggest that socially evoked fNIRS signals might be a suitable and predictive biomarker in early assessment of HR infants; however, further studies are needed to confirm these promising findings.

Growing evidence on the validity of fNIRS for studying early aberrant functional brain networks in HR infants emerged from the language domain as well.

Keehn et al. (2013) explored functional connectivity during the first year of life in HR infants using a task-related experimental paradigm (language processing task). They found that from 3 to 12 months HR children exhibited a pattern of decreasing connectivity with reduced intra-hemispheric connectivity for the left hemisphere compared to LR infants at 12 months.

Moreover, an alteration of language processing can be detected very precociously with fNIRS: Edwards et al. (2017) analyzed precursors of language development in HR and LR 3-month-old infants: they found that HR females exhibited significantly higher OxyHb responses to auditory stimuli containing syllable repetitions than HR males, LR females or LR males in both left and right anterior brain regions. It is worth noting that HR females showed different temporal response patterns with respect to LR females across different type of auditory pattern (repeating vs. non-repeating syllabic sequences) presented randomly (speech-like stimuli), indicating a potential gender endophenotype in ASD. Using the same experimental protocol, Pecukonis et al. (2021) performed a longitudinal study on functional specialization of language-related brain regions during the first year of life in HR and LR group, suggesting a possible role of language-evoked fNIRS measurements to predict the diagnostic outcome of infants at 24 months.

Altogether, these data demonstrate that fNIRS studies on siblings can provide a unique window into the earliest neurobiological atypical trajectories and have the potential to shed light on possible ASD-related endophenotypes and biomarkers of brain function. In particular, studies correlating fNIRS brain responses in the first year of life to the diagnostic outcome at 24–36 months (Lloyd-Fox et al., 2018; Pecukonis et al., 2021) highlight that atypical brain responses are detectable in ASD infants as early as 6 months, thus indicating that fNIRS might be the an useful tool to early predict ASD.

Is Hemispheric Asymmetry an Informative Index in fNIRS Recordings?

Theoretical speculations about possible left-hemisphere dysfunction (Fein et al., 1984) or predominant right-hemisphere impairment (Happé, 1999) have historical roots in autism research. Many reports of atypical hemispheric asymmetries in ASD came from anatomical (Herbert et al., 2005) and functional imaging studies (Kleinhans et al., 2008), and typically focused on the abnormal lateralization of language domain (Just et al., 2004), even at very early stages of infants' development (Eyler et al., 2012). Nevertheless, it is only in the last decade that attention on functional asymmetries related to non-verbal processing and the possibility to consider atypical brain asymmetry as a candidate for clinically meaningful stratification in ASD has grown (Floris et al., 2021). Indeed, recent evidence from resting-state fMRI studies supported the hypothesis that atypical rightward asymmetry shift may be a pervasive feature of functional brain organization in ASD, affecting not only the language network, but also the sensorimotor function and higher cognitive domains (Cardinale et al., 2013). Task-related – fNIRS studies focusing on hemispheric asymmetry in ASD individuals are still very limited and evidence reported are quite inconsistent (Doi and Shinohara, 2017). No systematic findings on hemispheric asymmetry in ASD population during resting-state fNIRS recording are currently available. Even in the studies included in this review, hemispheric asymmetry was not highlighted as a primary research purpose, allowing us to prompt only a few speculations on this topic.

Functional near-infrared spectroscopy studies on language perception and processing seem to confirm previous literature about ASD atypical lateralization for the language domain, with a reduced functional connectivity in the left hemisphere of HR infants compared to the LR peers, both at 6- (Pecukonis et al., 2021) and 12-months of age (Keehn et al., 2013). In contrast, Edwards and colleagues (Edwards et al., 2017) found left- or even hyper-lateralization of neural activity in 3-month-old HR females in response to a language auditory task, speculating about a gender-specific ASD endophenotype related to language processing. However, the limited spatial resolution of fNIRS prevent these results from being conclusive.

Similarly, task-related fNIRS studies of social processing were not consistent in defining hemispheric asymmetry. Indeed, Lloyd-Fox et al. (2013, 2018) found cortical activation patterns in response to social vs. non-social stimuli showing maximal differences between HR and LR groups in the left STS region. Moreover, Braukmann et al. (2018) detected different evoked responses in the right temporal region between groups. In contrast other studies, despite reporting cortical hypoactivation in HR both in naturalistic social interactions (Bhat et al., 2019) and face processing (Fox et al., 2013), did not highlight a clear hemispheric asymmetry of responses.

Finally, resting-state fNIRS studies found a hypoactivation with rightward asymmetry shift in the prefrontal cortical area of ASD preschool children (Li and Yu, 2016; Li et al., 2018). Only two studies reported ASD-related alterations bilaterally (Kikuchi et al., 2013; Jia et al., 2018). However, these results could be influenced by technical aspects, such as the different number of channels used.

Even if no systematic assessment of hemispheric asymmetry has been performed with fNIRS in preschoolers, these findings and evidence from other neuroimaging technique such as structural (Conti et al., 2016) and functional MRI (Müller et al., 2011) would encourage studying more in depth atypical lateralization in ASD population, also at early stage.

Could fNIRS Be the Right Tool to Dissect the Vascular Contribution to ASD Condition?

Over the last years of research, the biological dimension of ASD has been extensively investigated both in the preclinical (Ebert and Greenberg, 2013; Del Pino et al., 2018) and the clinical

settings (Billeci et al., 2016; Bralten et al., 2018). However, most studies only focused on neuronal mechanisms underlying ASD.

Preclinical studies on animal models can offer important insights about specific mechanisms underlying the pathophysiology of ASD (Bartz et al., 2008). Recent studies suggested the presence of structural and functional neurovascular abnormalities in ASD. Indeed, an early dysfunction of endothelial cells, altered angiogenesis and impaired vasodilation reactivity have been shown in a mouse model of 16p11.2 deletion ASD syndrome (Ouellette et al., 2020). Similarly, cerebrovascular deficiencies have been recognized in a mouse model of Creatine Transporter Deficiency, an inherited metabolic condition characterized by intellectual disability and autistic-like features (Mazziotti et al., 2020). The vascular hypothesis is also supported by clinical studies. Indeed, postmortem analysis of ASD brains suggested an impairment of cerebral angiogenesis (Azmitia et al., 2016) and resting-state imaging highlighted the alteration of cerebral blood flow in distinct brain regions (Jann et al., 2015; Bjørklund et al., 2018). Interestingly, it has been speculated that an exacerbated inflammatory response might be one of the possible causes of the deteriorated integrity of the blood-brain barrier (Fiorentino et al., 2016; Kumar and Sharma, 2016) and of the defective blood flow documented in the ASD brain (Morgan et al., 2010; Bjørklund et al., 2018). Moreover, there is evidence of glial dysfunction in ASD (Petrelli et al., 2016), with the alteration of astrocyte population (Fatemi et al., 2008; Edmonson et al., 2014; Wang et al., 2021) potentially contributing to the deregulation of neurovascular homeostasis.

We believe that the potential vascular contribution in the etiology of ASD should be explored more in-depth, since the interplay with the vascular system is crucial for the proper maturation and function of neuronal networks (Andreone et al., 2015; Segarra et al., 2018). In this framework, the combined analysis of hemodynamic responses measured with fNIRS and bioelectrical signals obtained through EEG recordings might allow dissecting vascular vs. neuronal aspects of ASD.

CONCLUSION

This review highlights theoretical and methodological advantages of fNIRS that encourage its application for identifying quantitative biomarkers in NDDs, and in particular in the autism field. Even if available papers in preschoolers are yet not conclusive to let us claim a "fNIRS signature" of autism, we believe that the use of this technique as an auxiliary diagnostic tool is very promising. Consistently, we recently reported that the variability of fNIRS visually evoked hemodynamic responses correlates with autistic traits in typically developing children, setting the background for testing the diagnostic value of fNIRS visual measurements in the ASD and HR clinical population (Mazziotti et al., 2022).

Nevertheless, some critical technical issues should be taken into account, because they might limit the interpretation of data and the comparison of results among different studies. First, the availability of reliable methods to detect and remove motion artifacts is fundamental for applying fNIRS in a very young population. Only a few of the studies reviewed (Edwards et al., 2017; Bhat et al., 2019; Pecukonis et al., 2021) systematically applied motion correction algorithms to the data recorded, while most of them simply removed corrupted data (caused by large head or body movements or other unexpected behaviors) by visual inspection of experiment video recordings. This methodological heterogeneity leads not only to a considerable failure rate of fNIRS data collection, but also to a possible bias in the results. Since this topic is debated, wide agreement on subtle technical parameters to apply is still missing even if they could significantly affect data outputs (Di Lorenzo et al., 2019). Moreover, in resting-state measurements complex algorithms to extract reliable data from the fNIRS raw output are often required, representing a potential bias and limiting factor for their clinical use. Indeed, several methodological models have been applied, like functional network efficiency (Li and Yu, 2016, 2018), detrended fluctuation analysis (Jia et al., 2018), coherence analysis of spontaneous hemodynamic fluctuations (Kikuchi et al., 2013) and a combination of principal component analysis and normalized entropy (Li et al., 2018). It is to note that all resting-state studies reviewed compared ASD vs. TD cohorts (3–7-year-old children) and included in the clinical sample only high-functioning autism, probably due to recording constraints (predictable low-grade of compliance during the experimental procedure) that prevent such experimental design in younger and more impaired subjects.

On the other hand, the analysis of articles reviewed in this survey offers several cues of topics to systematically stress in the future fNIRS research within the autism field. Only one of the 13 articles included a focus on the suitability of fNIRS to highlight gender differences in HR infants (Edwards et al., 2017) and the small sample size of other studies (or the lack of a gender-balanced population) did not allow separating gender analyses. However, since consistent findings reported sexual dimorphism of specific brain regions and networks in ASD (see Mo et al., 2021 for a recent review), a specific focus on gender-related brain differences in well-powered cohorts might be an added value of next fNIRS assessments. Similarly, more weight should be given to the extrapolation of asymmetry indexes from fNIRS signals that might represent intra-subject measures potentially useful as condition biomarkers. Moreover, previous studies (Burzi et al., 2015; Jones et al., 2015) explored the importance of imitative learning during early development, focusing on differences in brain activation evoked from live (real time actions) rather than artificial stimuli (videos or photos of actions). While the others used artificial stimuli was applied (Bhat et al., 2019), while in the others artificial stimuli were used. The putative role of stimulus "quality" is still challenging to assess and it might deserve attention in future studies.

Finally, the possibility to measure hemodynamic response with fNIRS would allow the investigation of the possible role of neurovascular coupling in the pathogenesis of ASD, in particular when paired with simultaneous EEG recordings. In this framework, the evolution of hemodynamic patterns from the developing to the adult brain (Kozberg and Hillman, 2016)

needs to be considered in the interpretation of typical and aberrant fNIRS signals.

AUTHOR CONTRIBUTIONS

EC and ES equally contributed to conceptualization of the manuscript, studies selection, interpretation of results, and manuscript writing. CB contributed to systematic search of the literature. VM, VC, VD, and RM contributed to the conceptualization of the study and interpretation of results. LD, CC, and FM supervised the manuscript. LB contributed to conceptualization, interpretation of technical results, and manuscript writing. SC and RB contributed to conceptualization and supervised the manuscript. All authors have read and agreed the final version of the manuscript.

FUNDING

This work has been supported by grant from the IRCCS Fondazione Stella Maris (Ricerca Corrente – RC 2021: Early functional and neurobiological markers of rare diseases associated to neurodevelopmental disorders -RB- and the 5 × 1000 voluntary contributions, Italian Ministry of Health), and from the University of Pisa, "Bando PRA-Progetti di Ricerca di Ateneo" (Institutional Research Grants) 2020–2021 "Multimodal non-invasive approach in young females with autism and their parents: a feasibility study."

ACKNOWLEDGMENTS

EC and SC acknowledge AIMS-2-TRIALS network.

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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.

frontiers | Frontiers in Neuroscience

ORIGINAL RESEARCH
published: 14 April 2022
doi: 10.3389/fnins.2022.844821

Functional Connectivity Underlying Symptoms in Preschool Boys With Autism: A Resting-State Functional Magnetic Resonance Imaging Study

Zhihong Lan[1†], Shoujun Xu[2†], Xiangrong Yu[1†], Zhenjie Yu[1], Meng Li[3], Feng Chen[3], Yu Liu[1], Tianyue Wang[3], Yunfan Wu[3], Yungen Gan[2‡] and Guihua Jiang[3*‡]*

[1] Department of Medical Imaging, Zhuhai People's Hospital, Zhuhai Hospital Affiliated With Jinan University, Zhuhai, China, [2] Department of Radiology, Shenzhen Children's Hospital, Shenzhen, China, [3] Department of Medical Imaging, Guangdong Second Provincial General Hospital, Guangzhou, China

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Edited by:
Eugenia Conti,
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Hironori Nakatani,
The University of Tokyo, Japan
Roma Siugzdaite,
Ghent University, Belgium

***Correspondence:**
Yungen Gan
gdszgyg-1@126.com
Guihua Jiang
GH.jiang2002@163.com

[†]These authors have contributed equally to this work and share first authorship

[‡]These authors have contributed equally to this work and share senior authorship

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: 28 December 2021
Accepted: 23 March 2022
Published: 14 April 2022

Citation:
Lan Z, Xu S, Yu X, Yu Z, Li M, Chen F, Liu Y, Wang T, Wu Y, Gan Y and Jiang G (2022) Functional Connectivity Underlying Symptoms in Preschool Boys With Autism: A Resting-State Functional Magnetic Resonance Imaging Study. Front. Neurosci. 16:844821. doi: 10.3389/fnins.2022.844821

Background: Single-sex children have been regarded as one of the best subjects to understand the abnormal development patterns of autism spectrum disorders (ASDs). However, the functional connectivity (FC) behind their symptoms is still unknown.

Methods: Based on FC analysis, the acquired resting-state functional magnetic resonance imaging (rs-fMRI) data sets, including 86 boys with ASD and 54 normal controls (NC), were used to detect the neural synchronous activity between brain regions. Pearson correlation analysis was used to evaluate the relationship between the abnormal FC value and clinical features.

Results: Individuals with ASD showed enhanced FC between the right calcarine and the right lingual gyrus (LG). The right medial orbital frontal cortex also showed increased FC with bilateral inferior temporal gyrus (ITG) [two-tailed, voxel-level $p < 0.001$, gaussian random field (GRF) correction, cluster-level $p < 0.05$]. We did not find a correlation between the abnormal FC value and clinical scales.

Conclusion: Our study reveals a possible relationship between atypical visual attention and poor learning ability in subjects with ASD, and delayed social language development may be a secondary symptom to ASD.

Keywords: autism spectrum disorders, functional connectivity, resting-state fMRI, preschool boys, visual

INTRODUCTION

Autism spectrum disorder (ASD), which includes two core symptoms, persistent defects in social communication and social interaction, and restricted, repetitive patterns of behavior, interests, or activities, is a heterogeneous neurodevelopmental disorder with many individuals affected. Recently, the first national survey of ASD in China has shown that the prevalence of ASD in

Abbreviations: FC, functional connectivity; ASD, autism spectrum disorder; NC, normal controls; GRF, gaussian random field; mOFC, medial orbital frontal cortex; IFG operc, inferior frontal gyrus; MTG, middle temporal gyrus; AG, angular gyrus; rs-fMRI, resting-state functional magnetic resonance imaging; ROI, region of interest; ABC, autism behavior checklist; CARS, childhood autism rating scale; ADOS, autism diagnostic observation schedule; DQ, development quotient; MPRAGE, magnetization-prepared rapid acquisition gradient echo; MNI, Montreal Neurological Institute.

children aged 6–12 years has been as high as 0.7% (Zhang and Han, 2020), of whom more than 40% receive more than one psychotropic drug treatment due to various symptoms (Henneberry et al., 2021). However, owing to the heterogeneity and high comorbidity rate of ASD (Leader et al., 2021), the typical developmental disorder model of ASD has not been explored so far, which poses great challenges to the treatment and prognosis of ASD. Thus, it remains a focus to explore the typical abnormal development pattern of ASD.

Existing studies have found that in early childhood, the brain volume of individuals with ASD is larger than that of normal children (Hazlett et al., 2017). However, owing to the stagnation or deterioration of cognitive and behavioral functions in late childhood, adolescence, or early adulthood, the brain volume of individuals with ASD seem to be no different to that of normal controls (NCs), known as "pseudo normalization" (Zielinski et al., 2014). Thus, the brain changes associated with "normalization" of brain volume in autism are likely inherently pathological or a complex mixture of pathology, compensatory mechanisms, relatively normal processes, and silent sculpting of the brain by the often atypical life experiences of individuals with autism. Moreover, an increasing number of studies have confirmed that sex differences in ASD are not only reflected in the incidence rate which is two to five times more common in men than in women, but also on the basis of clinical manifestations and anatomy (Lai and Szatmari, 2020). This means that using single-sex subjects in early childhood is one of the most promising ways to explore a more pure, uncompensated model of typical ASD developmental disorders. However, despite the fact that ASD can be early diagnosed by 24 months, the average age of diagnosis is 2 years later (Roberts et al., 2019), so preschool age may be the appropriate age to study ASD.

In our previous study on preschool boys with ASD using regional homogeneity analysis, we found that the right medial orbital frontal cortex (mOFC, which explains the ASD's repetitive stereotyped behavior), the opercular part of the left inferior frontal gyrus (IFG operc), the left middle temporal gyrus (MTG), and the left angular gyrus (AG, which can explain social language development defect) had abnormal spontaneous brain activity, as well as the right calcarine, a part of the primary visual cortex (Lan et al., 2021). Repetitive and rigid behaviors and social language development disorders are undoubtedly the most obvious clinical symptoms in ASD, and the strengthening of visual ability also seems to be important in the early stage of ASD. For example, an experiment demonstrated that if a toddler spent $\geq$69% looking at geometric scenes, the positive predictive effectiveness of accurately classifying children with ASD was 100% (Venker et al., 2021). Moreover, the enhanced visual ability of children at high-risk of ASD at 9 months was related to their severity of ASD symptoms at 15 and 24 months (Varcin and Jeste, 2017). Taken together, these findings suggest a link between increased visual ability and ASD symptoms.

We sought to investigate how the discovered brain regions could explain the important symptoms of ASD synergies with other brain regions? Functional connectivity (FC) of resting-state functional magnetic resonance imaging (rs-fMRI), an analytical method for identifying resting state neural networks between brain regions and elucidating abnormal brain activity from a comprehensive perspective, provides a good means of observation (Lowe et al., 1998). Chen et al. (2018) first explored atypical brain FC in preschool individuals with ASD, and found that brain regions involved in social cognition with ASD showed under-connectivity, while those involved in sensory and visual movement showed over-connectivity. McKinnon et al. (2019) found that the restricted behavior was associated with more positive FC between the default mode and dorsal attention networks at 24 months in individuals with ASD. However, some of these studies did not control the sex factor. Furthermore, most of the previous studies on FC in children with ASD investigated the integral network characteristics, but ignored the details of the possible effects of specific brain regions.

To understand the neural mechanism of male ASD symptoms, we attempted to explore the specific association between abnormal spontaneous activity brain regions and other certain brain regions in preschool boys with ASD using a relatively large sample size. Based on the results of a previous regional homogeneity of rs-fMRI study, we defined the right mOFC, left IFG operc, left MTG, left AG, and right calcarine as the region of interest (ROI) to analyze the FC between each of them and the whole brain. Then, correlation analysis was performed with the FC value and ASD scales. Based on a literature review, we speculated that there would be over-connectivity between visual-related brain regions and under-connectivity between the regions associated with social language in preschool-age boys with ASD.

MATERIALS AND METHODS

Participants

Eighty-six preschool boys (3–6 years old) with ASD were recruited from Shenzhen Children's Hospital, and 54 age-matched boys with typical development were recruited from local advertisements as the NC group. None of the NCs had reported a history of serious medical problems or neurological/mental illness. All participants were native Chinese speakers, their guardians fully understood the purpose of the study and written informed consent was obtained before enrolling in the group. The study was approved by the ethics review committee of Shenzhen Children's hospital.

Subjects with ASD were only enrolled after being jointly diagnosed by two or more deputy chief physicians of pediatrics or psychiatry. All individuals with ASD met the DSM-5 and were scored for symptom severity using the Autism Behavior Checklist (ABC) and the Childhood Autism Rating Scale (CARS). The CARS and ABC are the main diagnostic and screening tools for children with ASD in China. Although the diagnostic sensitivity of CARS for ASD is not as high as that of the Autism Diagnostic Observation Schedule (ADOS), which is considered as the gold standard, it has higher specificity and can avoid over diagnosed preschool children from being included in the ASD group (Randall et al., 2018). The CARS, which was scored by professionally trained pediatricians and psychiatrists, contains 15 items, and each item is divided into 4 levels. A total score $<$30 is classified as non-ASD, a total score of 30–36 is classified as mild to

moderate ASD, and a total score >36 is classified as severe autism. The ABC, which was completed by guardians (usually parents) who have spent a long time with the children, contains 57 items, each of which is divided into 4 levels. A total score of >30 indicates the suspected presence of ASD symptoms, and a total score of ≥67 indicates the presence of ASD symptoms. We also used the development diagnosis scale for children aged 0–6 years to evaluate the children's development quotient (DQ) (DQ < 70 is a low score). The NC group did not receive the corresponding scale score. Children with known mental, neurological (such as epilepsy and Tourette's syndrome), or genetic (such as fragile X and Rett syndrome) disorders were excluded from the ASD group. Children who had a history of unconsciousness for more than 5 min and were currently taking psychoactive drugs were also excluded.

Imaging Acquisition

Resting-state functional magnetic resonance imaging data were collected in the Radiology Department of Shenzhen Children's hospital using a 3.0T Siemens Skyra scanner. The Rs-fMRI acquisition parameters were as follows: repetition time/echo time, 2 s/30 ms; slice thickness, 3 mm with a 0.72 mm gap; field of view, 230 mm × 230 mm; flip angle, 90°; and matrix, 64 × 64. Thirty-five axonal slices covering the whole brain were positioned along the AC–PC line, and 240 volumes were acquired over approximately 8 min. Meanwhile, a T1-weighted sequence of magnetization-prepared rapid acquisition gradient echo (MPRAGE) prepared by three-dimensional magnetization covering the whole brain (176 sagittal sections) was obtained. Whole-brain T2-weighted images and T2-FLAIR images were also obtained to rule out the presence of organic brain lesions. After the MRI scan, the images of each participant were examined to ensure that the images met the experimental requirements.

Each child was sedated with 50 mg/kg chloral hydrate by trained and certified nurses in accordance with the guidelines and protocols developed by the hospital Radiation Sedation Committee. During the scan, a foam pad was used to prevent head movement, and adhesive earmuffs was used to protect hearing. Additionally, each participant was required to have a caregiver and their guardian present during the scan.

Functional Magnetic Resonance Imaging Data Pre-processing

Image pre-processing was performed using the data processing assistant in the resting state fMRI toolbox (DPARSF 3.0 Advanced Edition).[1] First, we eliminated the data with head movement >1.5 mm or >1.5° in any direction to minimize the influence of head movement. We also removed the first 10 time points of each subject to avoid the signal change before the magnetic field reached the stable state and let the subjects get accustomed to the noise of the fMRI. Next, the T1 image for each subject was co-registered with the functional images of the same subject. This co-registered T1 image was then segmented and normalized to the standard Montreal Neurological Institute (MNI) space (age 4.5–8.5 years) (Fonov et al., 2011) and the voxel size was resampled to 3 mm × 3 mm × 3 mm. After normalization, a full-width Gaussian kernel of 8 mm × 8 mm × 8 mm was used to smooth the image at the half-maximum value, and then the image was processed by linear detrending. Twenty-seven covariates were used for regression analysis, including 24 head motion parameters, white matter signal, global mean signal, and cerebrospinal fluid signal. Finally, bandpass filtering (0.01–0.08 Hz) was applied.

[1]http://rfmri.org/DPARSF

Functional Connectivity Analysis

The brain regions with abnormal spontaneous brain activity in previous studies of preschool boys with ASD (Lan et al., 2021) were defined as the ROI, with a radius of 6 mm. These five brain regions were the right calcarine (9, −90, 3), right mOFC (6, 51, −9), left MTG (−42, −48, 9), left AG (−42, −57, 27), and left IFG operc (−39, 3, 21). FC analysis was used to explore the patterns between the seed ROI and the whole-brain voxels. For each subject, FC correlation plots of each ROI were obtained by voxel multiple regression, and the resulting correlation coefficients were converted into Z-scores by Fisher's transformation.

Statistics

The two-sample *t*-test was used to assess age differences between the ASD and NC groups. The two-sample *t*-test was also executed by the toolbox in DPARSF to identify significant between-group differences in the FC of each ROI. Then, gaussian random field (GRF) was used for multiple correction to determine the brain regions with statistical differences (two-tailed, voxel-level $p < 0.001$, cluster-level $p < 0.05$). Finally, SPSS 20.0 software was used to conduct the Pearson correlation analysis between the FC value of abnormal brain region and the CARS and ABC ($p < 0.05$).

RESULTS

Demographic and Clinical Characteristics

As is shown in **Table 1**, there were no significant between-group differences in age ($T = -1.518$, $p = 0.13$). The values of the ABC, CARS, and DQ of the ASD group were consistent with the ASD standard.

TABLE 1 | Demographic and clinical characteristics of boys with ASD and NCs.

	ASD group (*n* = 86)	NC group (*n* = 54)	T	*P*
Age	3.92 ± 0.95	4.09 ± 0.96	−1.518	0.13
ABC	68.12 ± 15.15			
CARS	34.17 ± 2.08			
DQ	53.44 ± 7.90			

Data are presented as the mean ± standard deviation.
ABC, autism behavior checklist; CARS, childhood autism rating scale; DQ, developmental quotient.

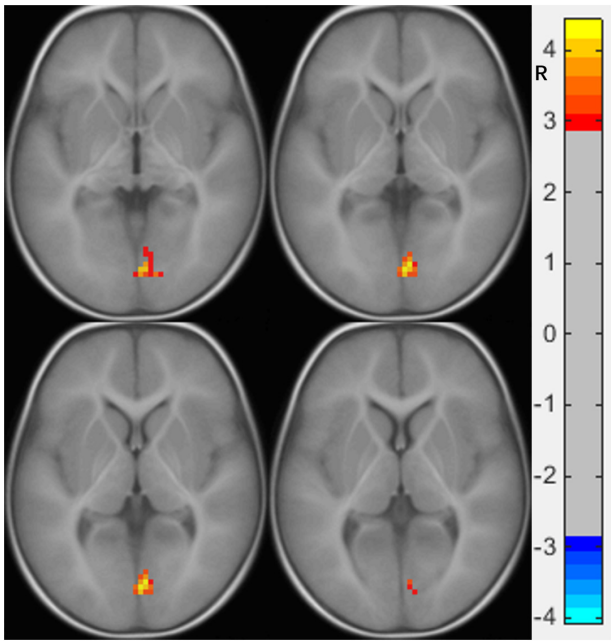

FIGURE 1 | Functional connectivity differences of the right calcarine between preschool boys with ASD and NCs. The warm color (right lingual gyrus) represents increased connectivity.

TABLE 2 | Brain regions showing abnormal FC with the right calcarine in the ASD group ($p < 0.05$).

Brain area (AAL)	VOXEL	MNI			Peak
		x	y	z	
Lingual_R	102	3	−81	0	4.4347

R, right; MNI, Montreal Neurological Institute; FC, functional connectivity.

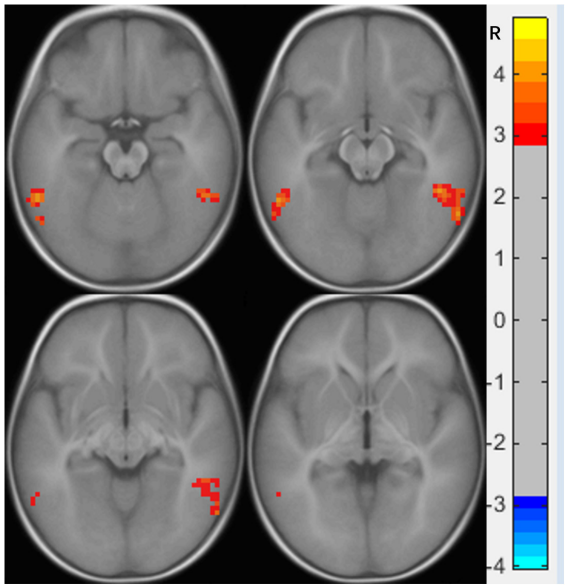

FIGURE 2 | Functional connectivity differences in the right medial orbital frontal cortex between preschool boys with ASD and NCs. The warm color (bilateral inferior temporal gyrus) represents increased connectivity.

TABLE 3 | Brain regions showing abnormal FC with the right medial orbital frontal cortex in the ASD group ($p < 0.05$).

Brain area (AAL)	VOXEL	MNI			Peak
		x	y	z	
Temporal_Inf_L	96	−57	−51	−15	4.9136
Temporal_Inf_R	201	54	−48	−21	4.5960

L, left; R, right; MNI, Montreal Neurological Institute; FC, functional connectivity.

Alterations of Functional Connectivity in Preschool Boys With Autism Spectrum Disorder

Compared to the NC group, the right calcarine-based FC analysis revealed increased FC with the right lingual gyrus (LG) (**Figure 1** and **Table 2**), the right mOFC-based FC analysis revealed increased FC with bilateral inferior temporal gyrus (ITG) (**Figure 2** and **Table 3**), and left MTG-, the left AG-, and the left IFG operc-based FC analyses did not find any significant difference in the brain region.

Correlation Between Functional Connectivity Values and Clinical Scales

The FC values of the three brain regions and the values of ABC and CARS had a normal distribution, so Pearson correlation analysis was used. However, we found no significant correlation between any brain region and ASD related scales.

DISCUSSION

In this study, we used the right calcarine-, right mOFC-, left IFG operc-, left MTG-, and left AG-based FC to compare the differences in the connectivity patterns between preschool boys with ASD and the NC group. We found that the FC between the right calcarine and the right LG, and the FC between the right mOFC and the bilateral ITG were increased in the ASD group. However, no abnormal brain regions were found in FC analysis based on ROI brain regions that are associated with social language. Moreover, no significant correlation was found between the abnormal brain regions and the ASD scale score.

We found that the FC of preschool boys with ASD was increased between the right calcarine and the right LG, both of which are important components of the visual system. The right calcarine belongs to the V1 area, which is responsible for projecting the received visual signals to higher-level processing areas, while the right LG belongs to zone V4, which is more inclined to process detailed color information (Johnson et al., 2015). The FC between the two was increased, which indicates that compared to the NC, the visual system of preschool boys with ASD showed abnormal enhancement in the processing of

color information. A previous study found that children with ASD completed the semantic task of picture-word matching more accurately than word-word matching (Turnbull et al., 2020). Another study found that compared to NC children, children with ASD (3 years old) showed faster response time and higher accuracy in completing the task of finding specific graphics hidden in colorful pictures (Nilsson Jobs et al., 2018). The advantage found in these studies in children with ASD in processing color-related details is consistent without our results showing FC enhancement in the right LG and right calcarine. Thus, our findings provide some evidence for visual advantage in children with ASD (Stevenson et al., 2018) from the perspective of functional imaging.

An FC increase between the right mOFC and the bilateral ITG of preschool boys with ASD was also found in our study. The right mOFC is part of the reward loop and is related to the ASD symptoms of restricted, repetitive patterns of behavior, and interests, which includes atypical visual attention (Carlisi et al., 2017), while the bilateral ITG is responsible for the key area of visual association learning (Zhang et al., 2016). The enhanced FC between the two suggests that most of the knowledge obtained by preschool boys with ASD from external visual information is limited to atypical visual attention, which is difficult to separate and transfer due to dysfunction of the reward loop. A previous study found that children with ASD prefer to focus on significant visual objects (such as color and geometric patterns) than NC children (spend more time looking at social stimuli), and the more severe the symptoms, the worse the learning ability of children with ASD (Venker et al., 2021). Another study also found that children with ASD who spend more time watching geometric images will show more serious ASD symptoms, a lower intelligence quotient, and lower adaptive skills (Bacon et al., 2020). These findings support our observed results that enhanced FC between the right mOFC and the bilateral ITG hinders the development of learning ability from the external visual environment in children with ASD. This also explains why children with ASD have visual advantages over NC, but their clinical symptoms show various social disorders. We speculate that the FC enhancement between the right mOFC and the bilateral ITG may be the key to the symptoms of ASD.

Additionally, in contrast to other FC studies of older subjects with ASDs (Chouinard et al., 2017; Kana et al., 2017; Lee et al., 2020), although the brain regions related to social language showed decrease spontaneous brain activity in our previous study, we did not find any other brain region with abnormal FC with them. We speculate that the previously observed abnormal FC in brain regions associated with social language might be functional compensation developed in children with ASD. In most cases, the behavioral symptoms of ASD did not completely appear before the age of two (Zwaigenbaum et al., 2015). However, atypical visual attention, which was not regarded as the core symptom, was manifested in individuals with ASD since infancy (Wang et al., 2015). This precursor symptom of atypical visual attention will interfere with word learning and lead to a delay in social language development in children with ASD (Venker et al., 2018). Therefore, we boldly speculated that the social language development disorder, which was the core symptom most easily observed by parents, may be a secondary result of the abnormal FC between the brain regions related atypical visual attention and visual association learning.

Our research has some limitations. First, our subjects were sedated in the scanner that the results we observed may not fully reflect the changes of FC in the awake state of ASDs. Second, our study was focused on male individuals with ASD and could not be extended to female individuals with ASD. To avoid the over-diagnosed preschool boys being included in the ASD group, the CARS and ABC were selected, but the weight of atypical visual attention was relatively low in these scales, and may mask the correlation between our observed results and the severity of ASD symptoms. Additionally, owing to the sample size, our research focused only on the group-level differences, thus limiting our ability to identify meaningful ASD subgroups, which may help us understand the convergence and divergence mechanism in the brain of preschool boys with ASD.

The current study found that preschool boys with ASD have a certain visual advantage, and that the increased FC between the right mOFC and the bilateral ITG, which caused a reduction in learning ability because of atypical visual attention, may be the key to the symptoms of ASD. Additionally, the social language development delay, which was a significant clinical symptom of ASD, may be a secondary result of atypical visual attention. Indeed, early defects in the field of visual behavior are beneficial to the early diagnosis of ASD and assist with the planning of corresponding treatment.

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 Ethics Review Committee of Shenzhen Children's hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

ZL, ZY, ML, FC, YL, and TW draft the manuscripts. SX, XY, YW, and GJ fund the project. YG scan the subjects. All authors contributed to the article and approved the submitted version.

FUNDING

This study has received funding by the National Natural Science Foundation of China (Grant Nos: U1903120, 81771807, 82071915, and 81901729), the Guangzhou Science and Technology Plan Project (Grant No: 202002030234), and the Sanming Project of Medicine in Shenzhen (Grant Nos: SZSM202011005 and SZSM201612036).

ACKNOWLEDGMENTS

We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript. We thank Qianyu Zhou for her support in daily work. We also wish to express our thanks to all the critics who have made great improvements to the article. We also thank all the volunteers who participated in this study.

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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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ORIGINAL RESEARCH
published: 03 May 2022
doi: 10.3389/fnins.2022.815933

Neural Markers of Auditory Response and Habituation in Phelan-McDermid Syndrome

*Emily L. Isenstein[1], Hannah E. Grosman[2], Sylvia B. Guillory[3,4], Yian Zhang[5], Sarah Barkley[3,4], Christopher S. McLaughlin[3,4], Tess Levy[3,4], Danielle Halpern[3,4], Paige M. Siper[3,4,6], Joseph D. Buxbaum[3,4,6,7,8], Alexander Kolevzon[3,4,6,9] and Jennifer H. Foss-Feig[3,4,6]**

[1] Department of Brain and Cognitive Sciences, University of Rochester, Rochester, NY, United States, [2] Department of Psychological and Brain Sciences, Drexel University, Philadelphia, PA, United States, [3] Seaver Autism Center, Icahn School of Medicine at Mount Sinai, New York, NY, United States, [4] Department of Psychiatry, Icahn School of Medicine at Mount Sinai, New York, NY, United States, [5] Center for Neural Science, New York University, New York, NY, United States, [6] Mindich Child Health and Development Institute, Icahn School of Medicine at Mount Sinai, New York, NY, United States, [7] Department of Neuroscience, Icahn School of Medicine at Mount Sinai, New York, NY, United States, [8] Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, United States, [9] Department of Pediatrics, Icahn School of Medicine at Mount Sinai, New York, NY, United States

OPEN ACCESS

Edited by:
Eugenia Conti,
Stella Maris Foundation (IRCCS), Italy

Reviewed by:
Ana Alves Francisco,
Albert Einstein College of Medicine, United States
Walter Erwin Kaufmann,
Emory University, United States

***Correspondence:**
Jennifer H. Foss-Feig
jennifer.foss-feig@mssm.edu

Specialty section:
This article was submitted to Neurodevelopment, a section of the journal Frontiers in Neuroscience

Received: *16 November 2021*
Accepted: *23 March 2022*
Published: *03 May 2022*

Citation:
Isenstein EL, Grosman HE, Guillory SB, Zhang Y, Barkley S, McLaughlin CS, Levy T, Halpern D, Siper PM, Buxbaum JD, Kolevzon A and Foss-Feig JH (2022) Neural Markers of Auditory Response and Habituation in Phelan-McDermid Syndrome. Front. Neurosci. 16:815933. doi: 10.3389/fnins.2022.815933

Phelan-McDermid Syndrome (PMS) is a rare genetic disorder caused by deletion or sequence variation in the *SHANK3* gene at terminal chromosome 22 that confers high likelihood of comorbid autism spectrum disorder (ASD). Whereas individuals with idiopathic ASD (iASD) can demonstrate diverse patterns of sensory differences, PMS is mainly characterized by sensory hyporesponsiveness. This study used electrophysiology and a passive auditory habituation paradigm to test for neural markers of hyporesponsiveness. EEG was recorded from 15 individuals with PMS, 15 with iASD, and 16 with neurotypical development (NT) while a series of four consecutive 1,000 Hz tones was repeatedly presented. We found intact N1, P2, and N2 event-related potentials (ERPs) and habituation to simple auditory stimuli, both in individuals with iASD and in those with PMS. Both iASD and PMS groups showed robust responses to the initial tone and decaying responses to each subsequent tone, at levels comparable to the NT control group. However, in PMS greater initial N1 amplitude and habituation were associated with auditory hypersensitivity, and P2 habituation correlated with ASD symptomatology. Additionally, further classification of the PMS cohort into genetic groupings revealed dissociation of initial P2 amplitude and habituation of N1 based on whether the deletions included additional genes beyond solely *SHANK3* and those not thought to contribute to phenotype. These results provide preliminary insight into early auditory processing in PMS and suggest that while neural response and habituation is generally preserved in PMS, genotypic and phenotypic characteristics may drive some variability. These initial findings provide early evidence that the robust pattern of behavioral hyporesponsiveness in PMS may be due, at least in audition, to higher order factors.

Keywords: phelan-mcdermid syndrome, autism spectrum disorder, EEG, auditory, perception, habituation, sensory, genetics

INTRODUCTION

Phelan-McDermid syndrome (PMS) is a rare neurodevelopmental disorder caused by haploinsufficiency of *SHANK3* either by pathogenic sequence variant or by deletion (Phelan and McDermid, 2012; Oberman et al., 2015). PMS is characterized by global developmental delay, absent or delayed speech, hypotonia, and dysmorphic features (Soorya et al., 2013). Comorbid autism spectrum disorder (ASD) is common in PMS, with up to 84% of individuals receiving a diagnosis (Soorya et al., 2013), though estimates vary substantially (Phelan and McDermid, 2012; Sarasua et al., 2014). Both PMS and ASD are associated with sensory reactivity differences. A recent study showed that the sensory reactivity symptoms associated with PMS are distinct from those typically associated with ASD, wherein individuals with PMS have greater hyporeactivity and fewer hyperreactivity and sensory seeking symptoms compared to individuals with idiopathic ASD (iASD) and typically developing controls (Tavassoli et al., 2021). Evidence of hyporeactivity also comes from clinical reports of many individuals with PMS displaying delayed response to auditory and verbal cues, despite having no hearing impairments (Phelan and McDermid, 2012). However, research to date has heavily relied on caregiver report.

Electroencephalography (EEG) offers a precise and objective tool to evaluate sensory processing. One method of assessing auditory perception is by measuring habituation, or the decrease in electrophysiological activity in response to repeatedly presented sounds. In neurotypical individuals (Fruhstorfer et al., 1970) and in animal models (Sambeth et al., 2004), this brain response is consistent: to the first tone in sequence, a large response is elicited; thereafter, the response is dampened with the strongest decline between the first and second repetitions (Budd et al., 1998; Ozesmi et al., 2000). Habituation paradigms have been used in ASD research on several occasions to explore auditory sensory differences but have yielded mixed results. Some studies found slower habituation to auditory stimuli in ASD (Ornitz et al., 1993; Hudac et al., 2018; Green et al., 2019; Jamal et al., 2021), while others found no differences between ASD and neurotypical (NT) children or adults (Kohl et al., 2014; Takahashi et al., 2016). These findings showcase the heterogeneity found within ASD and emphasize the difficulty in establishing unifying, biologically-based characteristics in the absence of stratifying variables.

A small body of research has used electrophysiology as an objective measure of auditory responsiveness in people with genetic disorders related to ASD, though not yet in PMS. For example, studies of auditory processing have identified atypical electrophysiologic signatures in neurodevelopmental disorders including Rett syndrome (Sysoeva et al., 2020) and Tuberous Sclerosis Complex (O'Brien et al., 2020). Notably, one study used a habituation paradigm in Fragile X syndrome (FXS), recording cortical activity during passive listening to repeated sequences of identical auditory tones. This study successfully captured electrophysiological evidence for cortical hyper-excitability in individuals with FXS that also correlated with parental reports of sensory sensitivity (Ethridge et al., 2016). This study's recapitulation of findings in *Fmr1* knockout mice (Frankland et al., 2004) suggests that similar parallels may exist between humans with PMS and *Shank3* rodent models.

Although no auditory electrophysiology work has been undertaken in humans with PMS to date, work in animal models provides clues to the underlying neurophysiology and expected phenotype. A recent manuscript utilized a *Shank3* mouse model and found reduced startle response across a variety of sound intensities (Drapeau et al., 2018). These mice showed normal Preyer reflexes, indicating aberrations in auditory processing rather than broader issues with sensory gating. Another study found weaker electrophysiological responses and decreased levels of spontaneous firing in the auditory cortex in *Shank3* heterozygous rats compared to wild-type rats (Engineer et al., 2018). These rodents also showed a decreased number of spikes evoked in response to noise burst trains, with the responses to the successive rapid noise burst showing the greatest reduction. Together, these results suggest that *Shank3* deficiency in animals confers a delayed and less vigorous cortical response to auditory stimuli, but this biomarker has not yet been translated to humans with PMS. Such translational work would not only offer the opportunity to more deeply understand the neurobiological alterations identified in patients, but also serve as biomarkers for potential treatment approaches that could be tested in animal models then brought back to human patients.

To bridge the gaps between pre-clinical and clinical work, the current study tested electrophysiological correlates of auditory response and habituation in individuals with PMS, as compared to those with iASD and NT controls. In addition, the present study examined the relation between EEG markers of auditory function and several clinical indices, including extent of 22q13 deletion, age, developmental quotient, autism diagnosis, and sensory symptoms. By investigating whether behavioral auditory hyposensitivity has detectable electrophysiologic correlates, we hoped to identify whether changes in early cortical processing drive this phenotype. If people with PMS display blunted electrophysiological responses to new or repeated sounds, it would implicate early sensory processing as the source of dysfunction. On the other hand, if electrophysiological habituation is not disrupted in PMS, this suggests alternative, perhaps higher level or more domain-general, parts of the sensory-perceptual pathway as the drivers of behavioral alterations. Based on both the clinical phenotype in PMS and on animal findings, we predicted that, as compared to the NT group, the PMS group would have smaller amplitude responses and longer latencies to initial tones, followed by weaker habituation over time. We also predicted that, compared to the NT group, the iASD group would have similar latencies to initial tones but habituate more slowly over time and have overall higher amplitudes, based on the trait of auditory hypersensitivity that many iASD individuals share with individuals with FXS. Finally, we also expected that clinician and parent reports of sensory hyporeactivity within the PMS group

would correlate with reduced amplitude and habituation of electrophysiological components.

MATERIALS AND METHODS

Participants

Written informed consent was obtained from all participants or their caregivers, as appropriate, and verbal assent was obtained from all participants under the age of 18 who were able to provide it, as approved by the Icahn School of Medicine Program for the Protection of Human Subjects.

Participants included 15 individuals with PMS (Mean = 14.7 years, SD = 6.4), 15 with iASD (Mean = 14.3 years, SD = 5.6), and 16 with NT development (Mean = 13.1 years, SD = 4.3), all between the ages of 8 and 26. The groups did not differ significantly in age [$F(2, 43) = 0.32$, $p = 0.73$]. A Chi Square analysis also identified no significant sex differences among groups [X^2 (2, $n = 46$) = 5.51, $p = 0.06$]. In the PMS group, chromosomal microarray or targeted sequencing of the *SHANK3* gene validated the genetic diagnosis. Confirmed genetic diagnoses of PMS were defined as having either a deletion encompassing *SHANK3* (MIM: 606230) or a pathogenic sequence variant in *SHANK3* according to standards established by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Microarray results were aligned to the hg19 reference genome and sequence variants to reference transcript NM_033517.1. Of the individuals with PMS, 11 had deletions encompassing *SHANK3* and four had pathogenic sequence variants in *SHANK3*. One of the individuals with a deletion had a ring chromosome 22. Participants in the iASD group had no known pathogenic genetic findings. Neurotypical controls had no psychiatric disorders and no first-degree relatives with iASD.

Phelan-McDermid Syndrome and iASD participants received autism diagnostic testing with the Autism Diagnostic Observation Schedule - 2 (ADOS-2, Lord et al., 2012) administered by a trained, research-reliable clinician. ADOS-2 social affect scores, restricted and repetitive behavior scores, and total scores did not differ between clinical groups (see **Table 1**), with 1 participant with iASD missing ADOS-2 data. The Autism Diagnostic Interview - Revised (ADI-R, Lord et al., 1994) was administered to the PMS group by a trained, research-reliable clinician to more deeply and precisely characterize developmental history of ASD symptoms, as this cohort is particularly hard to study with standardized assessments given the level of intellectual disability and other comorbidities. Clinical consensus among licensed psychiatrists and clinical psychologists confirmed final diagnoses with 33% of the PMS group receiving an ASD diagnosis. Sensory symptomatology in the PMS group was further characterized using the Sensory Assessment for Neurodevelopmental Disorders (SAND, Siper and Tavassoli, 2021), which incorporates clinician-administered observation with caregiver interview to measure sensory symptoms associated with DSM-5 criteria for ASD, and has been validated in both ASD and PMS (Siper et al., 2017; Tavassoli et al., 2021). SAND data was missing for one participant with PMS.

Intelligence Quotient (IQ) testing appropriate for age and developmental functioning was administered to the iASD and PMS groups, including the Wechsler Abbreviated Scale of Intelligence – Second Edition (Wechsler, 2011), Stanford-Binet Intelligence Scales – Fifth Edition (Roid, 2003), Mullen Scales of Early Learning (Mullen, 1995), Differential Ability Scales – Second Edition (Elliott, 2007), and Wechsler Intelligence Scale for Children – Fifth Edition (Wechsler et al., 2014). Five of 15 PMS participants did not meet basal threshold requirements to receive an IQ score, so developmental quotients [DQ: (mental age/chronological age) × 100] scores were computed from subtest-level age equivalent scores to provide a standardized DQ (ratio IQ) measure used in place of IQ, allowing us to compare data across the various IQ instruments. There was a significant difference in cognitive level between iASD and PMS groups [$t(2, 27) = 7.21$, $p < 0.001$]. One iASD participant was missing an IQ score. IQ testing was not done with the NT group, but none had history of learning, psychiatric, or neurodevelopmental disorders or concerns, thus we estimate their IQs to have followed a typical normative distribution and therefore also to be higher than both iASD and PMS groups.

Experimental Procedure

Participants completed a 16-min auditory habituation task during dense-array EEG, while seated in a chair, booster seat, or caregiver's lap, as best facilitated their remaining seated and still. Each of 150 trials contained a sequence of four 1,000 Hz, 50 ms tones (generated in Audacity), separated by 618 ms. Trials were separated by a 4,000 ms inter-stimulus-interval. Experimental flow was controlled using E-Prime 2.0. Tones were delivered at 80 dB. Participants were not instructed to attend to the tones, and each watched a silent video of their choice throughout the duration of the experiment.

Electroencephalography Data Acquisition and Analysis

Continuous EEG data were collected using a 128-channel Philips HydroCel Geodesic Sensor Net and NetStation Software Version 5.3. Data were re-referenced to average reference and high-pass filtered at 0.5 Hz. Data were then processed within Matlab using the Fully Automated Statistical Thresholding for EEG artifact Rejection (FASTER) Routine toolbox (Nolan et al., 2010) with EEGlab (Delorme and Makeig, 2004). The FASTER routine employs multiple measures for identifying statistical outliers within the data. Continuous EEG data were segmented into 3,000 ms epochs from −1,500 to 1,500 ms, and time-locked to the onset of each tone during FASTER pre-processing. The processing steps involve classifying and replacing outlier channels with interpolated values in the continuous data, removing outlier epochs from single participant data, removing outlier components through spatial independent component analysis, and correcting outlier channels by interpolating single channels within a single epoch. All participants were presented the same total of 600 tones (150 sets of 4). The NT, PMS, and iASD groups had averages of 3.95 ± 0.96, 3.17 ± 1.23, and 3.47 ± 1.12% trials removed, respectively. The groups did not vary significantly by

TABLE 1 | Clinical and demographic information.

	Group Mean (SE)				
	PMS (*n* = 15)	**iASD (*n* = 15)**	**NT (*n* = 16)**	**Statistic**	***p*-Value**
Age (year)	14.99 (6.61)	15.08 (0.81)	13.66 (4.38)	$F = 0.32$	0.73
Sex (M/F)	8M, 7F	11M, 4F	5M, 11F	$X^2(2) = 5.51$	0.06
Cognitive level	36.47 (16.77)	97.02 (27.51)		$t = 7.21$	$p < 0.001$
ADOS-2					
Social affect	10.92 (0.90)	10.20 (1.51)	–	$t = -0.39$	0.70
Restricted and repetitive behaviors	4.08 (0.85)	3.13 (0.39)	–	$t = -1.06$	0.30
Total	13.36 (1.35)	13.33 (1.83)	–	$t = -0.01$	0.99
ADI-R domain scores					
Language/communication	10.00 (1.40)				
Reciprocal social interaction	13.80 (2.42)				
Restricted and repetitive behaviors	3.80 (0.63)				

proportion of trials removed [$F(1,2) = 1.97$, $p = 0.15$]. Lastly, ERPs were averaged separately for each of the four tones in the trial's sequence and baseline corrected using a 100 ms pre-stimulus interval. A low-passed filter of 30 Hz was used to account for noise caused by considerable amounts of movement by some of the PMS and iASD participants. This filter level is higher than (Sysoeva et al., 2020) or consistent with (O'Brien et al., 2020) other research on auditory processing in genetic conditions. ERP averages were computed using a trimmed means method, discarding the top and bottom 5% of data from each time point to yield a robust mean estimate (Leonowicz et al., 2005).

Event-Related Potentials Analysis

N1, P2, and N2 mean amplitudes were calculated as the individual averages of the 10 ms surrounding each peak at electrode Cz at the vertex of the head, where auditory event related potential are easily detected (Rosburg et al., 2010). For each participant, the P2 component was first identified in the ERP as the maximum voltage within the latency range 120–210 ms post-stimulus onset. Next, using the computed P2 latency value, the N1 component was defined as the minimum voltage within the window from 70 ms until the P2 time point. The N2 peak amplitude was quantified as the minimum voltage in the time window that started at the computed P2 latency until 385 ms. Latency was defined as time to peak amplitude for each component.

Statistical analysis was done using SPSS software and included one-way ANOVAs to compare the effect of group on individual component amplitude and latency within each tone. Additionally, repeated measures ANOVAs were conducted to compare habituation of component amplitude across the four tones by group. Greenhouse-Geiser was used when there were violations in sphericity. When Levene's Test was significant, the Brown-Forsythe results or the combination of Friedman and Kruskal-Wallis Tests were used, as appropriate. Correlations were run between all clinical and electrophysiological variables to assess relationships. Bonferroni corrections for multiple comparisons were used in the analyses, with a significant *p*-value set at 0.008 for the initial tone analysis and 0.002 for analyses of habituation across tones.

RESULTS

Response to Initial Tone

Applying three separate one-way ANOVAs, one for each component, analysis of the ERP response to the initial tone found peak amplitude was not significantly altered across diagnostic groups (see **Figures 1**, **2** and **Table 2**; all p's > 0.13, all $\eta^2_p < 0.09$). Three one-way ANOVAs were conducted to determine group differences between latency values for each component in response to the initial tone, again with no significant differences among groups (see **Table 2**; all p's > 0.11, all $\eta^2_p < 0.12$).

Habituation of Amplitude Across Tones

To determine changes in the ERP waveform across the sequence of the four tones from habituation effects, amplitude of the N1, P2, and N2 were each subjected to a repeated measures ANOVA. Non-parametric tests, Friedman and Kruskal Wallis, were applied when violations of ANOVA assumptions were noted.

Analysis of the N1 found a significant effect of tone position [$F(1.65, 70.99) = 13.64$, $p < 0.001$, $\eta^2_p = 0.50$] where there was a marked reduction in amplitude between the 1st tone ($N1_1$: -2.49 ± 0.44 μV) and the 2nd, 3rd, and 4th tones in the sequence ($N1_2$: -1.25 ± 0.26 μV, $p < 0.001$, $d = 0.57$; $N1_3$: -0.74 ± 0.26 μV, $p < 0.001$, $d = 0.74$; $N1_4$: -0.95 ± 0.24 μV, $p < 0.001$, $d = 0.68$). Further habituation effects were also detected between the response to the $N1_2$ tone and the $N1_3$ tone ($p = 0.003$, $d = 0.29$), though the amplitude difference between the $N1_2$ and $N1_4$ amplitudes did not reach significance ($p = 0.12$, $d = 0.20$). There was no significant difference between the $N1_3$ and $N1_4$ amplitudes ($p = 1.00$, $d = 0.11$), suggesting response to repetition was maximally habituated by the third repetition. The effect of group [$F(2,43) = 0.62$, $p = 0.54$, $\eta^2_p = 0.03$] and the group by tone position interaction [$F(3.30, 70.99) = 0.33$, $p = 0.83$, $\eta^2_p = 0.02$] were not significant.

Similar to results for the N1 component, P2 amplitude showed a significant effect of tone position [$F(3, 70.92) = 76.82$, $p < 0.001$, $\eta^2_p = 0.64$], with a significantly larger $P2_1$ amplitude (4.60 ± 0.37 μV) as compared to the subsequent $P2_2$ (1.96 ± 0.22 μV, $d = 1.30$), $P2_3$ (2.16 ± 0.22 μV, $d = 1.20$), and $P2_4$ (1.76 ± 0.22 μV, $d = 1.41$) tones (all p's < 0.001, all d's > 1.20). Unlike for

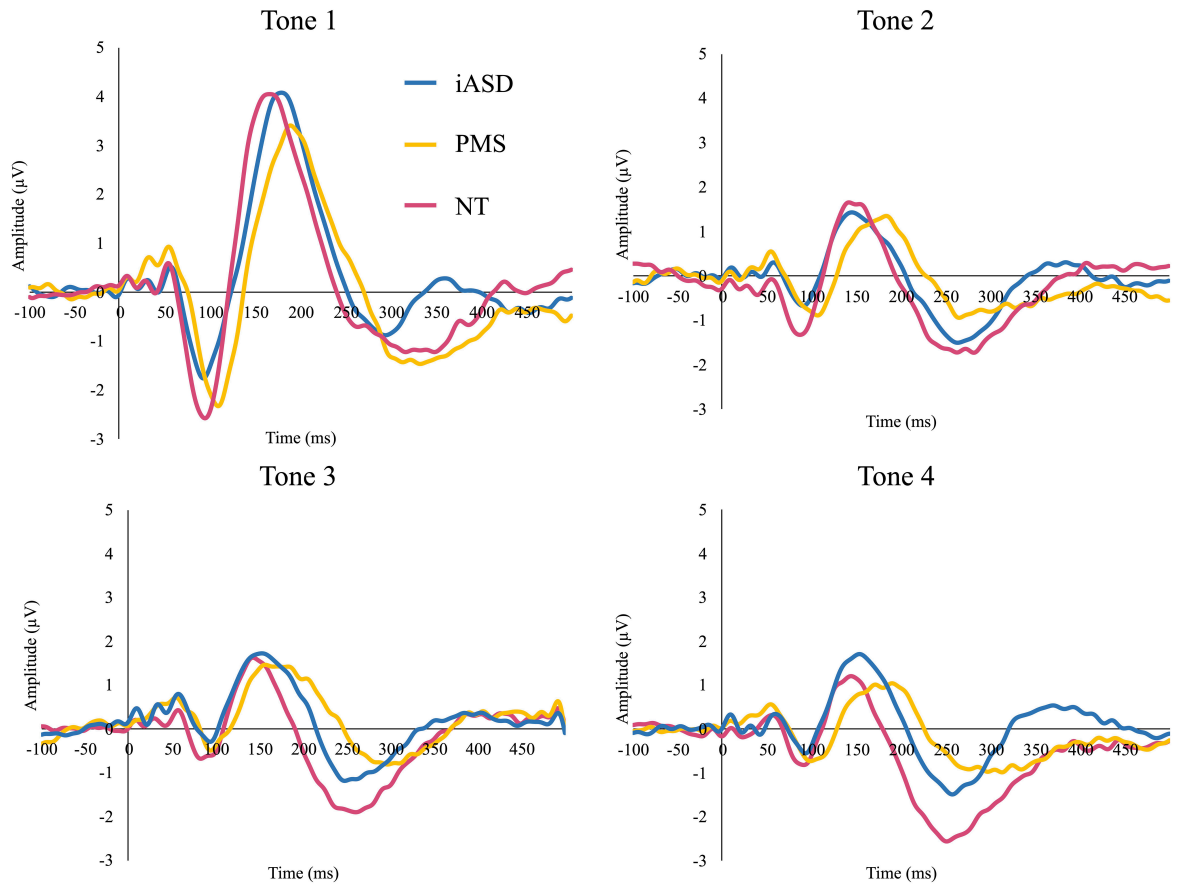

FIGURE 1 | Event-related potentials (ERP) response to consecutive four tones in individuals with idiopathic ASD (iASD), Phelan-McDermid Syndrome (PMS), and neurotypical development (NT) groups.

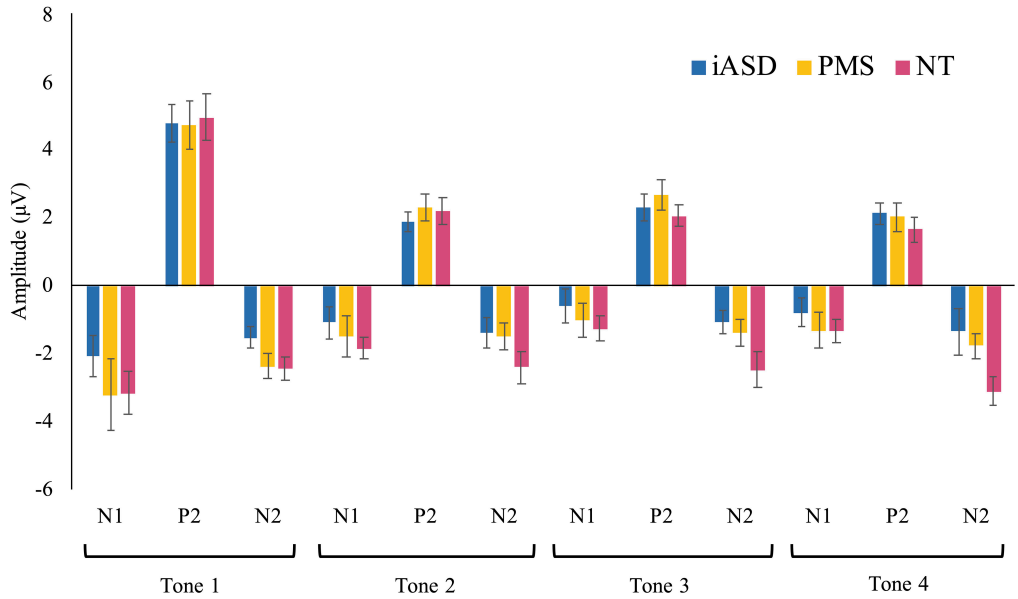

FIGURE 2 | Means of each ERP component in response to tones 1–4 with standard error bars in iASD, PMS, and NT groups.

TABLE 2 | Analysis of group differences to initial tone.

	Group mean (SE)			Statistics	
	PMS	**iASD**	**NT**	***F* Statistic**	***p*-value**
Initial tone					
N1 amplitude	−2.87 (1.00)	−1.81 (0.62)	−2.77 (0.62)	$F = 0.57$	0.57
P2 amplitude	4.53 (0.71)	4.60 (0.52)	4.68 (0.68)	$F = 0.02$	0.98
N2 amplitude	−2.49 (0.30)	−1.64 (0.18)	−2.29 (0.34)	$F = 2.13$	0.13
N1 latency	207.20 (6.26)	193.38 (2.65)	197.75 (3.61)	$F = 2.42$	0.11
P2 latency	298.53 (11.90)	276.69 (5.56)	279.13 (6.66)	$F = 2.02$	0.14
N2 latency	413.73 (7.92)	395.06 (7.73)	410.38 (10.78)	$F = 1.24$	0.30

N1, $P2_2$ amplitude did not differ from either $P2_3$ ($p = 1.00$, $d = 0.13$) or $P2_4$ ($p = 0.94$, $d = 0.15$) tones, but $P2_3$ was larger in amplitude compared to $P2_4$ ($p = 0.03$, $d = 0.27$). Again, there was no significant effect of group [$F(2,44) = 0.09$, $p = 0.92$, $\eta^2_p < 0.01$] and no significant tone position by group interaction [$F(6,70.20) = 0.62$, $p = 0.62$, $\eta^2_p = 0.03$].

Unlike the previous components, the effect of tone position on the N2 component did not withstand correction for multiple comparisons [$F(2.45, 105.40) = 3.18$, $p = 0.04$, $\eta^2_p = 0.07$]. All other comparisons were not significant, with no significant group effect [$F(2,43) = 3.19$, $p = 0.05$, $\eta^2_p = 0.13$] or group by tone position interaction [$F(4.90, 105.40) = 1.45$, $p = 0.21$, $\eta^2_p = 0.06$] for N2.

Exploratory Analyses

Correlation analyses were run for the PMS group with: (a) the mean amplitude and latency values for each component at tone 1, and (b) the difference between each component's amplitude at tone 1 and tone 2, used as a proxy measure of habituation. For negative components N1 and N2, a greater negative number indicates greater habituation. Correlations were not corrected for multiple comparisons due to the stringent threshold criteria driven by the limited sample size. With so little known about sensory processing in PMS, we believe that applying overly strict standards would obscure potential avenues for future research and slow progression of knowledge in this field. As the first study of auditory electrophysiological correlates of auditory hyposensitivity in a rare and difficult to test population, these data are important to include but should be interpreted cautiously as exploratory findings. See **Figure 3** for full statistics.

Genetics

Per the categorization scheme outlined in a recent manuscript on associations between genotype and phenotype in PMS, we divided participants with deletions into two classes (Levy et al., 2021). Class I was comprised of sequence variants as well as deletions including only *SHANK3,* or the combination of *SHANK3* with *ARSA* and/or *ACR* and *RABL2B* ($n = 11$); these final three genes are not thought to contribute to the phenotype of PMS. Class II was comprised of the remaining deletions that did not qualify as Class I deletions, i.e., larger deletions that extended beyond *SHANK3* and the three aforementioned genes ($n = 4$). Deletion size was not normally distributed so equal variance was not assumed.

The $P2_1$ amplitude in Class I (5.16 ± 0.89) was greater than Class II (2.80 ± 0.47, $p = 0.04$, $\Delta = 0.95$) and $N1_{1-2}$ habituation was greater in Class I (-2.30 ± 0.66) compared to Class II (0.49 ± 0.62, $p = 0.01$, $\Delta = 1.24$). No other electrophysiological measures differed between Classes. See **Table 3**.

Age

Age did not correlate with any of the metrics specified above (p's > 0.20).

Developmental Quotient

Non-verbal Developmental Quotient (NVDQ), Verbal Developmental Quotient (VDQ), and Full Scale Developmental Quotient (FSDQ) did not correlate with any ERP response or habituation metric (p's > 0.05). See **Figure 3**.

Autism Spectrum Disorder Diagnosis

There were no significant Pearson correlations between clinical consensus ASD diagnosis and any of the metrics specified above (p's > 0.09). See **Figure 3**.

Autism Diagnostic Observation Schedule - 2

A significant negative Pearson correlation was found in the PMS group between the $P2_1$ amplitude and ADOS-2 Restricted and Repetitive Behaviors (RRB) score ($r = -0.60$, $p = 0.02$), wherein lower P2 response was associated with higher levels of repetitive behaviors. There was also a significant negative relationship between the $P2_{1-2}$ habituation and the ADOS-2 RRB ($r = -0.67$, $p = 0.01$), Social Affect (SA) ($r = -0.55$, $p = 0.03$) and Total scores ($r = -0.60$, $p = 0.02$), with more ASD symptoms associated with reduced P2 habituation. No other electrophysiological measures were correlated with ADOS-2 scores. See **Figures 3**, **4A**.

Sensory Assessment for Neurodevelopmental Disorders

The total SAND scores as well as the three subdomains of the auditory domain (hypersensitivity, hyposensitivity, and seeking) were selected for analysis given our auditory task. The total, auditory hyposensitivity, and auditory seeking scores did not correlate with any of the ERP response or habituation metrics. However, the auditory hypersensitivity domain correlated with both the $N1_1$ amplitude ($r = -0.54$, $p = 0.04$) and

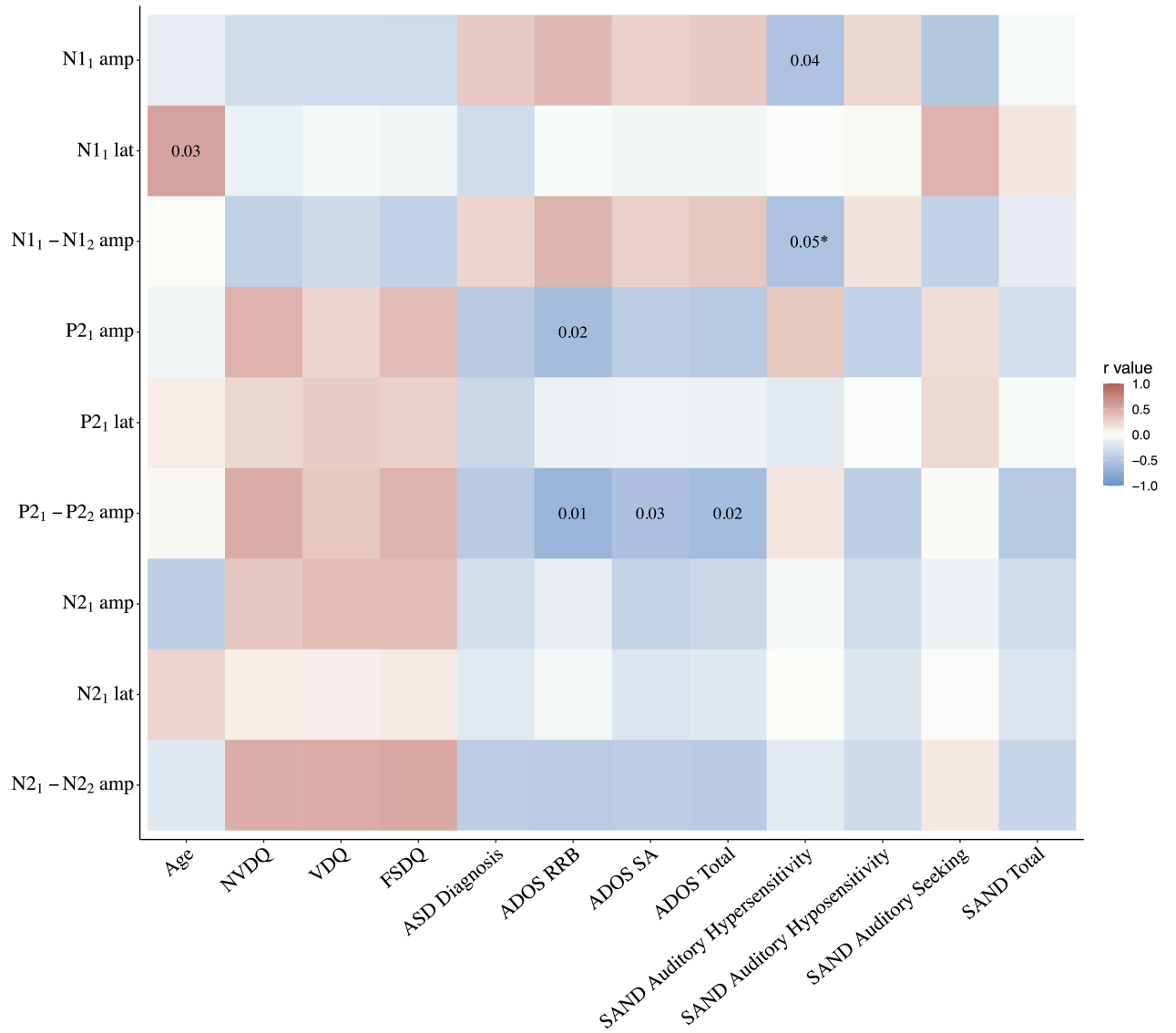

FIGURE 3 | Correlation heatmap of electrophysiologic findings and clinical indices in the PMS group. *Correlation between SAND auditory hypersensitivity and N1$_{1-2}$ habituation $p = 0.048$. amp, amplitude; lat, latency; NVDQ, Non-verbal Developmental Quotient; VDQ, Verbal Developmental Quotient; FSDQ, Full Scale Developmental Quotient; ADOS, Autism Diagnostic Observation Scale; RRB, Restricted and Repetitive Behaviors; SA, social affect; SAND, Sensory Assessment for Neurodevelopmental Disorders.

N1$_{1-2}$ habituation ($r = -0.54$, $p < 0.05$); greater auditory hypersensitivity was associated with both larger initial N1 response and greater N1 habituation. See **Figures 3**, **4B**.

DISCUSSION

In this study, we investigated electrophysiological markers of early auditory processing in groups of individuals with PMS, iASD, and NT controls. Our findings demonstrate preliminary evidence of intact response and habituation to simple auditory stimuli in PMS. Indeed, across both iASD and PMS, we found robust responses to initial tones, followed by a decay of response to the second tone and relatively asymptotic responses to the third and fourth tones. This response pattern corresponds with what has been shown in canonical habituation tasks in healthy controls (Budd et al., 1998; Ozesmi et al., 2000). These results were surprising, given the ample evidence for heightened sensory sensitivity in ASD (Karhson and Golob, 2016; Hudac et al., 2018), clinical reports of auditory hyporesponsiveness in PMS (Phelan and McDermid, 2012; Mieses et al., 2016), as well as findings in *Shank3* animal models showing reduced auditory startle (Drapeau et al., 2018). Latency of neural response to tones also did not differ among groups, despite prior studies in iASD showing slowed response to auditory stimuli (Roberts et al., 2010) and

TABLE 3 | Analysis of PMS genetic classes and electrophysiological measures.

	Group mean (SE)		Statistics	
	Class I	Class II	*F* Statistic	*p*-Value
Initial tone				
N1 amplitude	−3.93	0.08	$F = 4.58$	0.07
P2 amplitude	5.16	2.80	$F = 5.42$	0.04
N2 amplitude	−2.29	−3.04	$F = 2.51$	0.14
N1 latency	209.10	202.00	$F = 0.12$	0.75
P2 latency	303.09	286.00	$F = 0.29$	0.62
N2 latency	416.18	407.00	$F = 0.15$	0.72
Habituation				
N1 amplitude	−2.30	0.49	$F = 9.47$	0.01
P2 amplitude	2.80	1.37	$F = 2.38$	0.16
N2 amplitude	−0.99	−1.31	$F = 0.18$	0.68

others in animal models of PMS showing delayed response to auditory stimulation (Engineer et al., 2018). This divergence of the electrophysiological phenotypes of humans with PMS and equivalent animal models should be explored further to identify whether there are physiological differences driving the discrepancy.

With respect to PMS, our findings, though exploratory given the modest sample size, suggest that despite evidence for diminished behavioral response to auditory stimuli (Phelan and McDermid, 2012; Mieses et al., 2016), a key neural aspect of early auditory processing may be intact. A study that investigated the neurocognitive perception of communicative sounds using functional magnetic resonance imaging (fMRI) found similarly intact neural responses in PMS (Wang et al., 2016). Together, these results point toward unaffected early cortical processing of sounds in this population. If this is the case, alterations in *later* higher order stages of processing and the subsequent interpretation of auditory information that are beyond our measurement may contribute to the sensory phenotype of PMS. Such higher order cognitive processes could include extracting the relevance and meaning of stimuli, directing or sustaining auditory attention, and utilizing auditory input to direct behavior.

Our findings in PMS are distinct from those observed in FXS, where reduced habituation of the N1 was detected in a comparable sample size (Ethridge et al., 2016). Interestingly, the sensory phenotype of PMS and FXS differ; in PMS there is general sensory *hypo*sensitivity (Mieses et al., 2016), but FXS is characterized by sensory *hyper*sensitivity (Ethridge et al., 2019). Dissociable neural response patterns to auditory stimulation may indicate that the neuropathology of PMS and FXS diverge on a fundamental level. Whereas FXS demonstrates alterations in early cortical processing deficits, it may be that the sensory symptoms characteristic of PMS are perhaps driven instead by issues with interpreting and attending to sensory information or initiating an appropriate behavioral response. This observed preservation of early auditory habituation parallels pre-clinical findings in at least one *Shank3* mouse model that found no disruption of sensory gating (Drapeau et al., 2018). These results serve as a contrast to findings of reduced electrophysiological amplitude in response to visual stimuli in individuals with PMS (Siper et al., 2021), suggesting dissociation of cortical processing across at least these two sensory domains. Future research should also assess whether cortical responses to somatosensory stimulation resemble vision and parallel the behavioral tactile hyposensitivity found in PMS or if they are incongruent as with audition. This work on additional sensory modalities will help clarify the extent to which the neural bases of sensory processing abnormalities overlap or diverge across modalities in PMS.

Though we did not see overall group differences between PMS and NT controls, patterns of auditory response and habituation did nominally correlate with both genotype and phenotype. Class II deletions were associated with reduced initial P2 response and poorer habituation of the N1 response to repeated tones, indicating potential dissociation based on extent of genetic deletion. Likewise, associations between the ADOS-2 and electrophysiological measures indicate a possible

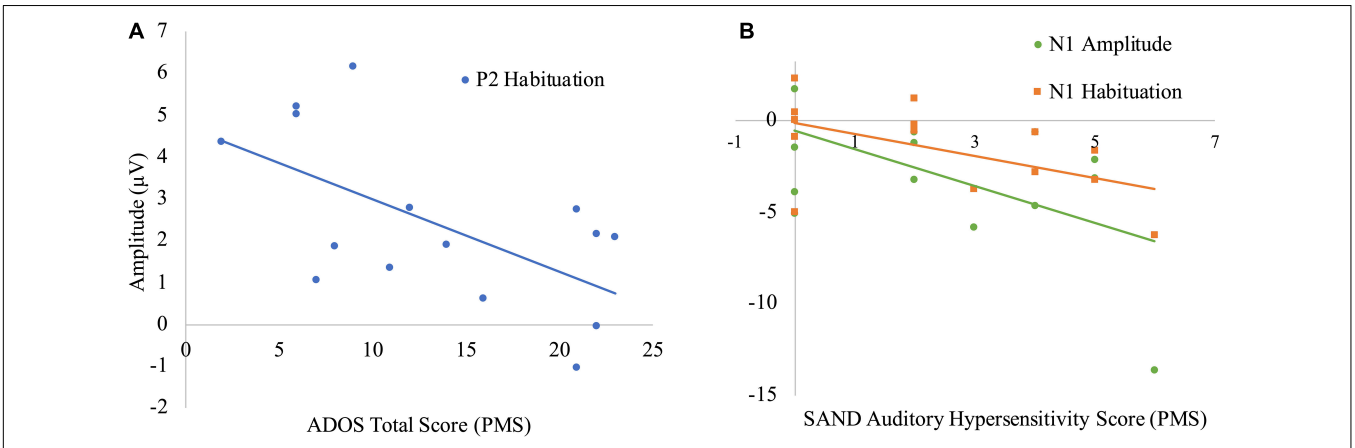

FIGURE 4 | Correlations between electrophysiologic and behavioral measures in PMS. **(A)** Negative correlation between $P2_{1-2}$ habituation and ADOS total score. **(B)** Negative correlation between $N1_1$ amplitude and SAND auditory hypersensitivity, as well as $N1_{1-2}$ habituation and SAND auditory hypersensitivity.

interaction between deletion size and ASD traits that could be explored further in larger samples. Finally, higher levels of clinically-observed auditory hypersensitivity were nominally associated with both larger N1 amplitudes to the initial tone and greater subsequent N1 habituation to the second tone. This pattern of greater habituation to sounds that initially evoke a particularly large response may result in behavioral hyposensitivity over time to sounds such as tones, or manifest as aversion responses. If it also extends to complex auditory input like speech or to socially-relevant sounds (e.g., having one's name called), it could be a contributor to reduced social engagement reported in PMS (Burdeus-Olavarrieta et al., 2021). With regard to the correlation with genotype, as the larger deletion sizes entail additional missing genetic material, it may be that other genes beyond *SHANK3* contribute to auditory habituation differences. These results must be interpreted cautiously and should be investigated in larger cohorts.

Finally, our findings of similar early processing of auditory habituation in groups with iASD as compared to both PMS and NT controls despite notably different sensory phenotypes imply that the level at which auditory processing diverges in these group is likely not in the most basic response and habituation to repeated simple tones. Recent studies of auditory habituation in ASD continue to yield inconsistent results, with some finding reduced habituation (Gandhi et al., 2020; Jamal et al., 2020) while others, like us, finding no differences between ASD and control groups (Top et al., 2018; Kuiper et al., 2019). Collectively, these studies exemplify the heterogeneous nature of ASD and how differences in samples and experimental parameters can influence results. The genetics-first approach used in this study offers a promising way to parse this heterogeneity and pave the way for greater understanding of sensory differences along the autism spectrum.

LIMITATIONS

Several limitations of this study stem from inherent issues with studying a rare genetic disorder, which makes recruitment, matching subjects, as well as obtaining large sample size and/or developmentally narrow age cohorts and high power results difficult. Although this research utilized a respectable sample size for an experimental study involving a rare disorder, a broader age range than is typical of most EEG studies also was needed to adequately sample the PMS population. The number of participants also remains small relative to typical EEG study samples. This small sample size also meant that we could not correct for multiple comparisons in correlations analyses, as overly stringent significance criteria in a sample of our size would be at high risk for obscuring small but meaningful results. As such, we report and discuss nominally significant findings to inform avenues for future work; replication in larger, independent samples is certainly needed.

Additional limitations stem from the complexity of our cross-group comparisons. First, groups varied considerably in terms of sex distribution; given limited research on the effect of sex on sensory processing (Osório et al., 2021), sex differences could be confounding. However, by and large, we saw few group differences for sex to have inadvertently driven. Second, we do not have IQ information for our NT group and our PMS sample had a significantly lower cognitive level than the iASD sample. However, at least within our PMS group, IQ did not correlate with any of the electrophysiological measures, and this observation is consistent with past research on IQ and sensory processing in ASD, which suggests a negligible – or at best inconsistent - relationship between the two domains (Rogers et al., 2003; Crane et al., 2009; Kargas et al., 2015; Sanz-Cervera et al., 2015). Thus, though intellectual level was not matched across groups, both our findings and past research supports that low-level detection of auditory information is decoupled from measured intelligence. Third, though our iASD sample is modest, particularly in light of the heterogeneity across ASD, our electrophysiological findings in iASD vs NT replicates earlier work (Kohl et al., 2014; Takahashi et al., 2016).

Finally, our PMS group was somewhat unusual in that only thirty three percent of our PMS sample also had an ASD diagnosis. This percentage is low given the higher prevalence typically reported in the literature (Kolevzon et al., 2014), though we note that ASD diagnosis did not interact with electrophysiological results in our PMS sample. Nonetheless, our results may not be generalizable to the PMS population as a whole, albeit given the ascertainment bias associated with genetic testing, it is possible that true ASD rates are lower than estimated among known cases. As genetic testing becomes less expensive and more widespread, additional diagnoses of PMS are expected to be made, enabling studies with larger sample sizes and smaller age ranges, as well as better matching based on functioning level, ASD diagnosis, comorbidities, and sensory phenotype. At that time, studies may be able to more clearly dissociate the neurophysiology of ASD, PMS with ASD, and PMS without ASD to better understand the interaction. At present, however, this study is worthwhile in offering the first-ever electrophysiological experimental look into brain processes subserving auditory function in the PMS population. Our findings of intact function are intriguing, particularly given the severity of deficits in this population.

CONCLUSION

This study is among the first to explore the neurocognitive basis of the PMS sensory phenotype and serves as the basis for future auditory neurophysiology work in this population. The preserved initial electrophysiological functioning shown here suggests that alterations in downstream processing of sounds, which deals more with extracting the relevance and meaning of stimuli and guiding behavioral response, may drive the hyporesponsive phenotype in PMS. Relations between the electrophysiological measures and ASD diagnosis, symptoms, and 22q13 deletion size in PMS point toward individual variability and genotype-phenotype relationships. Future studies that probe both early and late auditory processing in larger samples of people with PMS would help elucidate the source of these individual differences

and identify where in the auditory perceptual pathway the breakdown in processing occurs. This knowledge may aide in the development of targeted therapeutics that reduce the negative consequences of auditory hyporesponsiveness in this group.

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 Icahn School of Medicine Program for the Protection of Human Subjects. Written informed consent to participate in this study was provided by the participant or the participant's legal guardian/next of kin, as appropriate. Verbal assent was also provided by participants with capacity.

AUTHOR CONTRIBUTIONS

EI and JF-F designed the study and wrote the manuscript. EI, HG, SG, YZ, SB, and CM participated in data collection and processing. EI, SG, TL, and JF-F contributed to data analysis. DH, PS, AK, and JF-F characterized the participants and provided clinical supervision. SG, TL, PS, JB, and AK provided substantial revisions. All authors read and approved the manuscript.

FUNDING

This work was supported in part by a Seaver Foundation grant (JF-F) and NINDS R01NS105845 (AK).

ACKNOWLEDGMENTS

The time and effort of EI was supported by NIH T32 GM007356 during manuscript preparation.

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Conflict of Interest: The Sensory Assessment for Neurodevelopmental Disorders (SAND) was developed by PS and was licensed by Mount Sinai to Stoelting Co. AK received research support from AMO Pharma and consults to Ovid Therapeutics, GW Pharmaceuticals, Acadia, Alkermes, Ritrova Therapeutics, Jaguar Therapeutics, Neuren Pharmaceuticals, Clinilabs Drug Development Corporation, and Scioto Biosciences. JB consults to BridgeBio, holds a patent for IGF-1 for the treatment of Phelan-McDermid syndrome, holds an honorary professorship from Aarhus University Denmark, receives research support from Takeda and Oryzon, and is a journal editor for Springer Nature.

The remaining 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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