NEUROINFLAMMATION, METABOLISM, AND PSYCHIATRIC DISORDERS

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NEUROINFLAMMATION, METABOLISM, AND PSYCHIATRIC DISORDERS

Topic Editors:
Zachary Freyberg, University of Pittsburgh, United States
Ryan W. Logan, Boston University, United States
Marion Leboyer, Université Paris-Est Créteil Val de Marne, France
Brenda Penninx, Academic Medical Center, Netherlands

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EDITED AND REVIEWED BY
Virawudh Soontornniyomkij,
Mahidol University, Thailand

*CORRESPONDENCE
Zachary Freyberg
freyberg@pitt.edu
Marion Leboyer
marion.leboyer@inserm.fr
Brenda W. J. H. Penninx
b.penninx@amsterdamumc.nl
Ryan W. Logan
rwlogan@bu.edu

†These authors have contributed equally to this work

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Editorial: Neuroinflammation, metabolism, and psychiatric disorders

Zachary Freyberg[1,2*†], Marion Leboyer[3,4*†], Brenda W. J. H. Penninx[5*†] and Ryan W. Logan[6,7,8*†]

[1]Department of Psychiatry, University of Pittsburgh, Pittsburgh, PA, United States, [2]Department of Cell Biology, University of Pittsburgh, Pittsburgh, PA, United States, [3]Université Paris Est Créteil (UPEC), INSERM, IMRB, Translational Neuropsychiatry Laboratory, AP-HP, DMU IMPACT, FHU ADAPT (Hopital Henri Mondor), Créteil, France, [4]Fondation FondaMental, Hôpital Albert-Chenevier, Créteil, France, [5]Department of Psychiatry, Amsterdam UMC Location Vrije Universiteit Amsterdam, Amsterdam Neuroscience, Mood, Anxiety, Psychosis, Sleep and Stress Program, Amsterdam, Netherlands, [6]Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, Boston, MA, United States, [7]Center for Systems Neuroscience, Boston University, Boston, MA, United States, [8]Genome Science Institute, Boston University School of Medicine, Boston, MA, United States

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Editorial on the Research Topic
Neuroinflammation, metabolism, and psychiatric disorders

Growing evidence suggests that immunologic dysregulation is an important feature of both metabolic disease and psychiatric disorders including schizophrenia, major depressive disorder (MDD), bipolar disorder, anxiety disorders, anorexia nervosa, and neurodevelopmental disorders such as autism and attention-deficit/hyperactivity disorder (ADHD) (1–8). Indeed, chronic inflammation increases vulnerability to the onset of both psychiatric illnesses as well as metabolic syndrome (9, 10). However, the nature of the relationships between the psychiatric, inflammatory, and metabolic disturbances remains unclear. In this Research Topic, our goal was to pave the way for an improved understanding of these complex relationships. Consequently, we present a wide spectrum of original research and review papers that aim to: (1) draw attention to existing gaps in knowledge concerning the interplay of inflammation, metabolic dysfunction, and psychiatric disorders; (2) explore the bidirectional relationships between inflammation, metabolic disturbances, and psychiatric illness; and (3) discuss how inflammatory mechanisms are targeted to treat both metabolic dysfunction and psychiatric disorders.

Our Research Topic features the following original research studies:

DiCarlo et al. offered a novel mechanism for autism spectrum disorder (ASD) pathophysiology associated with dopamine transporter (DAT) function and its relationships to metabolism. The authors demonstrated that altered DAT function

in the context of a genetic variant associated with ASD changed dopaminergic neurotransmission and had significant impacts on metabolism, glucose handling, and the oral microbiome.

Lin and Huang examined the relationships between MDD and immune system activation. They discovered that MDD patients exhibited differences in the expression of markers of the innate immune response compared to non-depressed subjects. These results therefore suggest that altered regulation of innate immune activation may play a role in MDD pathophysiology.

Efthymiou et al. linked changes in gait to biomarkers of inflammatory states and metabolic syndrome in antipsychotic drug-treated individuals with psychosis. They showed that gait alterations were associated with metabolic syndrome in these patients. This raises the possibility that changes in gait can predict onset of metabolic syndrome in people with psychosis. Similarly, these data raise the intriguing possibility that adverse effects of some antipsychotic drugs may impact both metabolism and gait.

Chand et al. characterized sphingosine-1-phosphate receptor-1 (S1PR1), a gene expressed in astrocytes and microglia. The authors showed that S1PR1 was significantly upregulated in the dorsolateral prefrontal cortex of patients with one subtype schizophrenia while remaining unaffected in another subtype of the illness.

Pavlinek et al. showed that acute interferon-γ exposure significantly elevated gene expression of neuroimmune factors in human neuron iPSC-derived neurons, including major histocompatibility complex I and complement component 4A (C4A), while downregulating synapsins. Intriguingly, these C4A findings are consistent with C4's association with heightened schizophrenia risk (11).

Jiang et al. conducted metabolic profiling that compared cohorts of individuals with schizophrenia with or without cognitive impairment vs. matched unaffected comparison subjects. The authors found several differentially expressed metabolites in subjects with both schizophrenia and cognitive impairment including those associated with amino acid metabolism and the Krebs cycle, an important component of aerobic respiration.

Barko et al. examined microglial biology by RNA-sequencing TMEM119$^+$ microglia and found substantial brain region-specific expression differences. Microglia in midbrain were enriched in transcripts similar to disease-associated or immune-surveillant microglia, while prefrontal cortical microglia showed enrichment in synapse-associated pathways. In contrast, striatal microglia exhibited enrichment in microtubule polymerization-related pathways. There were also sex differences in microglial transcriptomes across all brain regions assayed, suggesting region and sex are crucial determinants of microglial signaling pathways and function. Consequently, these results may provide a novel mechanism that explains the brain region- and sex-specific differences in microglia-driven inflammation that contribute to the pathophysiology of psychiatric disorders.

Gustafsson et al. investigated alterations in polyunsaturated fatty acids alongside systemic inflammation associated with ADHD during pregnancy *via* a cross-sectional analytical observational research study in human subjects. The authors demonstrated that subjects with heightened ADHD symptoms possessed increases in the ratio of omega-6 to omega-3 polyunsaturated fatty acids as well as elevated levels of TNF-α, a pro-inflammatory cytokine. These findings suggest cause-and-effect relationships between factors such that there is an association between ADHD and changes in fatty acid metabolism that modify inflammatory states.

Freff et al. explored the relationships between anorexia nervosa (AN) and enhanced inflammation, showing increased expression of chemokine receptors CCR4, CXCR3, and CXCR4 on CD4$^+$ T-cells in AN vs. controls. Additionally, T-cell CXCR4 expression predicted body composition in adolescents. These data suggest important links between chemokine receptor expression, inflammatory states and AN, offering a new mechanism for AN pathogenesis.

We also feature the following reviews:

Tateishi et al. reviewed the therapeutic mechanisms underlying repetitive transcranial magnetic stimulation for treatment of cognitive dysfunction in depression, focusing on potential roles that neuroinflammation plays in these processes.

Rahimian et al. reviewed the involvement of microglia in the neuroinflammatory processes that contribute to the pathogenesis of MDD.

Author contributions

All 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.

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Autism-Associated Variant in the SLC6A3 Gene Alters the Oral Microbiome and Metabolism in a Murine Model

Gabriella E. DiCarlo[1,2†], Samuel J. Mabry[3,4†], Xixi Cao[5], Clara McMillan[6], Tiffany G. Woynaroski[1,2,7,8], Fiona E. Harrison[1,9], India A. Reddy[10], Heinrich J. G. Matthies[3], Charles R. Flynn[6], Mark T. Wallace[1,2,7,8,10], Hui Wu[5‡] and Aurelio Galli[3,4‡]*

[1] Vanderbilt Brain Institute, Vanderbilt University Medical Center, Nashville, TN, United States, [2] Department of Hearing and Speech Sciences, Vanderbilt University Medical Center, Nashville, TN, United States, [3] Department of Surgery, University of Alabama Birmingham, Birmingham, AL, United States, [4] Department of Neurobiology, University of Alabama Birmingham, Birmingham, AL, United States, [5] School of Dentistry, Oregon Health and Science University, Portland, OR, United States, [6] Department of Surgery, Vanderbilt University Medical Center, Nashville, TN, United States, [7] Vanderbilt Kennedy Center, Vanderbilt University Medical Center, Nashville, TN, United States, [8] Frist Center for Autism and Innovation, Vanderbilt University, Nashville, TN, United States, [9] Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, United States, [10] Department of Psychiatry and Behavioral Sciences, Vanderbilt University Medical Center, Nashville, TN, United States

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Edited by:
Zachary Freyberg,
University of Pittsburgh, United States

Reviewed by:
Peter Hamilton,
Virginia Commonwealth University, United States
Elisa L. Hill-Yardin,
RMIT University, Australia

***Correspondence:**
Aurelio Galli
aurelio.galli@gmail.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

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Background: Altered dopamine (DA) signaling has been associated with autism spectrum disorder (ASD), a neurodevelopmental condition estimated to impact 1 in 54 children in the United States. There is growing evidence for alterations in both gastrointestinal function and oral microbiome composition in ASD. Recent work suggests that rare variants of the SLC6A3 gene encoding the DA transporter (DAT) identified in individuals with ASD result in structural and functional changes to the DAT. One such recently identified *de novo* mutation is a threonine to methionine substitution at position 356 of the DAT (DAT T356M). The DAT T356M variant is associated with ASD-like phenotypes in mice homozygous for the mutation (DAT T356M$^{+/+}$), including social deficits, hyperactivity, and impaired DA signaling. Here, we determine the impact of this altered DA signaling as it relates to altered oral microbiota, and metabolic and gastrointestinal dysfunction.

Methods: In the DAT T356M$^{+/+}$ mouse, we determine the oral microbiota composition, metabolic function, and gastrointestinal (GI) function. We examined oral microbiota by 16S RNA sequencing. We measured metabolic function by examining glucose tolerance and we probed gastrointestinal parameters by measuring fecal dimensions and weight.

Results: In the DAT T356M$^{+/+}$ mouse, we evaluate how altered DA signaling relates to metabolic dysfunction and altered oral microbiota. We demonstrate that male DAT T356M$^{+/+}$ mice weigh less (Wild type (WT) = 26.48 ± 0.6405 g, DAT T356M$^{+/+}$ = 24.14 ± 0.4083 g) and have decreased body fat (WT = 14.89 ± 0.6206%, DAT T356M$^{+/+}$ = 12.72 ± 0.4160%). These mice display improved glucose handling (WT = 32.60 ± 0.3298 kcal/g, DAT T356M$^{+/+}$ = 36.97 ± 0.4910 kcal/g), and an altered oral microbiota. We found a significant decrease in *Fusobacterium* abundance.

The abundance of *Fusobacterium* was associated with improved glucose handling and decreased body fat.

Conclusions: Our findings provide new insights into how DAT dysfunction may alter gastrointestinal function, composition of the oral microbiota, and metabolism. Our data suggest that impaired DA signaling in ASD is associated with a number of metabolic and gastrointestinal changes which are common in individuals with ASD.

Keywords: autism, *Fusobacteria*, oral microbiome, dopamine transporter, mouse, metabolism

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental condition characterized by early-emerging differences in social communication and interaction, and by patterns of restrictive and repetitive interests, behaviors, or activities (1). This condition is estimated to affect 1 in 54 children (2), with males being 4 times more likely to be identified with ASD (2), and represents an economic burden of $11.5 billion—$60.9 billion in the United States (3). There is likely no single cause of ASD. Rather, its diagnosis represents a core set of behavioral symptoms that unifies individuals with a heterogeneous collection of genetic and phenotypic differences.

Within this framework, it is critical to ascertain how the various genetic and environmental risk factors associated with ASD ultimately translate to the core symptoms of this condition and its associated comorbidities. This highlights the importance of using animals to model rare inherited variants and *de novo* mutations associated with ASD to determine their contribution to the clinical presentation of the disorder. These animal models foster the uncovering of the molecular, neurobiological, and environmental contributors to ASD.

As previously shown, features of ASD might stem from or be exacerbated by abnormal dopamine (DA) signaling (4–8). Therefore, the study of dopaminergic dysfunction as it relates to this disorder is highly relevant. Recent work from our laboratories and other investigators has identified single nucleotide polymorphisms of DAT in individuals with ASD and associated comorbidities (6–11). Among those is an ASD-associated *de novo* mutation in the *SLC6A3* gene resulting in a threonine to methionine substitution at residue 356 (DAT T356M). This mutation impairs central DA signaling and DA-dependent behaviors, promoting repetitive behaviors and hyperlocomotion that reflect behavioral characteristics seen in ASD (6, 8).

In addition to its role in central neurotransmission, DA signaling is involved in regulating functions of the enteric nervous system (ENS), a system of neurons that spans the length of the digestive system and serves to regulate digestive function (12). Dopaminergic neurons are found in the ENS and are important for gut motility, insulin release, and metabolism (13–16). Altered DA signaling promoted by the T356M mutation might not only impact central nervous system (CNS) function, but also ENS function (17). Furthermore, catecholamines, including DA, have been shown to alter the growth of some gram negative microbial species (18), including *E. coli* and *Y. enterocolitica*. Thus, changes in DA turnover (as observed in the DAT T356M$^{+/+}$ mouse) could underlie changes in gut health, microbiota, and metabolism.

Problems with the ENS are reported to co-occur with a number of CNS disorders linked to dysregulation of the DA system. Children with ASD show multiple gastrointestinal (GI) abnormalities (19, 20). Also, they are more likely to experience abdominal pain, constipation, and diarrhea than those without ASD (21). Specifically, 30–70% of individuals with ASD have a functional GI disorder (fGID) (22). The Rome Foundation defines fGIDs as disorders of gut-brain interaction (DGBI). fGIDs are classified by GI symptoms related to any combination of motility disturbances, visceral hypersensitivity, altered gut microbiota, mucosal composition, immune function, and/or central nervous system processing (23). Evidence has suggested that these GI symptoms may exacerbate the behavioral symptoms exhibited by children with ASD by promoting emotional distress (24). Notably, maladaptive behaviors directly correlate with GI issues in individuals with ASD (25). For example, in children with ASD, behavior scores for irritability, social withdrawal, stereotypy, and hyperactivity are significantly higher in children with frequent abdominal pain, gaseousness, diarrhea, and constipation (25).

Beyond alterations in GI function, children with ASD also have an altered oral microbiota. Specifically, they have an increase in *Streptococcus* levels concomitant with a significant decrease in *Fusobacterium* (26) relative to neurotypical controls. *Fusobacterium nucleatum* is an anaerobic filamentous gram-negative bacterial species from the *Fusobacterium* genus (27, 28). It is a bacterial species implicated in a variety of infections ranging from appendicitis to osteomyelitis, as well as acting as an oncogenic bacterium implicated in colon cancer (28, 29). It is one of the most common oral bacteria found associated with a wide variety of periodontal health conditions, including gingivitis and periodontitis (30). Interestingly, *Fusobacterium* abundance has been shown to be associated with patients that display obesity and insulin resistance (31) and preterm birth (32), demonstrating a potential role of *Fusobacteria* in host metabolism.

Abbreviations: DA, dopamine; DAT, dopamine transporter; WT, wild type; ASD, autism spectrum disorder; CNS, central nervous system; ENS, enteric nervous system, GI, gastrointestinal; fGID, functional GI disorder; DGBI, disorder of gut brain interaction.

Evidence is emerging to suggest the DA system is implicated in ASD phenotypes. Furthermore, there is ample data supporting altered gastrointestinal function and both intestinal and oral microbiome composition in ASD. To further probe this, we have generated a mouse harboring a point mutation in the SLC6A3 gene identified from a proband with ASD that displays altered DA neurotransmission (8). In this study, we explore the degree to which oral microbiome composition, metabolism, and glucose tolerance are altered by DA dysfunction in a mouse model of ASD.

MATERIALS AND METHODS

Generation of DAT T356M$^{+/+}$ Mouse

Mice were generated by GenOway S.A. These mice were generated and used in a previous study by the lab (8). Briefly, the point mutation was inserted into the exon 8 of the mouse SLC6A3 gene and was expressed under the control of the endogenous SLC6A3 promoter. PCR and southern blot were used to validate the proper integration of the gene. All animals used in the study were derived from matings of DAT T356M$^{+/-}$ parents.

Body Composition

Bruker's minispec Body Composition Analyzer was used to determine the body composition of mice based on Time Domain NMR (TD-NMR). This equipment acquires and analyzes TD-NMR signals from all protons in the entire sample volume and provides a precise method for measurement of lean tissue, fat, and free body fluid in living mice. Body composition was analyzed in mice between 14 and 19 weeks of age. Nineteen male mice and 10 female mice were utilized in these experiments.

Caloric Expenditure

The Promethion from Sable Systems (Las Vegas, NV) was used to assess energy expenditure in 16–19 week-old mice. Ten male mice were used in these experiments. Mice were individually housed in Promethion System cages for 5 days, during which numerous parameters were continuously measured (including O_2 consumption, CO_2 production, food and water intake, weight, and activity). One week prior to the experiment start date, mice were singly housed for acclimation. The cages were housed in a light and temperature-controlled chamber. The light cycle was set on a 12:12 h cycle (6 am−6 pm). The temperature was maintained at 23°C for the duration of the test.

Oral Glucose Tolerance Test

Oral glucose tolerance testing was performed in mice aged 14–20 weeks of age. 30 mice (20 males and 10 females) were used for these experiments. Animals were fasted for 4 h prior to testing. Fasted blood glucose levels were determined before a solution of 20% dextrose was administered by oral gavage. Mice were given 2 g dextrose/kg body mass. Blood glucose levels were measured at the following time points following oral gavage: 10, 20, 30, 45, 60, 75, 90, and 120 min.

Fecal Measurements

Both male and female mice were used for these experiments. Seven mice were used in these experiments with 21 total fecal pellets measured. Mice were placed in individual transfer buckets for 5 min during the first hour of the light cycle. At the end of the 5-min period, the feces were collected. Each fecal sample was weighed using an analytical scale and measured along the longest axis using a digital caliper.

Oral Microbiome

Sterile swabs were used to collect samples from mice oral cavity at 15–18 weeks of age. Twelve total mice were used for these experiments. Swabs were snap frozen and stored at −80°C until DNA extraction was performed.

DNA samples were extracted from swab samples with ZR Fecal/Soil DNA Miniprep Kit (Zymo Research). PCR was performed with primers specific for the V4 region of the 16S rRNA gene for amplification (33, 34). Sequences were performed on an Illumina Miseq as described previously (35, 36). Sequences were analyzed using the Quantitative Insight into Microbial Ecology (QIIME) suite v1.7 (33) and a QIIME wrapper called QWRAP (35). Operational Taxonomic Units (OTUs) were clustered at 97% sequence similarity. Taxonomic groups were assigned by using the Ribosomal Database Project (RDP) classifier (37) as well as the May 2013 Greengenes 16S rRNA sequence database (38). Samples were tested to determine whether samples clustered differently between the two groups of mice by the permutational multivariate analysis of variance (PERMANOVA) test for significant differences in clustering ($p < 0.05$). OTUs were grouped by phyla, classes, orders, families, genera and species.

Statistics

All statistical analyses were performed using GraphPad Prism software (version 8.3.0). Data were tested for normality and homogeneity of variance when necessary. Statistical methods are indicated in the figure legends and the results section. All *t*-tests were run as two-tailed. Data are presented as mean ± SEM. Differences are considered statistically significant at $p < 0.05$.

Ethics Approval and Consent to Participate

All behavioral and metabolic experiments were performed under a protocol approved by the Vanderbilt University Animal Care and Use Committee.

RESULTS

The DAT T356M$^{+/+}$ Mouse Has Increased Energy Expenditure

We have previously shown that the DAT T356M$^{+/+}$ mouse has impaired central DA neurotransmission (8). Since DA is implicated in regulating metabolism, most notably in insulin release (14–16), and ENS function (13), we first measured body weight and body composition of DAT T356M$^{+/+}$ mice and their wild type (WT) littermates. Body weight was significantly reduced in male DAT T356M$^{+/+}$ mice compared to male WT mice at 14–19 weeks of age (**Figure 1A**. WT = 26.48 ± 0.6405 g,

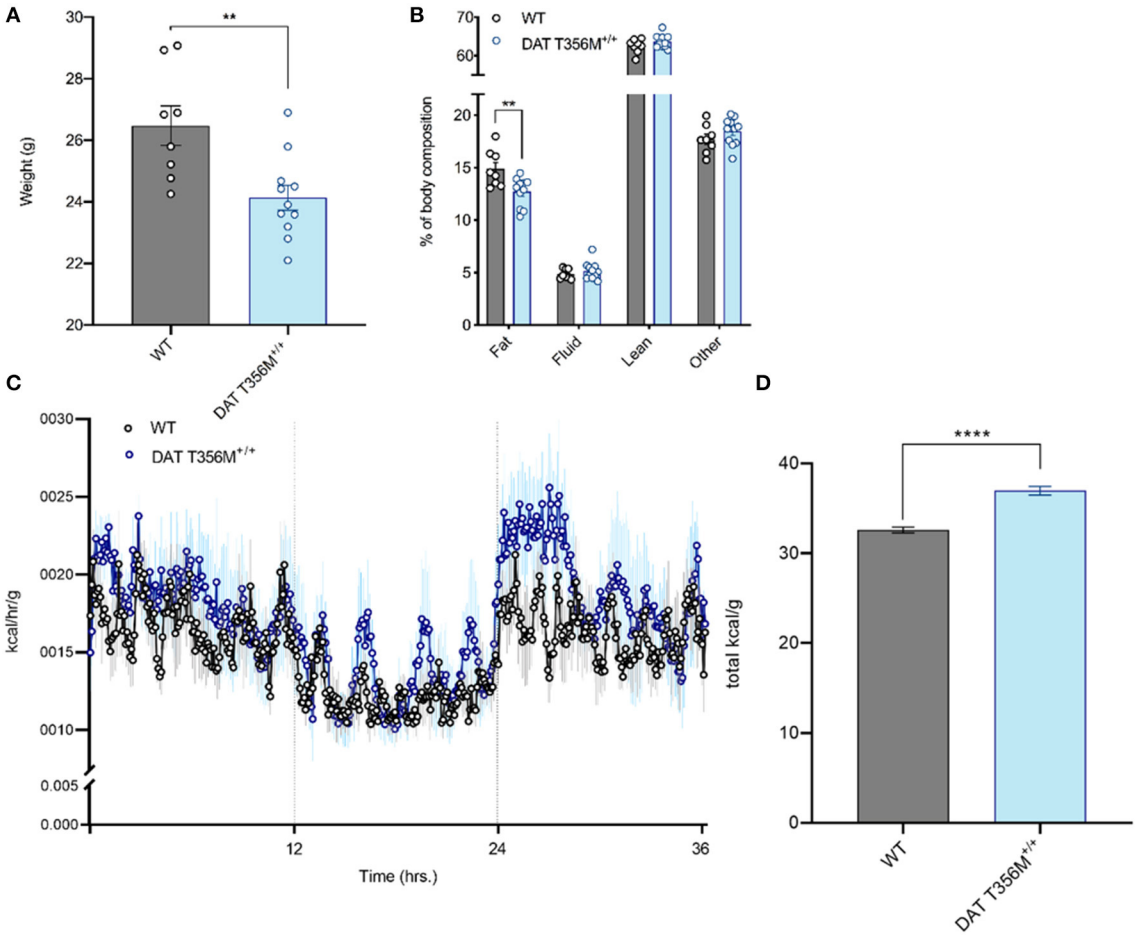

FIGURE 1 | Male DAT T356M$^{+/+}$ mice have reduced body weight, reduced percent body fat, and increased total caloric expenditure. **(A)** Total body weight is reduced in male DAT T356M$^{+/+}$ mice (WT = 26.48 ± 0.6405 g, DAT T356M$^{+/+}$ = 24.14 ± 0.4083 g; n = 8 WT, 11 DAT T356M$^{+/+}$; t = 3.226, df = 17, ** = p = 0.0050 by Student's t-test). **(B)** Percent body fat is reduced in male DAT T356M$^{+/+}$ mice (WT = 14.89 ± 0.6206%, DAT T356M$^{+/+}$ = 12.72 ± 0.4160%; n = 8 WT, 11 DAT T356M$^{+/+}$; $F_{(3,68)}$ = 5.373, ** = p = 0.0047 by two-way ANOVA followed by Sidak's multiple comparisons test). **(C)** Caloric expenditure per gram of body weight binned in 5-min intervals over the course of 36 h. Vertical lines at 12 and 24 h represent the start and end of the light cycle, respectively. The blue circles represent the mean value for DAT T356M$^{+/+}$ mice (n = 5) and the black circles represents the mean value for WT mice (n = 5). The light gray and light blue lines represent the SEM. **(D)** Total caloric expenditure is increased in male DAT T356M$^{+/+}$ mice as measured by indirect calorimetry (WT = 32.60 ± 0.3298 kcal/g, DAT T356M$^{+/+}$ = 36.97 ± 0.4910 kcal/g, n = 5 WT, 5 DAT T356M$^{+/+}$, t = 7.388, df = 8, **** = p < 0.0001 by Student's t-test).

DAT T356M$^{+/+}$ = 24.14 ± 0.4083 g; n = 8 WT, 11 DAT T356M$^{+/+}$; t = 3.226, df = 17, p = 0.0050 by Student's t-test). However, there was no difference in body weight between female DAT T356M$^{+/+}$ and WT mice (WT = 22.78 ± 0.8938 g, DAT T356M$^{+/+}$ = 22.18 ± 0.7658 g; n = 4 WT, 6 DAT T356M$^{+/+}$; t = 0.5015, df = 8, p = 0.6296 by Student's t-test), suggesting that this effect is sexually dimorphic.

To better understand how the T356M mutation influences mouse body weight, we measured body composition of male and female DAT T356M$^{+/+}$ mice and WT mice. In males, we found significantly reduced percent body fat in DAT T356M$^{+/+}$ mice compared with WT mice (**Figure 1B**. WT = 14.89 ± 0.6206%, DAT T356M$^{+/+}$ = 12.72 ± 0.4160%; n = 8 WT, 11 DAT T356M$^{+/+}$; $F_{(3,68)}$ = 5.373, p = 0.0047 by two-way ANOVA followed by Sidak's multiple comparisons test). No other components of the body composition (i.e., lean mass, fluid, or other) were different between male DAT T356M$^{+/+}$ mice and WT mice (**Figure 1B**). In contrast, no differences in body composition were observed between female DAT T356M$^{+/+}$ mice and WT mice (Body fat: WT = 13.23 ± 0.5502%, DAT T356M$^{+/+}$ = 13.17 ± 0.2128%, Fluid: WT = 5.987 ± 0.1256%, DAT T356M$^{+/+}$ = 5.752 ± 0.3474%, Lean: WT = 64.17 ± 0.776%, DAT T356M$^{+/+}$ = 63.15 ± 0.3714%; n = 4 WT, 6 DAT T356M$^{+/+}$, $F_{(1,24)}$ = 1.631 and p = 0.2137 for genotype, $F_{(2,24)}$ = 0.7398 and p = 0.4878 for interaction between genotype and tissue type by two-way ANOVA). In male DAT T356M$^{+/+}$ mice, this decrease in body weight is driven, at least in part, by a decrease in percent body fat and not lean mass (**Figure 1B**). Exercise can regulate body composition (i.e., decreased body fat and increased lean mass) (39).

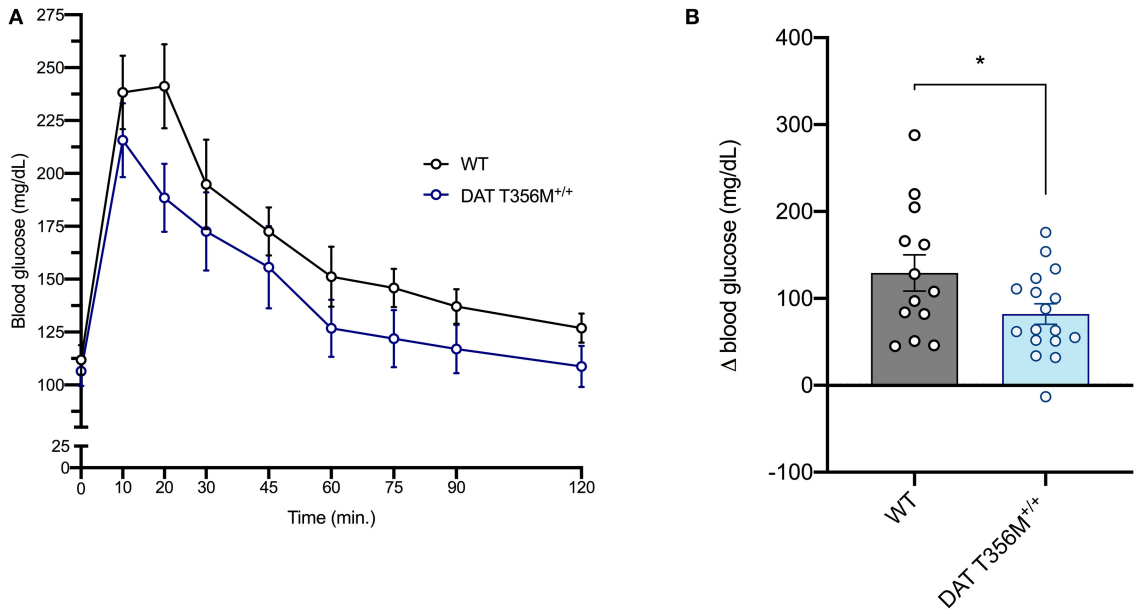

FIGURE 2 | DAT T356M$^{+/+}$ mice have improved glucose handling after glucose challenge. **(A)** Blood glucose levels during a glucose tolerance test in WT and DAT T356M$^{+/+}$ mice. **(B)** Change in blood glucose from baseline to 20 min (Δ blood glucose) is significantly reduced in DAT T356M$^{+/+}$ mice compared to WT mice (WT = 129.4 ± 20.80 mg/dL, DAT T356M$^{+/+}$ = 81.94 ± 11.78 mg/dL; n = 13 WT, 17 DAT T356M$^{+/+}$; t = 2.100, df = 28, * = p = 0.0448 by Student's t-test).

In 7 week old heterozygous animals (DAT T356M$^{+/-}$) we did not observe differences in body weight with respect to WT. To note, is that these animals were younger than the animals used in other experiments in this study. This cohort was made of entirely male mice and there was no significant difference in weight between WT and DAT T356M$^{+/-}$ mice (WT = 21.42 ± 3.57 g, DAT T356M$^{+/-}$ = 20.85 ± 2.75g, n = 13 WT, 11 DAT T356M$^{+/-}$, t = 0.776, df = 22, p = 0.446 by Students t-test).

We used a metabolic cage (Promethion, Sable Systems, Las Vegas, USA) to measure energy expenditure continuously over 36 h both in male DAT T356M$^{+/+}$ mice and WT mice (**Figure 1C**). Total energy expenditure over 36 h was significantly increased in male DAT T356M$^{+/+}$ mice (**Figure 1D**. WT = 32.60 ± 0.3298 kcal/g, DAT T356M$^{+/+}$ = 36.97 ± 0.4910 kcal/g, n = 5 WT, 5 DAT T356M$^{+/+}$, t = 7.388, df = 8, p < 0.0001 by Student's t-test). As the DAT T356M$^{+/+}$ mice are hyperactive (8), one would expect lean mass to be increased. However, since we saw no change in lean mass our data indicate that this decrease in body mass is not likely promoted by hyperactivity, but rather by metabolic changes.

DAT T356M$^{+/+}$ Mice Have Improved Glucose Tolerance

It is well-established that DA also plays an important role in glucose and insulin regulation (16, 40, 41). Insulin-secreting pancreatic β-cells express the enzymes required for DA synthesis and catabolism, as well as all five DA receptors (40, 42). In these cells, DA functions as a negative regulator of glucose-stimulated insulin secretion (GSIS) (41). The D2 receptor (D2R) and D3 receptor (D3R) signaling act in concert to inhibit GSIS (14–16). Consistent with these findings, β-cell-selective D2R knockout mice exhibit marked postprandial hyperinsulinemia (16).

DAT T356M$^{+/+}$ mice have impaired D2 receptor signaling (8). Thus, we sought to determine whether glucose homeostasis was also affected in these animals. After glucose loading, there was a rapid decrease in blood glucose in DAT T356M$^{+/+}$ mice, while this rapid decrease in blood glucose is not present in WT mice (**Figure 2A**). Maximum blood glucose levels were lower in DAT T356M$^{+/+}$ mice than WTs and returned to baseline faster than WTs, which still had slightly elevated levels even after 120 min. These results highlight that there is significantly improved glucose handling in the DAT T356M$^{+/+}$ mice (**Figure 2B**. WT = 129.4 ± 20.80 mg/dL, DAT T356M$^{+/+}$ = 81.94 ± 11.78 mg/dL; n = 13 WT, 17 DAT T356M$^{+/+}$; t = 2.100, df = 28, p = 0.0448 by Student's t-test).

The DAT T356M$^{+/+}$ Mouse Displays Reduced Abundance of Oral *Fusobacterium*

A number of changes in gut and oral microbial populations have been reported in individuals with ASD (26, 43). Strong evidence points to a decrease in the abundance of oral *Fusobacterium* in persons with ASD (26). Changes in these bacterial populations have also been associated with alterations in various metabolic processes. For example, increased *Fusobacterium* in the gut correlates with insulin resistance (31). Thus, we sought to determine the composition of the oral microbiota and, specifically, the abundance of *Fusobacterium* in DAT T356M$^{+/+}$ mice compared to WT mice. In **Figure 3A**, we show the top 10 operational taxonomic units (OTUs) isolated from

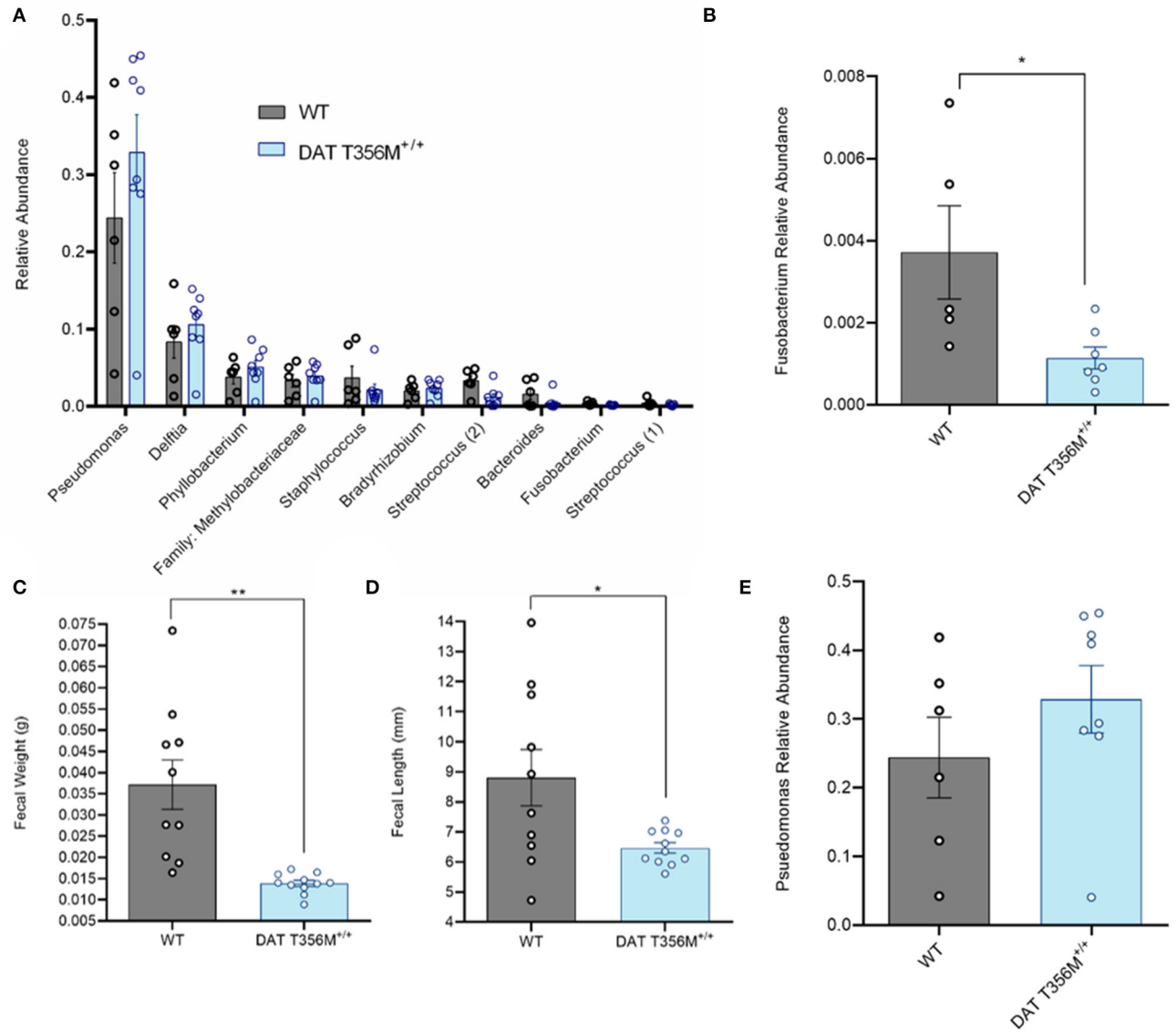

FIGURE 3 | DAT T356M$^{+/+}$ mice have altered oral microbiota. **(A)** Relative abundance of top 10 OTUs isolated from oral swabs from WT and DAT T356M$^{+/+}$ mice. **(B)** DAT T356M$^{+/+}$ mice have significantly lower abundance of oral *Fusobacterium* compared to WT mice (WT = 0.003716 ± 0.001136 relative units, DAT T356M$^{+/+}$ = 0.001 ± 0.0003 relative units; n = 5 WT, 7 DAT T356M$^{+/+}$; t = 2.589, df = 10, * = p = 0.027 by Student's t-test). **(C)** DAT T356M$^{+/+}$ mice have significantly decreased fecal weight compared to WT mice (WT = 0.0371 ± 0.0058 g, DAT T356M$^{+/+}$ = 0.0138 ± 0.0139 g; n = 10 fecal pellets from 3 WT mice, 11 fecal pellets from 4 DAT T356M$^{+/+}$ mice; t = 3.981, df = 9, ** = p = 0.0032 by Welch's t-test). **(D)** DAT T356M$^{+/+}$ mice have significantly decreased fecal length compared to WT mice (WT = 8.805 ± 0.9381 mm, DAT T356M$^{+/+}$ = 6.469 ± 0.1727 mm; n = 10 feces from 3 WT mice, 11 feces from 4 DAT T356M$^{+/+}$ mice; t = 2.449, df = 9, * = p = 0.0368 by Welch's t-test). **(E)** There is no difference in the relative abundance of *Pseudomonas* between WT and DAT T356M$^{+/+}$ mice (WT = 0.2438 ± 0.05866 relative units, DAT T356M$^{+/+}$ = 0.3286 ± 0.0491 relative units; n = 6 WT, 8 DAT T356M$^{+/+}$; t = 1.113, df = 12, p = 0.2873 by Students t-test).

oral swabs from DAT T356M$^{+/+}$ and WT mice. We found that *Fusobacterium* was significantly decreased in the DAT T356M$^{+/+}$ mice (**Figure 3B**. WT = 0.003716 ± 0.001136 relative units, DAT T356M$^{+/+}$ = 0.001 ± 0.0003 relative units; n = 5 WT, 7 DAT T356M$^{+/+}$; t = 2.589, df = 10, p = 0.027 by Student's t-test). However, the decrease in the abundance seen in *Fusobacterium* is not observed for other bacteria, such as *Pseudomonas*, the most abundant OTU isolated from our samples (**Figures 3A,E**. WT = 0.2438 ± 0.05866 relative units, DAT T356M$^{+/+}$ = 0.329 ± 0.0491 relative units; n = 6 WT, 8 DAT T356M$^{+/+}$; t = 1.113, df = 12, p = 0.2873 by Student's t-test).

To evaluate the impact of the DAT T356M on GI function, we analyzed fecal weight and length. In a mouse model of constipation, there is a decrease in both fecal weight and length (44), demonstrating that changes in these parameters can be

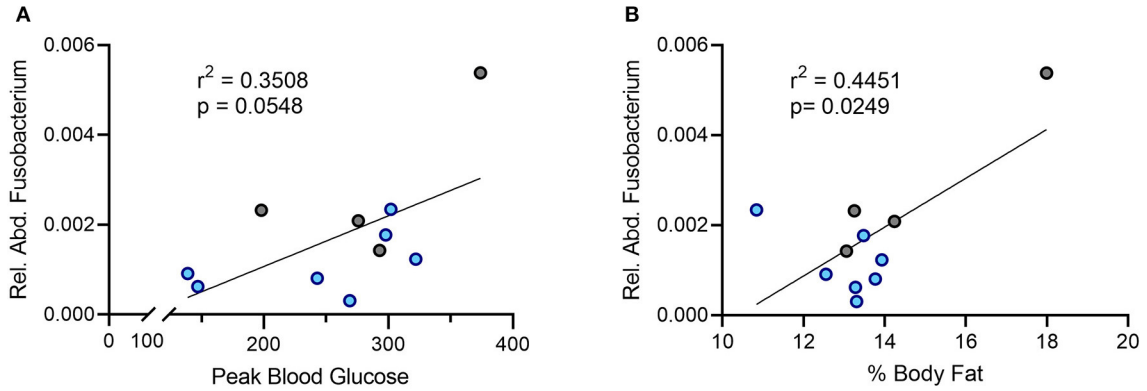

FIGURE 4 | There is a positive correlation between the relative abundance of Fusobacterium and both peak blood glucose and percent body fat. **(A)** Peak blood glucose is positively correlated with relative abundance of oral *Fusobacterium* (Pearson's $r = 0.5923$; $n = 4$ WT, 7 DAT T356M$^{+/+}$; $p = 0.058$). **(B)** The relative abundance of *Fusobacterium* in the oral cavity and peak blood glucose are positively and significantly correlated (Pearson's $r = 0.6671$; $n = 4$ WT, 7 DAT T356M$^{+/+}$; $p = 0.0249$). Blue dots represent T356M$^{+/+}$ mice and gray dots represent WT mice.

used as a partial readout of GI function. DAT T356M$^{+/+}$ mice displayed reduced fecal weight (**Figure 3C**. WT = 0.037 ± 0.0058 g, DAT T356M$^{+/+}$ = 0.01388 ± 0.01388 g; $n = 10$ from 3 WT, $t = 3.981$, df = 9, 11 from 4 DAT T356M$^{+/+}$; $p = 0.0032$ by Welch's t-test) and length (**Figure 3D**. WT = 8.805 ± 0.9381 mm, DAT T356M$^{+/+}$ = 6.469 ± 0.1727 mm; $n = 10$ from 3 WT, 11 from 4 DAT T356M$^{+/+}$; $t = 2.449$, df = 9, $p = 0.0368$ by Welch's t-test), suggesting constipation in DAT T356M$^{+/+}$ mice (44).

Relative Abundance of Oral *Fusobacterium* Is Positively Correlated With Percent Body Fat and Peak Blood Glucose

In humans, an increased abundance of *Fusobacterium* correlates with insulin resistance (31). Given the improved glucose handling in the DAT T356M$^{+/+}$ mice, we sought to determine the relationship between relative abundance of oral *Fusobacterium* and metabolic parameters in our sample. We observed a positive association between the relative abundance of *Fusobacterium* in the oral cavity and peak blood glucose during a glucose challenge (**Figure 4A**, Pearson's $r = 0.5923$; $n = 4$ WT, 7 DAT T356M$^{+/+}$; $p = 0.058$). Although this correlation did not reach statistical significance, it was notably strong in magnitude. We similarly observed a strong, positive and significant association between the relative abundance of *Fusobacterium* in the oral cavity and percent body fat (**Figure 4B**, Pearson's $r = 0.6671$; $n = 4$ WT, 7 DAT T356M$^{+/+}$; $p = 0.0249$). To determine aforementioned relations were limited to *Fusobacterium,* we additionally analyzed the associations for relative abundance of *Pseudomonas* with both percent body fat and peak blood glucose. We found no significant correlation between relative abundance of *Pseudomonas* and either percent body fat (Pearson's $r = 0.1208$; $n = 6$ WT, 8 DAT T356M$^{+/+}$; $p = 0.2233$) or peak blood glucose (Pearson's $r = 9.7 \times 10^{-6}$; $n = 6$ WT, 8 DAT T356M$^{+/+}$; $p = 0.99$). These correlations were negligible to small in magnitude.

DISCUSSION

The mechanistic understanding of proteins, such as DAT, that regulate DA neurotransmission is vital to pharmacologically target DA dysfunction in autism and other conditions. DAT variants, dysregulation of DA, and altered developmental trajectory of dopaminergic structures have been associated with ASD (6, 9–11, 45, 46). Beyond the importance of proper DA neurotransmission for central function, increasing evidence highlights the role of DA signaling in gut motility, insulin release, and metabolism. In the pancreas, DA is stored in secretory granules in pancreatic beta-islet cells and regulates GSIS (14, 15). Moreover, in the intestines, dopaminergic neurons are found in the ENS and are important for proper intestinal motility (13). As such, it is not surprising that dysfunction in the DA system can affect both metabolism and GI function. Thus, studying DAT variants associated with ASD will facilitate understanding of their role in central and peripheral neurotransmitter homeostasis and will help to determine how transporter dysfunction contributes to GI and metabolic disorders.

Here we explore the impact of an ASD-associated *de novo* mutation of the DAT (6, 8), which impacts DA neurotransmission, on metabolism, glucose handling, and the oral microbiome. We demonstrate that DAT T356M$^{+/+}$ mice exhibit altered body composition, energy expenditure, glucose handling, and composition of oral microbiota. This study links altered dopaminergic signaling due to DAT dysfunction caused by an ASD-associated genetic variant with pathophysiological changes that recapitulate aspects of the human disorder in a murine model.

We first explored the impact of the DAT T356M mutation on energy homeostasis. We found that male DAT T356M$^{+/+}$ mice have reduced body weight and that this reduction in body weight is characterized predominantly by reduced body fat, without a significant change in fluid, lean, or other body tissues. Interestingly, no difference in body composition were observed in female mice. Males have been shown to have sex specific deficits in other animal models of autism (47–49). Thus, a more pronounced phenotype in our male mice is in line with previous data. Since no change in lean mass was observed, it is unlikely that this change in body fat is driven by hyperactivity. Rather, this reduction in body fat is more likely associated with increased total energy expenditure.

As DA signaling regulates insulin release (16), we additionally asked whether the observed reduction in body fat was associated with altered glucose handling. We found that DAT T356M$^{+/+}$ mice display significant differences in glucose handling (specifically, a significantly lower change in blood glucose 20 min after glucose challenge in male mice). Furthermore, in pancreatic beta-islet cells, the D2 autoreceptor negatively regulates the release of insulin (14, 16). Considering that DAT T356M$^{+/+}$ mice have reduced D2 receptor signaling (8), we therefore suggest that this improvement in glucose handling may be due to increased insulin release. Furthermore, it is important to note that, when compared to skeletal muscle, adipose tissue is not generally thought as being a major user of enteral (i.e., gastrointestinal) glucose. Therefore, we suggest that this altered glucose handling in the DAT T356M$^{+/+}$ mice is likely not due to their lowered fat composition, but rather changes in the sensitivity of the D2 receptor. Future studies should directly assess the insulin levels seen in these mice, as well as the sensitivity and expression of D2 receptor in beta-islet cells in the pancreas to determine the underlying physiology of the improved glucose handling seen in our animal model.

In addition to insulin release, DA plays a critical role in GI function (13, 17). Children with ASD display multiple GI abnormalities (19, 20) with 30–70% of individuals with ASD having a fGID (22), a group of disorders classified by motility disturbances, altered mucosal function, and altered gut microbiota (23). As a read out for proper GI function, we measured both the length and weight of feces in DAT T356M$^{+/+}$ mice and discovered that their feces are lighter and shorter than WT mice. These alterations have been previously observed in mouse models of constipation (44). Notably, children with ASD are more likely to experience abdominal pain and constipation when compared to those without ASD (21).

Many studies have reported differences in the composition of the oral and gut microbiota in patients with ASD (26, 50) and it is possible that these differences in the flora may contribute to both behavioral and GI symptoms associated with autism (21, 24). As communication between the brain and the gut is bidirectional, it is conceivable that dysfunction of key regulators of CNS function associated with ASD may also drive dysfunction of the ENS and thus changes in the composition of the oral and GI microbiota (51, 52). Here, we point to the DAT as one such key regulator. We found that DAT T356M$^{+/+}$ mice have reduced abundance of *Fusobacterium* in the oral cavity and no difference in *Pseudomonas*, the most abundant bacteria in the oral cavity of our animals, as compared to WT mice. It is possible that this change in oral microbiome is due directly to the anomalous DA release seen in this mutant (8), as there is evidence that DA can alter the growth of certain gram-negative bacterial species (18). However, we cannot rule out the possibility that this change is due to dietary differences or differences in metabolism leading to an altered microbiome. Consistent with our data, increased *Fusobacterium* abundance has been associated with insulin resistance and impaired glucose handling in obese patients (31). Of note, we found a positive association between the relative abundance of *Fusobacterium* and both peak blood glucose and percent body fat in our mouse model. However, there was no relation between the abundance of *Pseudomonas* and either peak blood glucose or percent body fat.

Our data demonstrate that there is less *Fusobacterium* in the oral microbiome in the DAT T356M$^{+/+}$ model of ASD, a finding that is in agreement with human data from Qiao and colleagues (26). *Fusobacterium nucleatum* (*F. nucleatum*) is one of the most common species in periodontal diseases, such as gingivitis (30). These findings, collectively, point toward a potential explanation for the fact that, despite having no difference in quality of teeth brushing (53), children with ASD have been observed to display better oral health and less caries when compared to a control population (53, 54). Given that both humans and our murine model display significantly less oral *Fusobacterium,* this could potentially be a driving factor in the superior oral hygiene seen in children with ASD, despite no differences in teeth brushing habits (53). Consistent with our hypothesis, a recent study from Schoilew and colleagues demonstrated that patients without a history of caries have significantly less *Fusobacterium* than patients that previously had caries (55). Alternatively the reduction in *F. nucleatum* level may mediate some of the phenotypes seen in ASD as *F. nucleatum* is known to produce butyrate and this is a short chain fatty acid that has been shown to be present at decreased levels in individuals with ASD (56). It is speculated that the alterations in the microbiome and the decrease in butyrate is one of the drivers of the constipation and other GI issues observed in ASD (56). Clearly, this observation requires more research on both human patients and alternative animal models of ASD to corroborate differences presented in other studies and explore underlying mechanisms (57).

Here, we present new evidence of how DAT dysfunction may translate to altered GI function, composition of the microbiota of the oral cavity, and metabolism. This model suggests that impaired DA signaling in ASD drives a number of pathophysiological changes that could explain, in part, at least some of the observed metabolic and GI phenotypes in ASD. This work also suggests that DA signaling, or specific microbial populations in the microbiome, may represent a tractable target for the treatment of GI and metabolic disturbances in persons effected by ASD, and potentially in individuals with other conditions associated with DA dysfunction.

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 animal study was reviewed and approved by Vanderbilt University Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

GD, SM, XC, and CM designed and performed experiment and contributed to the writing of the manuscript. TW, FH, IR, HM, CF, MW, HW, and AG designed the experiments and contributed to writing the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.655451/full#supplementary-material

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SIGIRR and TNFAIP3 Are Differentially Expressed in Both PBMC and TNF-α Secreting Cells of Patients With Major Depressive Disorder

Chin-Chuen Lin[1†] *and Tiao-Lai Huang*[1,2*†]

[1] *Department of Psychiatry, Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine, Kaohsiung, Taiwan,* [2] *Genomic and Proteomic Core Laboratory, Department of Medical Research, Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan*

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***Correspondence:**
Tiao-Lai Huang
a540520@adm.cgmh.org.tw

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

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Background: Major depressive disorder (MDD) is associated with the activation of the immune/inflammatory system. TNF-α is associated with MDD and poor treatment response. Toll-like receptors (TLR) are responsible in innate immune response, and is associated with MDD and antidepressant response. Some negative regulators of TLR pathway such as SOCS1, TOLLIP, SIGIRR, TNFAIP3, and MyD88s, are reported to be differentially expressed in the peripheral blood samples of patients of MDD.

Methods: We recruited patients with MDD and healthy controls, collect their demographic data, and measured their mRNA levels of negative TLR regulators, using peripheral blood mononuclear cells (PBMC) and isolated TNF-α secreting cells. Clinical symptoms were evaluated using Halmiton Depression Rating Scale (Ham-D). Some patients were evaluated again after 4 weeks of antidepressant treatment.

Results: Forty-seven patients with MDD and 52 healthy controls were recruited. Between the PBMC samples of 37 MDD patients and 42 controls, mRNA levels of SOCS1, SIGIRR, TNFAIP3, and MyD88s were significantly different. Between TNF-α secreting cells of 10 MDD patients and 10 controls, mRNA levels of SIGIRR and TNFAIP3 were significantly different. Change of Ham-D score only correlated significantly with TOLLIP mRNA level after treatment.

Conclusion: SIGIRR and TNFAIP3, two negative regulators of TLR immune response pathways, were differentially expressed in both PBMC and TNF-α secreting cells of patients with MDD as compared to healthy controls. The negative regulations of innate immune response could contribute to the underlying mechanism of MDD.

Keywords: MDD, SIGIRR, TLR, TNFAIP3, TNF-α secreting cells

INTRODUCTION

Major depressive disorder (MDD) has been associated with the activation of the immune/inflammatory system, including changes in serum acute phase protein (1, 2) and cytokine levels (3–5). Antidepressant treatment has also been shown to normalize the inflammatory state, by decreasing serum levels of proinflammatory cytokines such as IL-12 and increasing serum levels of anti-inflammatory cytokines such as IL-4 and TGF-β1 (6). Increased plasma levels of IL-6 and TNF-α before treatment predicted poor antidepressant response (7, 8). A meta-analysis has shown that increased serum levels of TNF-α and IL-6 are the most replicated findings in MDD (9).

Toll-like receptors (TLRs) are the pattern recognition receptors that recognize pathogenic exogenous and endogenous molecular patterns and play an important role in the innate immune system. In humans, 10 TLRs (TLR-1 to 10) were characterized. TLR-1, 2, 4, 5, and 6 are at the cell membrane, detecting bacteria. For example, TLR-4 binds lipopolysaccharide in gram-negative bacteria. TLR-3, 7, 8, and 9 are located on intracellular endosomes, and detect nucleic acids from bacteria and viruses that have penetrated the cell. For example, TLR-7 binds single-stranded RNA from viruses as well as some endogenous proteins. After receptor binding, a cascade is initiated, leading to transcriptions of inflammatory cytokines (10). Prior studies have shown that TLR expressions were associated with MDD diagnosis (11) and depressive symptoms (12). Antidepressant treatment could normalize elevated TLR expressions prior to medications (13). In the recent years, attention has turned to negative regulators of TLR pathway, suggesting that dysfunction in the negative feedback loop could also contribute to the psychopathology of MDD (13, 14). Some of the more frequent investigated negative regulators include suppressor of cytokine signaling 1 (SOCS1), Toll-interacting protein (TOLLIP), single immunoglobulin interleukin-1-related receptor (SIGIRR), TNF-α-induced protein 3 (TNFAIP3), and the short form of MyD88 (MyD88s) (15).

Earlier studies regarding TLR expressions and their negative regulators used peripheral blood mononuclear cells (PBMC) as the analyzed sample (14), which contain a variety of cells. Recent advances in technology allowed isolation of specific types of cells, such as monocytes (16) and TNF-α secreting cells (17). As mentioned earlier, TNF-α is an important cytokine in MDD (9). Therefore, in this study, we intended to investigate the mRNA levels of five negative TLR regulators (SOCS1, TOLLIP, SIGIRR, TNFAIP3, and MyD88s) in PBMC and TNF-α secreting cells from patients of MDD, compared to the healthy controls and after antidepressant treatment.

MATERIALS AND METHODS

Study Samples

From September 2017 to July 2018, hospitalized patients diagnosed with MDD were recruited at the Chang Gung Memorial Hospital. MDD was diagnosed by a psychiatrist according to the criteria of the *Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition* (DSM-5). Only patients aged between 20 and 65 years old were included. Patients with systemic diseases, such as cardiovascular diseases, liver diseases and thyroid diseases, smokers, or patients with alcohol dependence were excluded. The severity of depression was assessed by the 17-item Halmiton Depression Rating Scale (Ham-D) (18). The choice of antidepressants depended on what the clinicians considered best for the patients. Healthy controls were recruited and assessed by semi-structured interviews to rule out psychiatric disorder according to DSM-5 criteria. Written informed consent was provided by all participants after the content and context of the study was fully explained. The institutional review board (IRB) of Chang Gung Memorial Hospital approved the study design (IRB 201602052B0C501).

Laboratory Data

Venous blood of 15 ml was drawn from each participant in the morning following a 6-h fast. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque medium. TNF-α secreting cells were further isolated from PBMC using TNF-α Secretion Assay-Cell Enrichment and Detection Kit (Miltenyi Biotec, #130-091-269). Isolated cells were stored at −80°C until assay.

Quantitative Reverse Transcription-Polymerase Chain Reaction Analysis

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using the following sets of primers: SOCS1 5′-GACCCCTTCTCA CCTCTTGA-3′ (sense) and 5′-GTAGGAGGTGCGAGTTCAGG-3′ (antisense); TOLLIP, 5′-GACAACTGTCTCCGTCGCA-3′ (sense) and 5′-CGGGAGCTCACCGATGTA-3′ (antisense); SIGIRR, 5′-CCCAGCTCTTGGATCAGTCT-3′ (sense) and 5′-AGTCAGGGGCCCTATCACAG-3′ (antisense); TNFAIP3, 5′-GGACTT TGCGAAAGGATC G-3′ (sense) and 5′-TCACAGCTTTCCGCATATTG-3′ (antisense); MyD88s, 5′-TCATCGAAAAGAGGTTGGCT-3′(sense) and 5′-GATGGG GATCAGTCGCTTCT-3′ (antisense); glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-TGCACCACC AACTGCTTAGC-3′ (sense) and 5′-GGCATGGACTGTGGTCATGAG-3′ (antisense).

The relative abundance of mRNAs was calculated with the comparative Ct method using GADPH as the housekeeping gene, represented by -ΔCt, to make comparisons with earlier studies possible. Fold changes were calculated with $2^{\wedge} - \Delta\Delta Ct$.

Statistical Analysis

All results are represented as mean ± standard deviation. Comparisons of study groups were calculated using independent *t*-test, Wilcoxan sign rank test, or Mann-Whitney *U*-Test. Pearson correlation was used to assess the relationship with the associated parameters. Data analysis was performed using SPSS 19 (Chicago, IL, U.S.A.). *p*-values of <0.05 were considered statistically significant.

TABLE 1 | Demographic data and mRNA levels of PBMC.

	MDD ($n = 37$)	Controls ($n = 42$)	Fold change	p
Age (years)	49.6 ± 13.0	42.6 ± 7.8		0.006*
Onset (years)	41.6 ± 14.4			
Duration (years)	8.0 ± 8.3			
BMI (kg/m^2)	25.3 ± 4.9	23.8 ± 3.6		0.141
Ham-D score	16.4 ± 20.0			
SOCS1 (-ΔCt)	−5.9 ± 1.5	−7.2 ± 0.9	3.8 ± 3.1	1.1×10^{-5}*
TOLLIP (-ΔCt)	−10.6 ± 0.9	−10.5 ± 0.5	1.2 ± 0.7	0.823
SIGIRR (-ΔCt)	−7.2 ± 1.6	−5.3 ± 1.1	0.4 ± 0.4	2.0×10^{-8}*
TNFAIP3 (-ΔCt)	−7.0 ± 1.7	−4.3 ± 0.9	0.3 ± 0.30	6.0×10^{-14}*
MyD88s (-ΔCt)	−9.5 ± 2.0	−6.8 ± 0.9	20.5 ± 1.8	7.2×10^{-12}*

$p < 0.05$.BMI, body mass index; Ham-D, 17-item Halmiton Depression Rating Scale; MDD, major depressive disorder; MyD88s, the short form of MyD88; PBMC, peripheral blood mononuclear cells; SIGIRR, single immunoglobulin interleukin-1-related receptor; SOCS1, suppressor of cytokine signaling 1; TLR, Toll-like receptors; TNFAIP3, TNF-α-induced protein 3; TOLLIP, Toll-interacting protein.

TABLE 2 | Demographic data and mRNA levels of PBMC before and after antidepressant treatment.

	Baseline ($n = 13$)	After treatment ($n = 13$)	Fold change	p
Age (years)	45.6 ± 15.3			
BMI (kg/m^2)	24.1 ± 6.2	24.3 ± 6.0		0.301
Ham-D score	36.2 ± 20.7	16.2 ± 10.3		0.002*
SOCS1 (-ΔCt)	6.1 ± 1.2	6.4 ± 1.5	1.4 ± 1.6	0.480
TOLLIP (-ΔCt)	11.2 ± 0.7	11.2 ± 0.7	1.1 ± 0.5	0.638
SIGIRR (-ΔCt)	5.7 ± 0.8	5.8 ± 0.8	1.1 ± 0.7	0.754
TNFAIP3 (-ΔCt)	6.3 ± 1.6	6.6 ± 1.4	1.2 ± 1.0	0.239
MyD88s (-ΔCt)	7.8 ± 0.9	7.8 ± 0.9	1.2 ± 0.9	0.937

$p < 0.05$.BMI, body mass index; Ham-D, 17-item Halmiton Depression Rating Scale; MDD, major depressive disorder; MyD88s, the short form of MyD88; PBMC, peripheral blood mononuclear cells; SIGIRR, single immunoglobulin interleukin-1-related receptor; SOCS1, suppressor of cytokine signaling 1; TLR, Toll-like receptors; TNFAIP3, TNF-α-induced protein 3; TOLLIP, Toll-interacting protein.

RESULTS

Forty-seven patients with MDD and 52 healthy controls were recruited. Samples of 37 patients and 42 controls were analyzed for PBMC data. Thirteen patients were treated with antidepressants for 4 weeks, and their PBMC data were analyzed both at baseline and after treatment. Samples of 10 patients and 10 controls were analyzed for TNF-α secreting cells. Medications of the 37 MDD patients were summarized in **Supplementary Table 1**.

Between the PBMC samples of 37 MDD patients (12 males and 25 females) and 42 controls (18 males and 24 females), mRNA levels of SOCS1, SIGIRR, TNFAIP3, and MyD88s were significantly different, using independent t-test ($p = 1.1 \times 10^{-5}$, 2.0×10^{-8}, 6.0×10^{-14}, and 7.2×10^{-12}, respectively). Their demographic data and mRNA levels were summarized in **Table 1**. Fold changes of MDD group were relative to the controls. No significant difference in GPDPH expression was found between MDD patients and controls. SOCS1 level correlated significantly with levels of TNFAIP3 and MyD88s ($p = 0.001$ and 0.014, respectively). SIGIRR level correlated significantly with BMI, Ham-D score, TNFAIP3 level, and MyD88s level ($p = 0.026$, 0.000, 0.000, and 0.000, respectively). TNFAIP3 level correlated significantly with BMI, SOCS level, SIGIRR level, and MyD88s level ($p = 0.011$, 0.001, 0.000, and 0.000, respectively). MyD88s level correlated significantly with BMI, Ham-D score, SOCS level, SIGIRR level, and TNFAIP3 level ($p = 0.010$, 0.000, 0.014, 0.000, and 0.000, respectively).

In the 13 treatment completers (1 male and 12 females), mRNA levels of PBMC did not differ significantly before and after treatment using Wilcoxan sign rank test, despite the significant decrease of Ham-D score. Their demographic data and mRNA levels were summarized in **Table 2**. Fold changes of post-treatment group were relative to the baseline. No significant difference in GPDPH expression was found before and after treatment. The demographic data of the treatment completers do not differ significantly from the 37-patient group, though the treatment completers had more severe depression at baseline. Change of Ham-D score only correlated significantly with TOLLIP mRNA level after treatment ($p = 0.021$).

TABLE 3 | Demographic data and mRNA levels from TNF-α secreting cells.

	MDD ($n = 10$)	Controls ($n = 10$)	Fold change	p
Age (years)	44.5 ± 10.8	42.3 ± 9.4		0.634
Onset (years)	37.2 ± 13.0			
Duration (years)	7.4 ± 8.2			
BMI (kg/m^2)	23.6 ± 2.9	24.2 ± 3.6		0.678
Ham-D score	14.2 ± 14.9			
SOCS1 (-ΔCt)	6.9 ± 0.6	6.0 ± 1.0	0.6 ± 0.2	0.063
TOLLIP (-ΔCt)	10.9 ± 0.7	11.4 ± 0.5	1.5 ± 0.6	0.089
SIGIRR (-ΔCt)	7.6 ± 0.3	5.6 ± 2.1	0.2 ± 0.5	0.023*
TNFAIP3 (-ΔCt)	0.50 ± 0.4	0.7 ± 0.7	2.4 ± 0.7	3.2×10^{-4}*
MyD88s (-ΔCt)	6.7 ± 0.7	7.0 ± 0.7	21.8 ± 1.5	0.631

*$p < 0.05$.
BMI, body mass index; Ham-D, 17-item Halmiton Depression Rating Scale; MDD, major depressive disorder; MyD88s, the short form of MyD88; SIGIRR, single immunoglobulin interleukin-1-related receptor; SOCS1, suppressor of cytokine signaling 1; TLR, Toll-like receptors; TNFAIP3, TNF-α-induced protein 3; TOLLIP, Toll-interacting protein.

Between TNF-α secreting cells of 10 MDD patients (1 male and 9 females) and 10 controls (2 males and 8 females), mRNA levels of SIGIRR and TNFAIP3 were significantly different, using Mann-Whitney U Test ($p = 0.023$ and 3.2×10^{-4}, respectively). Their demographic data and mRNA levels were summarized in **Table 3**. Fold changes of MDD group were relative to the controls. No significant difference in GPDPH expression was found between MDD patients and controls. SIGIRR level correlated significantly with SOCS1 level and TNFAIP3 level ($p = 0.000$ and 0.001, respectively). TNFAIP3 level correlated significantly with SOCS1 level, SIGIRR level, and MyD88s level ($p = 0.042$, 0.001, and 0.004, respectively).

TABLE 4 | Summary of recent findings of negative regulators of TLR pathway (-ΔCt).

Publication	Sample cells	MDD vs. controls	SOCS1	TOLLIP	SIGIRR	TNFAIP3	MyD88s
Hung 2017	PBMC	100 vs. 53	↓	↑	↓	↓*	↓*
Hung 2018	monocytes	34 vs. 33	↑*	↓*	↑*	↓*	↓*
Huang 2019	TNF-α secreting cells	30 vs. 30				↑	
Present study	PBMC	37 vs. 42	↑*	↓	↓*	↓*	↓*
	TNF-α secreting cells	10 vs. 10	↓	↑	↓*	↑*	↑

*$p < 0.05$.

MDD, major depressive disorder; MyD88s, the short form of MyD88; SIGIRR, single immunoglobulin interleukin-1-related receptor; SOCS1, suppressor of cytokine signaling 1; TLR, Toll-like receptors; TNFAIP3, TNF-α-induced protein 3; TOLLIP, Toll-interacting protein. Up arrow refers to the values of MDD are greater than those of controls, and vice versa.

DISCUSSION

The most important finding of this study is that SIGIRR and TNFAIP3, two negative regulators of TLR immune response pathways, were differentially expressed in both PBMC and TNF-α secreting cells of patients with MDD as compared to healthy controls. While both SIGIRR and TNFAIP3 mRNA levels had been investigated in the PBMC of patients with MDD in the past (14), only TNFAIP3 mRNA level was reported in TNF-α secreting cells of patients with MDD before (17). To our knowledge, this is the first study to report the differential expression of SIGIRR in TNF-α secreting cells of patients with MDD as compared to healthy controls.

SIGIRR is a transmembrane TLR regulator, which binds to TLR4 and interleukin-1 receptor associated kinase (IRAK) to inhibit the downstream TLR signal pathways (15). In this study, SIGIRR were significantly lower in patients in MDD whether the samples were PBMC or TNF-α secreting cells. Previously, lower SIGIRR was found in PBMC in patients with MDD, but not statistically significant (14). In another study analyzing SIGIRR in monocyte sample of patients of MDD, significantly higher level of SIGIRR was found (16). We speculate that the lower SIGIRR in PBMC and TNF-α secreting cells indicate a deficiency to prevent TLR inflammation. Given most of the studies, including our own, had limited sample size, further investigations would be needed to confirm the significance of SIGIRR in TLR regulation in MDD.

TNFAIP3, also known as A20, is an intracellular TLR regulator, which deubiquitylates tumor-necrosis factor-receptor-associated factor 6 (TRAF6), thus inhibiting the downstream activation of NF-κB inflammatory pathway (15). TNFAIP3 is a potent regulator of dendritic spine remodeling and synapse efficacy in neurons (19). TNFAIP3 had been investigated in various samples from patients of MDD in the past, including PBMC, monocytes, and TNF-α secreting cells (14, 16, 17). In this finding, we found significantly lower TNFAIP3 mRNA level in patients with MDD, which was in line with earlier studies on PBMC (14) and monocytes (16). However, in the TNF-α secreting cells, we found significantly higher mRNA levels of TNFAIP3 in patients of MDD, while an earlier study also found higher TNFAIP levels, no statistical significance was found (17). While lower TNFAIP3 levels in PBMC and monocytes in patients of MDD could indicate a failed defense against TLR inflammation, the higher level found in TNF-α secreting cells in this study might also be an exaggerated response from the overall inflammatory state in patients of MDD. There had also been reports of abnormalities of TNFAIP3 in other psychiatric disorders. In the PBMC of adolescents diagnosed bipolar I disorder, TNFAIP3 mRNA level correlated with pediatric inpatient aggression prediction score, as well as functional brain activations of right anterior part of anterior cingulate gyrus, a part of aggression pathway (20). Significantly higher levels TNFAIP3 mRNA levels were found in the monocytes of patients of bipolar disorder (21). The abnormalities of TLR pathway negative regulators could also be found in psychiatric disorders other than MDD.

We also found SOCS1 to be statistically higher and MyD88s to be statistically lower in PBMC of patients of MDD, though no statistical significance was found in TNF-α secreting cells. SOCS1 suppresses IRAK to prevent inflammatory response initiated by TLR 4 and 9 (15, 22, 23). In our study, significantly higher SOCS1 level was found in PBMC of patients with MDD. Previously, significantly higher SOCS1 was also found in the monocytes of patients of MDD (16), but not in PBMC (14). SOCS1 had also been investigated in other mood disorder, namely bipolar disorder. SOCS1 mRNA levels were also significantly higher in patients with bipolar disorder, but this finding remained positive in male patients only if different genders were analyzed separately (24). SOCS1 could also contribute to the innate immune responses associated with MDD.

MyD88s is the short form of MyD88, which is the most crucial adaptor in TLR signaling (15). MyD88s antagonizes MyD88 functions, preventing IRAK4 to phosphorylate IRAK1, thus halting the inflammatory pathway. In our study, we found significantly lower MyD88s mRNA levels in the PBMC in patients with MDD, similar to past findings in PBMC (14) and monocytes (16). The negative regulators of TLR signaling could form a complex web, and further investigations are warranted. The recent findings of negative TLR regulators of MDD are summarized in **Table 4**.

In the patients treated with antidepressants for 4 weeks, we did not find significant changes in the levels of those negative regulator mRNA. Earlier studies could not find significant changes in levels of SOCS1, TOLLIP, SIGIRR, or MyD88s in PBMC or monocytes of patients with MDD, but TNFAIP3 levels showed significant increase after treatment (14, 16). TNFAIP3 level is also associated with psychological

anxiety in MDD (25), baseline Ham-D score (14), and decreases in Ham-D score (14). While in another study, SOCS1 level correlated with changes in Ham-D score (16). In our study, only TOLLIP mRNA levels after treatment correlated significantly with changes in Ham-D score. The exact mechanism of how those regulators contribute to clinical symptoms remained elusive.

There are several limitations in this study. The sample sizes of both PBMC and TNF-α secreting cells investigations were small. The antidepressants were not controlled. Different classes of antidepressants could interact with the targeted outcomes in various ways. The treatment duration was merely 4 weeks, which is relatively short compared to other studies involving antidepressant treatment, which usually lasted 8–12 weeks. Due to limited fund, we were unable to analyze negative TLR regulators of TNF-α secreting cells after antidepressant treatment. Lastly, TNF-α-secreting cells include several cell types, such as monocytes, macrophages, and T cells, which could confound the findings. The readers are warned against over-interpret our study results because of those limitations, and a larger sample size with more controlled variables will be needed before a firm conclusion could be made.

SIGIRR and TNFAIP3, two negative regulators of TLR immune response pathways, were differentially expressed in both PBMC and TNF-α secreting cells of patients with MDD as compared to healthy controls. The negative regulations of innate immune response could contribute to the underlying mechanism of MDD.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Chang Gung Memorial Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

T-LH contributed substantially to conception, design, and approved the final draft. C-CL and T-LH contributed to acqusition of data, helped with analysis, and interpretation of data. C-CL drafted the article and revised it critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.698257/full#supplementary-material

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Gait Alterations in the Prediction of Metabolic Syndrome in Patients With Schizophrenia: A Pilot Study With PODOSmart ® Insoles

Dimitris Efthymiou[1], *Dimitrios X. Zekakos*[2], *Evangelia Papatriantafyllou*[3], *Efthimis Ziagkas*[4], *Alexandros N. Petrelis*[2†] *and Emilia Vassilopoulou*[3*†]

[1] Division of Neurosciences, Department of Psychiatry, School of Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece, [2] Digitsole SAS, Nancy, France, [3] Department of Nutritional Sciences and Dietetics, International Hellenic University, Thessaloniki, Greece, [4] Laboratory of Motor Behaviour, Aristotle University of Thessaloniki, Thessaloniki, Greece

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Brenda Penninx, Academic Medical Center, Netherlands

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***Correspondence:**
Emilia Vassilopoulou
vassilopoulouemilia@gmail.com
orcid.org/0000-0002-2665-5908

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

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Background: Second-generation antipsychotics (APs) are associated with metabolic syndrome (MetS), characterized by abnormal pro-inflammatory cytokine production and oxidative stress due to the reduced antioxidant systems, and neurological effects, including mobility impairment. This pilot study investigated relationships between inflammatory-metabolic biomarkers, MetS and gait alterations in patients with psychosis treated with APs.

Methods: Patients with psychosis treated with APs, 20 with MetS (MPS group) and 20 without MetS (PS group) were studied, usinganthropometric data, blood measurements and gait analysis performed with the PODOSmart ® gait analysis device.

Results and Discussion: The MPS group had significantly higher mean body mass index (BMI) and arterial blood pressure (BP) than the PS group. PODOSmart ® gait analysis recorded significant differences between groups in pronation-supination at Heel Off (HO), gaitline HO and gaitline Toe Off (TO). Multifactorial elastic net regression models demonstrated significant association with MetS of inflammatory markers, specific AP2 treatment, gender, age; BMI; BP and smoking (accuracy $\lambda = 0.08$), and in relation to gait parameters (accuracy $\lambda = 0.750$), the three pronation- supination variables, i.e., at HO, flat foot in (AP2 related) and TO, and propulsion speed. The gait parameters were at the edges of the model, thus indicating a more significant role of these parameters compared to the other clinical variables. Early diagnosis of MetS in patients with schizophrenia via identification of gait alterations can be a screening measure for serious cardiovascular complications related to psychosis and APs, to enable timely dietary intervention that can control the pro-inflammatory state and reduce oxidative stress.

Keywords: antipsychotics, metabolic syndrome, gait analysis, inflammation, gaitline, speed, weight, PODOSmart ®

INTRODUCTION

"Don't walk behind me, I may not lead.
Don't walk ahead of me, I may not follow.
Walk next to me and be my friend."
Albert Camus

Schizophrenia is a chronic psychotic disorder characterized by disorganization of thought and behavior, with possible delusions and/or hallucinations, possible signs of negative symptoms and cognitive deficits (1, 2). Second-generation, or atypical antipsychotics (APs) for the treatment of schizophrenia have offered significant benefits to patients, providing greater effectiveness than traditional APs in treating the negative and emotional symptoms of psychosis, with a lower risk of extrapyramidal side effects (3). Other serious side-effects, however, have been documented for the second generation or atypical APs, which are associated with a reduction in life expectancy of up to 20 years in comparison with the general population (4).

One significant complication is the metabolic syndrome (MetS), which is observed in 40% of patients taking second generation APs, and which carries the risk of cardiovascular disease (CVD) and diabetes mellitus (DM), contributing to a decrease in life expectancy (5). MetS is associated with a low-grade inflammation and oxidative stress, that may be modulated with early proper dietary intervention (5).About 50% of patients with schizophrenia are obese, due to a variety of factors, including reduced mobility, drugs that cause significant weight gain (WG), poor eating habits and difficulty in understanding the meaning of proper nutrition (6). Apart from the APs medication, metabolic complications result from the disease itself. WG, type II DM and insulin resistance were first reported to be associated with schizophrenia by Sir Henry Maudsley in 1879, long before the use of second-generation APs (7).

Among the second-generation APs, olanzapine and clozapine are reported to cause the most severe metabolic complications, while aripiprazole, brexpiprazole, cariprazine, lurasidone, and ziprasidone produce milder effects. The metabolic side-effects are correlated with overweight or obesity at baseline weight, male sex, and non-white ethnicity, but also with the effectiveness of the APs in the treatment of psychosis (8).

In addition, both psychosis and APs are associated with a variety of adverse neurological effects, among which is impairment of mobility (9).Changes in mobility can be observed even before the onset of psychosis, and have been documented in children with a family history of schizophrenia (10). Studies have shown that the main gait deficit in schizophrenic bradykinesia is a disturbed regulation of stride length (11). Treatment with conventional APs exacerbated this deficit, but treatment with atypical APs showed no additional effects on the gait of patients with schizophrenia, and their pace (steps per minute) remained largely unaffected (12). The reasons for the impairment of motion in schizophrenia have not yet been fully elucidated. Studies of motor disorders in patients with schizophrenia indicate that at least part of the reduction in motor function may be due to impairment of internal control mechanisms, interfering with the automatic execution of motor tasks (13).

Traditional antipsychotics are reported to cause drug-induced mobility disorders, including neuroleptic-induced parkinsonism, neuroleptic-induced acute dystonia, neuroleptic-induced dementia, neuroleptic-induced late dyskinesia, neuralgia-induced malignant syndrome, and malignant neoplastic syndrome, all of which affect the gait, resulting in slowness, dragging of the feet and impaired balance. Fine movements are also affected, leading to difficulties in the physical execution of daily activities. With the second-generation APs, these neurological side-effects are significantly less common (14).

Gait consists of a series of rhythmical, alternating movements of the trunk and limbs that result in the forward progression of the center of gravity, and is a reliable indicator of overall functionality. Gait analysis includes accurate measurements of spatiotemporal and kinematic parameters, for which various devices have been developed, including both laboratory based and portable systems (15). Although these devices have gained popularity in scientific research, they also present shortcomings. Specifically, this type of equipment is not usually portable and it can be used only in laboratory-based measurements. In addition, its configuration is capable of capturing only a limited number of steps (16, 17). The high cost of these devices is also a factor to be considered (18, 19).

Portable smart devices have been developed recently that appear to overcome the limitations of the classical gait analysis devices. The PODOSmart ® device, developed by Digitsole SAS, is a low-cost portable system that consists of insoles with completely wireless sensors and integrated internal storage. They can fit into any shoe and can measure spatial, temporal, and kinematic gait parameters in general and specific populations. The device and the validation of the measured and calculated parameters is described in detail in a paper that is currently under review.

An increasing body of research has used analysis of gait to predict metabolic complications, including type II DM and CVD. A recent meta-analysis suggested the use of gait speed as a predictor of CVD onset and mortality (20).The limited research on gait alterations in MetS revealed an association between slower gait and low levels of high-density lipoprotein cholesterol (HDL) and high fasting glucose levels in women (21), but no further exploration was made.

Aim of the Study

The purpose of the current pilot study was to investigate whether the MetS in patients with psychosis treated with second-generation APs is associated with alterations in gait, the type of APs medication and other inflammatory-metabolic biomarkers.

METHODS

Participants

Patients diagnosed with schizophrenia, who were being treated with second-generation APs and monitored at the Thessaloniki Psychiatric Hospital (Greece), were recruited from January to April 2021. The inclusion criteria were: (1) age $\geq$ 18years, (2)

a diagnosis of psychosis according to the ICD-10 classification system, and (3) long-term treatment with second-generation APs medication (≥5 years). The exclusion criteria were: (1) a clinical history of substance misuse (dual diagnosis patients), (2) current or past use of typical AP medication, (3) pregnancy, (4) intellectual disability, and (5) a diagnosed chronic medical/metabolic condition other than MetS, such as CVD and DM.

The patients were classified in two groups, based on the following criteria:

1. Patients With Psychosis who met at Least 3 of the Criteria for the Diagnosis of MetS (the MPS Group).
2. Patients With Psychosis Without MetS (the PS Group).

Before inclusion in the study, all the participants were informed in detail about the study protocol and they provided their written informed consent. The study was approved by the Research Ethics Committee of the Aristotle University of Thessaloniki (code number 4/26.01.2021) and complied with the International Code of Medical Ethics of the World Medical Association and the Helsinki Declaration.

Clinical Assessment

Schizophrenia classification (F20) was made according to the ICD-10 classification by the medical team of the Thessaloniki Psychiatric Hospital and cross-checked by the psychiatrist in the study team. The APs medication prescribed to each of the patients in the study was recorded in detail and subdivided into two categories: AP1, related to a higher risk of WG of ≥ 7% from the baseline weight, namely aripiprazole, amisulpride quetiapine XR, paliperidone, and ziprasidone, and AP2, related to a higher risk of WG ≥ 7% from the baseline weight, namely olanzapine, asenapine, clozapine and risperidone (22–24), as we described previously in detail (25).

Blood Pressure Determination

Arterial blood pressure (BP) was recorded to the nearest 2 mmHg, using a mercury sphygmomanometer with the arm supported at heart level, after the subject had been sitting quietly for 10 min. One trained member of the research team took three separate readings at 1-min intervals. The average of the last two readings was used for analysis.

Anthropometric Assessment

Anthropometric measurements were made on all participants on the morning of gait analysis, after fasting for at least 8 h, by one trained investigator. Height was measured to the nearest 0.1 cm, using a commercial stadiometer (Leicester Height Measure, Invicta Plastics Ltd, Oadby, UK) with the participants barefoot, their shoulders in a relaxed position, their arms hanging freely and their heads in the Frankfort horizontal plane. The participants were weighed barefoot and in light clothing to the nearest 0.1 kg, using a TANITA RD-545 ("RD-545-Connected smart scale | Tanita Official Store," n.d.). Body mass index (BMI) was calculated from the current weight and height [weight (kg) by height squared (m2)]. The waist circumference (WC) was measured with a SECA flexible, inextensible measuring tape with an accuracy of 1 mm, on a horizontal plane, after exhalation, at a point equidistant from the lowest floating rib and the upper border of the iliac crest.

Biochemical/Hematological Assessment

Venous blood samples were collected from all participants, as part of their routine monitoring procedure on a day independent of the gait analysis, after overnight fasting, and analyzed on the hospital premises, using automatic biochemical analyzers, under standard conditions. The white blood cell count (WBC), and concentrations of blood glucose (GL), serum total cholesterol, triglycerides (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), high-sensitivity C reactive protein (hsCRP) and B12 were measured by an automatic analyzer (Toshiba TBA 120FR; Toshiba Medical Systems Co., Ltd., Tokyo, Japan).

Definition of MetS

MetS was defined according to the International Diabetes Federation (IDF) criteria (25), according to which patients need to have at least three of the following: BMI ≥ 30 or WC ≥ 94 cm in men and ≥ 88 cm in women; TG ≥ 150 mg/dl, or receiving drug treatment for hypertriglyceridaemia; HDL < 40 mg/dl in men and HDL < 50 mg/dl in women, or receiving drug treatment for hyperlipidemia; BP ≥ 130/80 or receiving drug treatment for hypertension; fasting GL ≥ 100 mg/dl, or known DM, or receiving drug treatment for hyperglycaemia (26).

Gait Analysis

The PODOSmart ® gait analysis device allows measurement of walking and running parameters under real-world conditions. The PODOSmart ® insoles (Digitsole SAS, Nancy, France) consist of a pair of insoles (available in most shoe sizes) connected to a mobile application via a Bluetooth connection box. The PODOSmart ® smart insole is rechargeable via USB and can be used continuously for more than 33 h. Each PODOSmart ® insole has an inertial platform that can record walking or running steps and the placement of each foot in 3D space. The Bluetooth connection box is used to collect data captured by the smart insoles. The data are processed into a clinically usable dataset by artificial intelligence algorithms to extract spatiotemporal, kinematic, and biomarker parameters that are presented in the interface.

PODOsmart insoles offer gait analysis parameters based on both monopedal and bipedal gait data. Monopedal gait parameters include: the angles of the foot during heel strike (HS), heel-off (HO), flat foot in (FFI) and toe off (TO), the stride length in meters (i.e., the distance of foot displacement between two consecutive steps on the same side); stride duration in milliseconds (i.e., the duration of foot displacement between two consecutives steps on the same side); stance time (i.e., the percentage of duration of contact between the foot and the ground during a one stride cycle); swing time as a percentage of swing duration during a one stride cycle; foot progression angle in degrees (i.e., the angle defined between the orientation of the foot and the user's trajectory). Three gait parameters are calculated from the recorded data on both feet (bipedal): cadence,

which represents the number of steps per minute; gait speed (km/h), which is the average walking speed of the user; double contact duration (%), that refers to the duration of simultaneous contact between both feet and the ground.

Procedure

Prior to gait analysis, the subjects removed their shoes and wiped their feet with alcohol. A study team member placed an insole in each shoe, according to the subject's shoe size. The subjects walked for 1 min at their preferred velocity when walking with comfort, on flat ground, straight ahead, and they were then asked to make a U-turn at the half-way point and return to the starting line.

Statistical Analysis

Statistical analysis included statistical tests, inferential analysis and modeling. The R software (version 4.04) and R studio (version 1.4.1106) were used. For the descriptive analysis, data were shown as mean and standard deviation (mean±SD) or median and interquartile range, as appropriate. Normality was checked using the Shapiro-Wilk test.

Normally distributed data were compared between the MPS and PS groups by *t*-test and non-normally distributed data by the Wilcoxon test. In all cases, the level of significance (a) was set at 0.05. *P*-values ≤ 0.05 were considered statistically significant.

To further investigate the association of MetS in relation to gait analysis parameters, APs medication, demographic, anthropometric, clinical, and biochemical data, the elastic net regression model was used. This is a regularized regression methodology that combines Lasso and Ridge regression (26). Logistic regression model indicates the conditional probabilities through a linear function of the predictors:

$$Pr(G = 1/\mathrm{x}) = \frac{\mathbf{1}}{\mathbf{1} + \mathbf{e}^{-(\beta_0 + \boldsymbol{x}^T\beta)}}$$

$$Pr(G = 2/\mathrm{x}) = \frac{\mathbf{1}}{\mathbf{1} + \mathbf{e}^{+(\beta_0 + \boldsymbol{x}^T\beta)}} = 1 - \mathrm{Pr}(\mathrm{G} = 1/\mathrm{x})$$

Alternatively, this implies that

$$\log \frac{Pr(G = 1/x)}{Pr(G = 2/x)} = \beta_0 + \boldsymbol{x}^T\beta$$

In the regularized maximum binomial likelihood, $\mathrm{p}(x_i) = \mathrm{Pr}(\mathrm{G} = 1/x_i)$ is the probability for the i observation at a specific value for the parameters (β_0,β). To maximize the penalized log-likelihood

$$\max_{(\beta_0,\beta)\in\mathbb{R}^{p+1}} [\frac{1}{N}\sum_{i=1}^{N} \mathrm{I}\{(g_i = 1)^*\mathrm{logp}(x_i) + \mathrm{I}(g_i = 2)^*\log(1 - p(x_i))\} - \lambda P_a(\beta)]$$

In the final step of the elastic net, the use of coordinate descent for solving the penalized weighted least-squares problem has as an objective function, the penalized negative binomial log-likelihood, and is:

$$\min_{(\beta_0,\beta)\in\mathbb{R}^{p+1}} -[\frac{1}{N}\sum_{i=1}^{N} y_i(\beta_0 + x_i^T\beta) - log(1 + e^{(\beta_0 + x_i^T\beta)})] + \lambda[(1-\alpha)||\beta||_2^2/2 + \alpha||\beta||_1]$$

where λ is a parameter that controls shrinkage, 0 is no penalty and ∞ is entirely penalty, and α regulates how much of the ridge vs. lasso, 0 is the ridge, and 1 is the lasso. In our process, we run multiple cross-validations for different values inside this interval [0,1] to define the optimal α for our model. To apply this methodology in R software, we used the glmnet package (version 4.1-1).

RESULTS

For the purposes of this pilot study, 20 patients with schizophrenia (80% males) were recruited who were taking second-generation APs and who fulfilled the criteria of MetS (the MPS group), and 20 patients with schizophrenia taking second-generation APs, but without MetS (85.7% males) (the PS group). All the study patients had been taking second-generation APs for a mean of 12.9 ± 7.2years: An AP2 was the selected treatment for 12/20 MPS (60%) and 6/20 of PS, while the rest of the patients were receiving an AP1.

Table 1 shows the demographic characteristics, anthropometric measurements, and clinical data of the patients in the MPS and PS groups. The mean age of the participants was similar in the two groups (48.8 ± 10.98 years for MPS vs. 48.05 ± 15.07 years for PS, $p > 0.05$). The MPS patients (those with MetS) had a significantly higher mean body weight ($p = 0.04$) and BMI ($p < 0.01$), mean diastolic arterial pressure (DAP) ($p = 0.01$) and median systolic arterial pressure (SAP) ($p < 0.01$) than the PS patients. **Table 2** summarizes the chemical biomarkers in the two groups. The mean serum level of HDL was lower, and the WC and the mean serum levels of TG and fasting GL were higher in the MPS than in the PS group, but the differences were not statistically significant (p>0.05).

Table 3 shows the comparison of parameters measured with PODOSmart ® during free gait, that presented a statistically significant difference between the MPS and the PS group: pronation and supination HO (p = 0.04), in gaitline HO (p = 0.01) and gaitline TO ($p = 0.03$). The comparison of all measured parameters with PODOSmart ® during free gait are provided in **Supplementary Table A** of the **Supplementary Material**.

Elastic net regression was used to construct a multifactorial model to explore the APs, anthropometric factors and biomarkers associated with the risk of MetS, as shown in **Figure 1**. The model presented good accuracy (0.857) and the AUC showed ability of the model to distinguish factors in affecting MetS (AUC: 0.94; 95% CI: 0.74, 1). According to the model, factors associated with a higher risk of MetS in patients with psychosis were: higher levels of hsCRP, GL, TG, and WBC;

TABLE 1 | Study participants' demographic and clinical characteristics.

Characteristic	MPS	PS	*p*-value
n	20	21	
Gender	16M/4F	18M/3F	0.780
Age (years)	48.8 (±10.98)	48.05 ± 15.07	0.857
Weight (kg)	88.7 (±15.15)	78.57 ± 15.79	0.043
Height (m)	1.73 (±0.11)	1.72 ± 0.11	0.718
BMI (kg/m^2)	29.9 (±6.32)	26.58 ± 4.52	0.059
Waist circumference (cm)	102.85 (±14.14)	100.81 ± 14.02	0.645
Systolic arterial pressure (mmHg)	130 (±9.64)	110 ± 10.38	0.004
Diastolic arterial pressure (mmHg)	81.5 (±9.64)	74.71 ± 8.93	0.014
Cigars/day	15.1 (±8.72)	12 ± 9.17	0.216

BMI, Body Mass Index.

TABLE 2 | Biochemical parameters between MPS and PS.

	MPS	PS	*p*-value
HT (%)	43.05 (±3.66)	42.32 ± 4.19	0.563
RBC (M/ml)	4.94 (±0.5)	4.8 ± 0.52	0.389
PLT (K/ml)*	231 (264)	208 (270)	0.285
B12 (pg/ml)*	297 (484)	298 (542)	0.979
WBC (K/ml)*	8.57 (7.14)	7.24 (8.19)	0.103
hsCRP (mg/dl)*	0.29 (1.02)	0.25 (1.12)	0.441
T-Chol (mg/dl)	162.1 (±440.3)	182.33 (±48.54)	0.170
HDL (mg/dl)	39.15 (±11.52)	42.86 (±9.81)	0.273
LDL (mg/dl)	91.3 (±38.01)	114.47 (±39.6)	0.063
TG (mg/dl)*	123.5 (520)	108 (244)	0.230
Glucose (mg/dl)*	101.5 (127)	93 (57)	0.137

HT, hematocrit; RBC, Red Blood Cells; PLT, Platelets; WBC, White blood cells; hsCRP, high sensitive C-reactive protein; T-Chol, Total Cholesterol; HDL, High Density Cholesterol; LDL, Low Density Cholesterol; TG, triglycerides. Values are Mean (±Standard Deviation) or Median (Interquartile Range) in non-normally distributed variables indicated by (Wilcoxon Test).*

TABLE 3 | Gait analysis variables analyzed with PODOSmart ® which presented statistically significant difference ($p < 0.05$) among the MPS and PS.

	MPS	PS	*p*-value
Right.pro_sup_HO.avg	0.06 (±0.11)	0 (±0.08)	0.043
Right.gaitline_TO.avg*	0.06 (3)	0 (3)	0.035
Right.gaitline_TO.std*	0.14 (0.54)	0 (0)	0.035
Left.gaitline.HO.std*	0.11 (0.41)	0.19 (0.49)	0.012

*Pro, pronation; Sup: supination; HO, Heel off; TO, Toe off; avg: average; std, standard deviation. Values are Mean (±Standard Deviation) (t-test) or Median (Interquartile Range) in non-normally distributed variables indicated by * (Wilcoxon test).*

use of AP2 treatment; female gender; greater age; higher BMI; higher arterial BP; and smoking a greater number of cigarettes. In this case series, AP1 medication, male gender, WC, and high levels of LDL, total cholesterol and B12 were not correlated with MetS.

A second elastic net model (**Figure 2**) was constructed to assess the MetS risk in relation to gait parameters measured with PODOSmart ®, along with anthropometric, clinical and biochemical markers. This second model demonstrated accuracy at good levels (0.750), along with an acceptable AUC (AUC: 0.8; 95% CI: 0.6, 1). Of the gait parameters, the two pronation and supination variables (HO and FFI) and gaitline TO, and the propulsion speed, showed a close association with the risk of MetS. Among the clinical and anthropometric factors and biochemical markers, AP2, and higher levels of weight, BMI, systolic and diastolic arterial BP and WBC showed significant positive association with the MetS risk.

Conversely, the MetS risk was reduced when the progression angle, gaitline HO and the pronation-supination FFI increased. AP1 medication was inversely related to MetS, while the levels of LDL and TG showed no effect. The gait parameters were found in the edges of the model with higher positive and negative coefficients compared to the clinical, anthropometric and biochemical biomarkers.

DISCUSSION

Patients with schizophrenia present various metabolic and inflammatory disturbances related to both the disease itself and the APs medication used (27), leading to an increased risk of MetS (28). Alterations in gait have been studied only rarely, in either schizophrenia (29, 30) or MetS (21), with results suggesting the need for further research. In this, our first pilot study, we aimed to investigate factors in the clinical, metabolic and inflammatory profile of patients with psychosis, through their routine screening, to determine their possible association with gait alteration and MetS and to explore whether gait analysis could be used as an early indicator for metabolic and inflammatory abnormalities in psychosis. We developed two prediction models that detected a close relationship between MetS and known metabolic and inflammatory markers, depending on the APs medication used and lifestyle factors. Alteration in the gait parameters measured with PODOSmart ® showed a strong relationship with MetS in the prediction model; increase in the two parameters of pronation-supination (HO and FFI), gaitline TO, and propulsion speed were positively associated with the MetS. Conversely, increases in gaitline HO, progression angle and the pronation-supination FFI were inversely correlated with MetS.

We observed that patients receiving AP2were at greater risk of developing MetS. A recent meta-analysis reflected the wide variation in the impact of different APs on metabolic dysregulation (8). Clozapine and olanzapine, sub-categorized in the AP2 medication sub-group, have been observed to produce higher rates of WG and greater increase in BMI than AP1 medication, such as aripiprazole and paliperidone. MetS has been documented to contribute to the increased risk of CVD in both the general population (31) and patients with psychosis receiving atypical APs therapy (32, 33); as such the last are

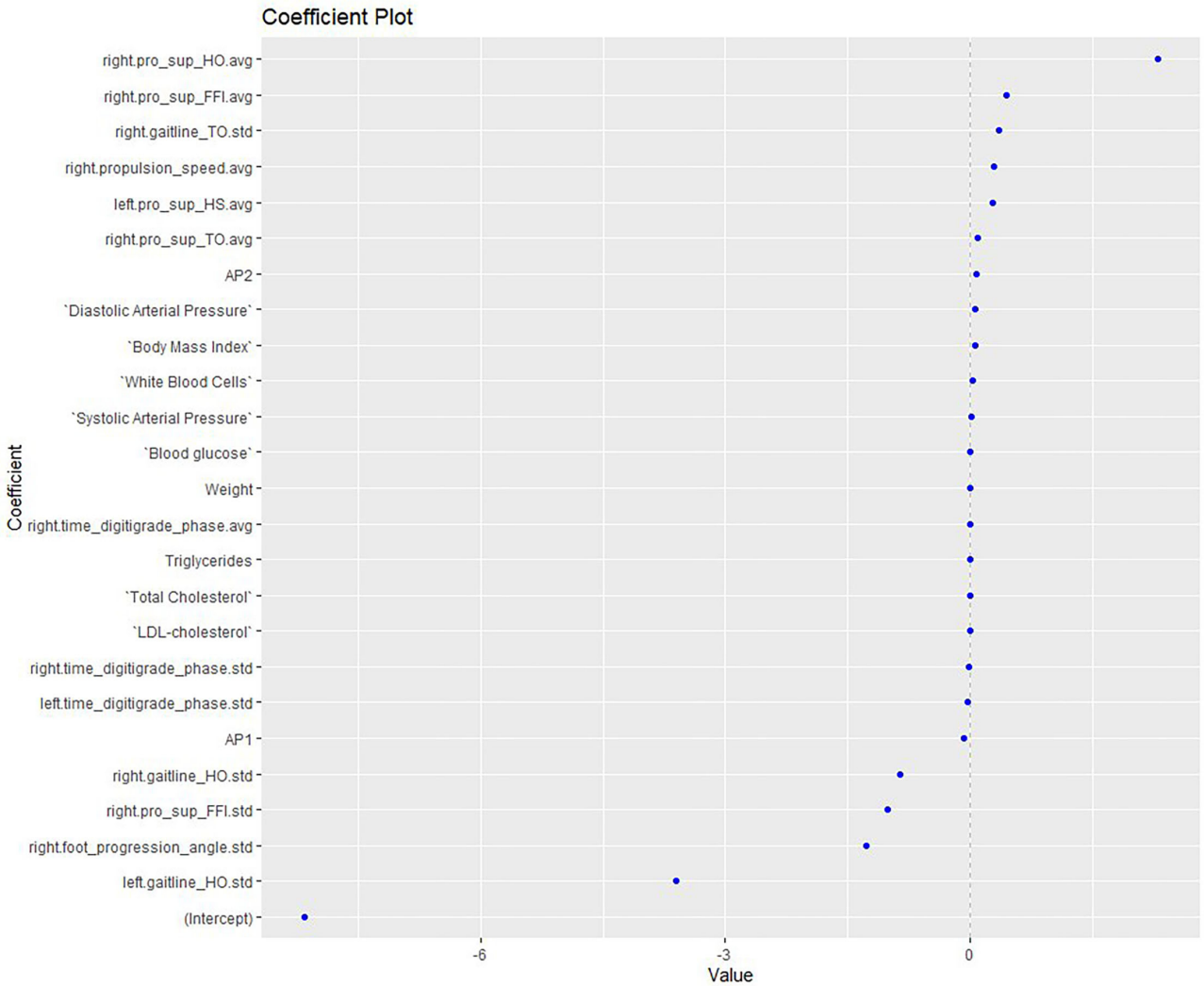

FIGURE 1 | Elastic net model exploring the APs, anthropometric and biomarkers associated with the risk of MetS. HO, Heel off; FFI, Flat foot in; TO, Toe off; HS: heel strike; Pro, pronation; Sup, supination; avg, average; std, standard deviation; AP, antipsychotics; LDL, low-density lipoprotein.

suggested to be monitored constantly for metabolic dysregulation in the clinical routine (32). Metabolic dysregulation is evident in drug naive patients with psychosis, indicating that factors other than medication are implicated, including genes (34) and lifestyle choices (35, 36). Studies in recently diagnosed, untreated patients with psychosis indicate that they have up to three times more intra-abdominal fat than the general population. This can be explained by the involvement of the hypothalamic-pituitary-adrenal axis, which regulates, among other functions, the body metabolism and its response to the stress of the development of psychosis (37). Half of the patients have signs of MetS when first diagnosed with psychosis, even before initiation of APs treatment. Medication is also implicated, as, according to Eskelinen and colleagues (2015), the risk of metabolic disorders in clozapine users is doubled (37).The duration of APs treatment, also, is related to an increase in BMI and the occurrence of MetS. In our study population, the prediction model endorses as significant factors for MetS the specific APs treatment, increased BMI, arterial BP, and raised blood levels of GL and TG. Longer duration of psychosis is related to the development of obesity and metabolic symptoms, while according to Dehelean and colleagues (2019) the use of risperidone appears to lead to hypertension and an increase in WC (38).

Although HDL level and WC have been recognized as factors in the diagnosis and prognosis of MetS (39), they did not appear as significant factors in our population. Conversely, two inflammatory markers, hsCRP and WBC, were found to be significantly related with MetS. Patients with psychosis suffer from low-grade chronic inflammation, probably as a stress response of the immune system (40). Various immune and inflammatory alterations have been found, in both brain and blood, among which increases in WBC and hsCRP relevant to

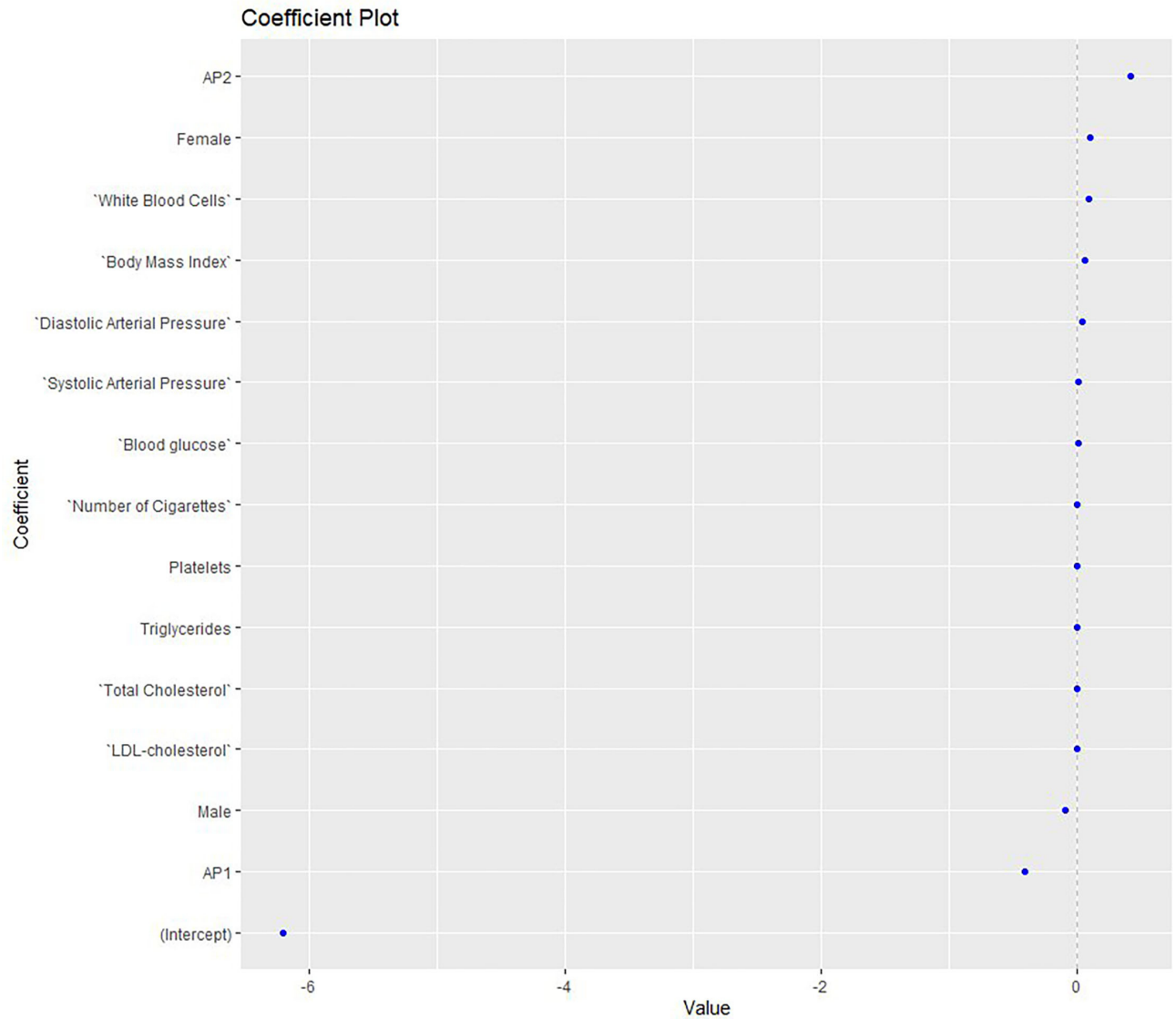

FIGURE 2 | Elastic net model exploring the MetS risk in relation to gait parameters measured with PODOSmart ®, along with anthropometric, clinical and biochemical markers. AP, antipsychotics; LDL, low-density lipoprotein.

metabolic disturbances in psychotic patients (41). A high level of hsCRP during adolescence is a risk factor for psychosis in later life (42). In under-treated patients with psychosis, the type of drug therapy is related to the level of hsCRP and the WBC (43) and under-response to treatment has also been correlated with inflammatory markers (44). As shown by Vassilopoulou et al. (25) the long-term use of AP2 is relevant to increases of hsCRP and WBC. Similarly, Jacomb et al. (45) suggested that hsCRP is elevated in acute psychosis, but also significantly elevates in chronically ill patients with psychosis.

In parallel, schizophrenia and APs both disturb gait parameters in complex ways, as a variety of factors are involved in the alterations. In 1999, Flyckt and colleagues referred to heredity as a factor in the occurrence of neurological abnormalities that gait (44), while other researchers have reported that differences in mobility can be partly explained by the cognitive level of an individual (46). The impact on motor activities and gait performance are often depicted by the speed and balance (47). Analysis of gait using PODOSmart ® uncovered significant differences in gait variables between the two study groups, with and without MetS, including the gaitline at the TO phase and the pronation and supination HO. During the TO phase, pressure on the heel switch is released, activating the stimulator and evoking dorsiflexion of the foot during the swing phase of gait and altering the knee flexion during the swing phase of normal gait (48).

The three pronation and supination variables in PODOSmart ®, namely pronation-supination at heel strike (HO), pronation-supination at flat foot (FFI) and pronation-supination at toe off (TO) represent ankle varus or vagus in the three single limb support phases (initial contact, mid stance, terminal stance).

Ankle valgus and varus belong to an insidious deformity that results in pronation or supination of the foot. The causes of varus or valgus ankle vary and may occur due to neuromuscular disorders or skeletal dysplasia (49–53).

The prediction model also correlated propulsion speed with MetS. Propulsion speed as a PODOSmart ® variable refers to the speed of the foot at the TO phase. Propulsion and stance stability, shock absorption and energy conservation all belong to the basic locomotor functions of gait (54).Consequently, regardless of the initial standing phase of the limb, initiation of a step begins with a shifting of body weight and anterior displacement at the ankle joint of the supporting limb. Swinging of the lower limb involves a change in body posture for the propulsion. Hip flexion and ankle dorsiflexion lead to lifting of the swing limb, creating anterior forces that modify the standing balance. Rapid hip flexion offers further acceleration that augments this effect (55, 56).

Motor side-effects significantly affect the autonomy and functionality of the individual in their daily living activities. Psychosis and some types of APs medication have been associated with a variety of mobility side-effects, but also with metabolic and inflammatory dysregulation, all of which can adversely affect the quality of life and morbidity and mortality rates. Gait disturbances can reduce mobility and therefore increase MetS and inflammatory complications (57, 58). In general, the assessment of motor dysfunction and gait disorders using clinical tools is a challenging process (58, 59). We have demonstrated in our model that gait parameters calculated by PODOSmart ® have a stronger association with MetS than clinical, anthropometric and biochemical variables.

A limitation of the current investigation is the small number of participants and the absence of statistically significant differences in the biomarkers determining MetS between the two subgroups of patients taking different second-generation APs. In view of the documented link between psychosis, APs and inflammatory and metabolic disturbances and altered gait patterns, however, it is expected that further investigation with larger samples will provide valid information and better understanding, enhancing the ability to interpret these alterations.

Future investigation of the metabolic and inflammatory parameters related to schizophrenia and APs medication, and their association with impairment of motor-gait parameters may provide information about the pathophysiological complications of schizophrenia and the mechanisms leading to the differences in motor performance. Gait analysis with PODOSmart® is a low-cost method, rapid and simple to use method and replicated in any space available. Early diagnosis of MetS via gait alterations in patients with schizophrenia can be implemented for timely preventive measures against serious cardiovascular complications, primarily adaptation of a healthier antioxidant and anti-inflammatory diet and enhancement on the compliance with guidelines for relevant lifestyle changes in patients with psychosis, such as smoking cessation and increase in physical activity.

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 Research Ethics Committee of the Aristotle University of Thessaloniki. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DE and EV designed the project and wrote the original article. DE and EP collected the data. DZ and AP analyzed the data. EV, DZ, EZ, and AP interpreted the data. All authors critically revised and approved the final version of the manuscript.

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REVIEW
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Is the Therapeutic Mechanism of Repetitive Transcranial Magnetic Stimulation in Cognitive Dysfunctions of Depression Related to the Neuroinflammatory Processes in Depression?

Hiroshi Tateishi, Yoshito Mizoguchi and Akira Monji*

Department of Psychiatry, Faculty of Medicine, Saga University, Saga, Japan

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Ryan Logan,
Boston University, United States

Reviewed by:
Adam Stern,
Beth Israel Deaconess Medical Center and Harvard Medical School, United States
Reiji Yoshimura,
University of Occupational and Environmental Health, Japan

***Correspondence:**
Hiroshi Tateishi
tateishh@cc.saga-u.ac.jp

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The lifetime prevalence of depression is reported to be >10%, and it is an important illness that causes various disabilities over a long period of life. Neuroinflammation process is often reported to be closely linked to the pathophysiology of depression. Approximately one-third of depression is known to be treatment-resistant depression (TRD), in which the symptoms are refractory to adequate treatment. Cognitive dysfunction is one of the most important symptoms of depression that impedes the rehabilitation of patients with depression. Repetitive transcranial magnetic stimulation (rTMS) is a minimally invasive and effective treatment for TRD and is also known to be effective in cognitive dysfunction in depression. Since the details of the therapeutic mechanism of rTMS are still unknown, we have been conducting studies to clarify the therapeutic mechanism of rTMS, especially focusing on cognitive dysfunction in depression. In the present review, we present our latest results and discuss them from the standpoint of the neuroinflammation hypothesis of depression, while citing relevant literature.

Keywords: depression, repetitive transcranial magnetic stimulation, cognitive dysfunction, neuroinflammation, tryptophan metabolites, white matter integrity

INTRODUCTION

The lifetime prevalence of depression is 21%. It is an important illness that causes various disorders over a long period of time (1). Approximately one-third of patients are known to have treatment-resistant depression (TRD), in which the symptoms are refractory to adequate treatment (2).

Patients with major depression have been found to exhibit increased levels of peripheral blood inflammatory biomarkers including inflammatory cytokines (3). The association between inflammation and major depression has been one of the leading hypotheses for over a decade (3). Recent studies have also reported that the inflammatory process is involved in the pathophysiology of depression (4, 5). A schema of the neuroinflammation hypothesis of depression is shown in

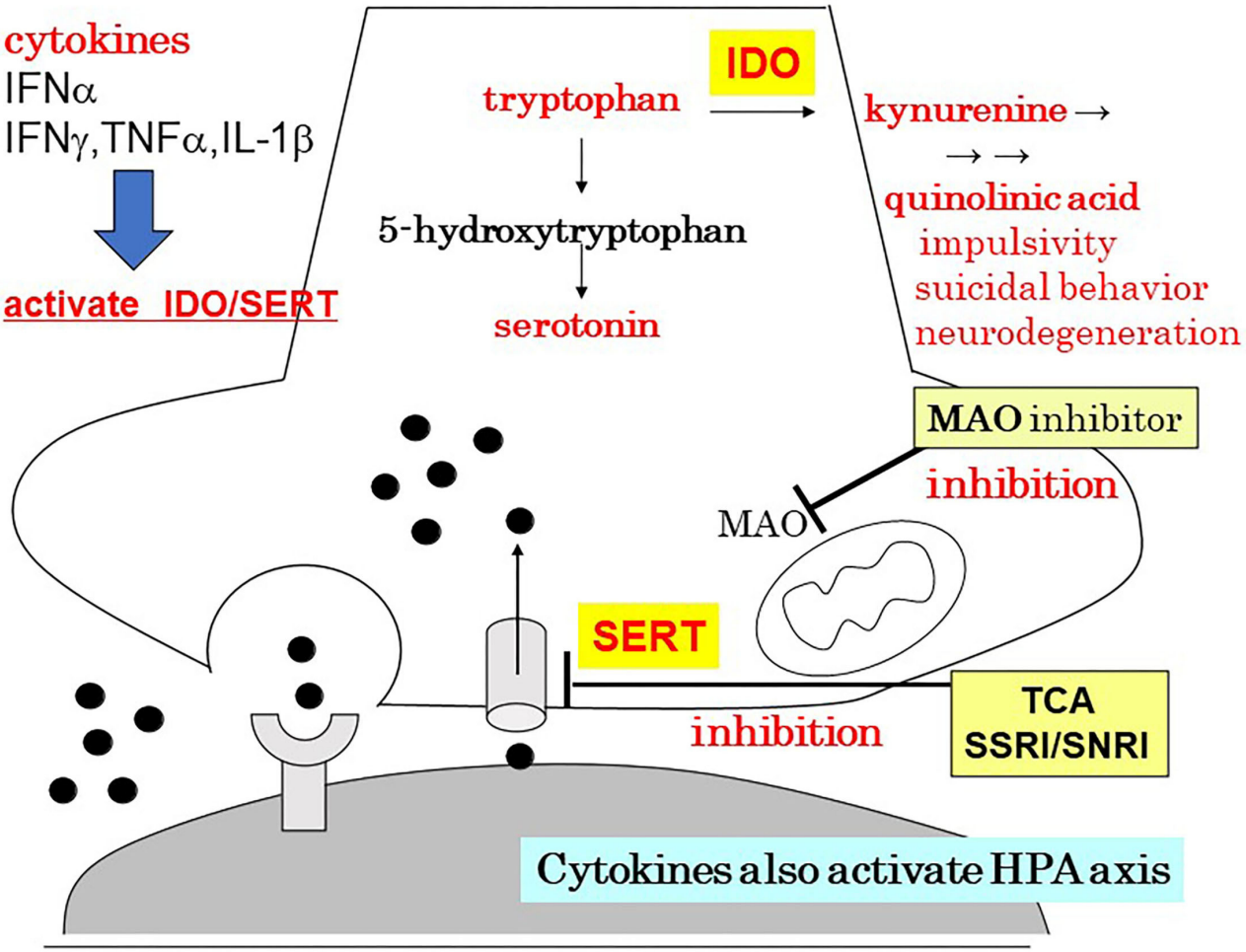

FIGURE 1 | The neuroinflammation hypothesis of depression. Author's modification from Monji (6). HPA, hypothalamic–pituitary–adrenal axis; IDO, indoleamine 2,3-dioxygenas; IFN, interferon; IL, interleukin; MAO, monoamine oxidase; SERT, serotonin transporter; SNRI, serotonin/noradrenaline reuptake inhibitor; SSRI, serotonin reuptake inhibitor; TCA, tricyclic antidepressant; TNF, tumor necrosis factor.

Figure 1 (6). Recently, TRD was shown to be associated with increased inflammatory processes (7, 8). The concentrations of many inflammatory proteins were higher in patients with TRD than in the control group, and poorer responses to treatment were associated with elevated levels of interleukin (IL)-6 and 8, tumor necrosis factor (TNF), C-reactive protein (CRP), and macrophage inflammatory protein-1 (7). There is a significant relationship between higher plasma levels of inflammatory cytokines, including IL-6, and the number of failed treatment trials (8). Patients who failed three or more trials in the current episode revealed significantly higher plasma levels of inflammatory cytokines, including IL-6, compared to patients with less than one trial by *post-hoc* pairwise comparisons with correction for multiple testing (8). More treatment failures were also associated with high-sensitivity CRP only in models with body mass index excluded (8).

A meta-analysis investigated changes in inflammatory cytokines due to selective serotonin reuptake inhibitor (SSRI) treatment in patients with depression (9). This meta-analysis included 22 eligible studies of 827 patients with major depressive disorder (MDD): seven studies for IL-1β, 15 for IL-6, 11 for TNF-α, six for IL-4, and four for interferon-γ. The pooled effect estimate indicated that the levels of the pro-inflammatory markers IL-1β, IL-6, and TNF-α were reduced by SSRI treatment. However, there was a high degree of heterogeneity among the studies included in this meta-analysis.

Antidepressants, psychostimulants, and non-pharmacological therapies improve cognitive dysfunction in patients with MDD. However, no reports have investigated the relationship between inflammatory cytokines and their therapeutic effects. Anti-cytokine drugs such as tocilizumab (targeting IL-6), infliximab, etanercept, and adalimumab (all targeting TNF-α) significantly improve depressive symptoms in humans (10–12), but little is known about the ameliorating effect of anti-cytokine treatment on cognitive dysfunction.

Cognitive dysfunction is closely associated with disorders experienced by many patients with depression (13). Cognitive dysfunction is persistent and observable in many patients with depression from the initial onset of depression to remission (14). Cognitive deficits affect several areas of social functioning, such as employment, social life, family life, and family responsibilities (13). More favorable outcomes, including depression-related psychosocial disorders, are associated with a shorter duration of untreated depression (15). In several neuropsychological studies, cognitive dysfunction has been found to involve a wide range of cognitive areas of depression, including executive function. Executive dysfunction associated with frontal lobe dysfunction has been reported to be prominent in patients with depression (16). Cytokines may affect cognition via various mechanisms. The roles of IL-1, IL-6, and TNF-α have been highlighted in most studies investigating the mechanisms by which cytokines are involved in cognitive dysfunction (17). Recent reports have shown that enhanced inflammatory processes reduce functional brain connectivity that is closely associated with cognitive dysfunction (18–20).

Repetitive transcranial magnetic stimulation (rTMS) is a minimally invasive and effective treatment for TRD and is known to be effective in treating cognitive dysfunction in depression. A systematic review and meta-analysis have shown that rTMS treatment targeting the prefrontal cortex in patients with MDD may moderately enhance cognitive function in set-shifting ability, visual scanning, and psychomotor speed (21). Tong et al. showed that rTMS might improve executive function in patients with MDD (22). Repetitive transcranial magnetic stimulation is considered promising and valuable for improving cognitive dysfunction in TRD (23).

Since the details of the therapeutic mechanism of rTMS are still unknown, we have been conducting studies to clarify the therapeutic mechanism of rTMS, especially focusing on the effect of rTMS on cognitive dysfunction in depression. In the present review, we present our latest results and discuss them from the standpoint of the neuroinflammation hypothesis of depression, while citing relevant literature.

rTMS AND NEUROINFLAMMATION

In MDD, the release of increased levels of pro-inflammatory cytokines and hormones in the plasma and indicators of oxidative stress have been identified as consequences of the activation of inflammatory pathways in the brain (4). The major supply of IL-1β in the central nervous system is provided by microglia. Many studies have reported that microglia are closely associated with the pathophysiology of depression in animal models (24). Administration of lipopolysaccharide stimulates the expression of IL-1β mRNA primarily in the cortical regions (the frontal and parietal cortex), hypothalamus, hippocampus, pituitary gland, thalamic nuclei, and cerebellum of rat (25) and mouse brains (26). In animal models, IL-1β injection causes hippocampal-dependent learning, memory impairment, and long-term potentiation impairment (27). In rodent models, IL-1β has been shown to have stress-induced anhedonic and anti-neurogenic effects (28).

It has been reported that rTMS decreases serum IL-1β and TNF-α levels in elderly patients with TRD (29).

We have demonstrated that 6 weeks of rTMS treatment significantly improved the Hamilton Depressive Rating Scale (HAM-D), Beck Depression Inventory, total errors of the Wisconsin Card Sorting Test (WCST), category of the Word Fluency Test (WFT), and part 3 of the Color Stroop Test (CST) scores (30). Although serum IL-1β, IL-6, and TNF-α levels were not significantly changed by rTMS, serum IL-1β tended to decrease (30). This study suggested that rTMS tended to decrease serum IL-1β independently of improvement in depressive symptoms and that rTMS improved partial cognitive dysfunction independently of improvement in depressive symptoms (30). Decreased serum IL-1β by rTMS was correlated with partial improvement in cognitive dysfunction (CST score part 3) (**Figure 2**) (30). Changes in various cytokines have been associated with cognitive dysfunction observed in depression in human and animal models (17). The ameliorating effect of rTMS on cognitive dysfunction in patients with TRD may be related to changes in IL-1β, but further studies on the involvement of inflammatory cytokines other than IL-1β are needed in the future.

rTMS AND TRYPTOPHAN METABOLITES

Tryptophan (TRP) is metabolized to several bioactive molecules, the most famous of which is serotonin. However, only a small portion of the TRP is converted to serotonin. Kynurenine (KYN) and its degradation products are metabolites of 95% or more TRP via the kynurenine pathway (KP). These are collectively known as KYNs (31). The KP scheme for TRP metabolism is shown in **Figure 3** (31).

Numerous studies have reported changes in TRP metabolite levels in patients with depression. Patients with MDD have significantly lower plasma TRP and KYN levels than the controls (32). The higher concentration of plasma KYN to KYNA and KYN/TRP to KYNA/KYN ratios during pregnancy and lower concentration of plasma 3-hydroxyanthranylic acid during the postpartum period have been shown to be closely associated with postpartum depressive symptoms (33). Profound changes in TRP metabolism have been reported in patients with late-onset depression, whereas low TRP levels and changes in KYN metabolism have been reported in patients with early-onset depression (34). It has been suggested that the increased risk of depression observed after interferon-α administration in patients with chronic medical illness is likely to be mediated by the KP (35). Erabi et al. demonstrated that KYN and KYNA levels were significantly and negatively associated with reduced HAM-D in 62 patients with MDD treated with escitalopram for approximately 6 weeks (36). Patients with MDD have lower KYNA levels, in which only one of the 73 metabolites is detected, and lower KYNA levels are associated with better treatment responsiveness to escitalopram (36). Patients with MDD, especially the less personality-biased group,

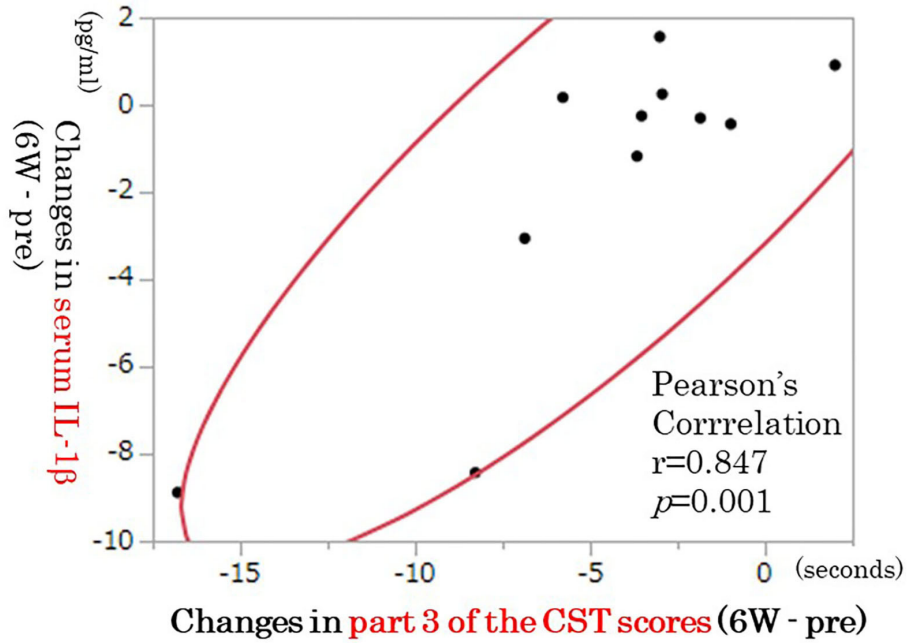

FIGURE 2 | Correlation between changes in IL-1β and part 3 of the CST scores ($n = 11$). The decrease in IL-1β was correlated with improvement in part 3 of the CST scores ($p = 0.001$, $n = 11$). IL, interleukin; CST, color Stroop test.

showed significantly lower levels of plasma metabolites in the TRP pathway, including TRP, serotonin, and KYN (37). Antidepressant treatment may affect plasma levels of KYN-related metabolites. Antidepressant treatment has been reported to be likely to normalize KYN pathway dysfunction both in preclinical and clinical trials (38). Eskelund et al. found that vortioxetine reduced quinolinic acid levels in many brain regions in both genetic rat models and mouse models, with increased inflammation-induced depression-like behavior (39).

Electroconvulsive therapy (ECT) is an effective treatment for TRD, and KP is involved in the therapeutic mechanism of ECT. Several recent studies have investigated the changes in KYN metabolism during ECT in patients with MDD. Guloksuz et al. showed that treatment with ECT increases KYNA levels and KYN/TRP, KYNA/KYN, and KYNA/3-hydroxyquinurenin ratios during ECT and up to 6 weeks after the last ECT (40). In a study by Schwieler et al. ECT significantly reduced the plasma levels of TRP, KYN, and quinolinic acid, but did not change the plasma levels of KYNA (41). Electroconvulsive therapy treatment significantly reduces the quinolinic acid/KYNA ratio (41). In a study by Ryan et al. an increase in KYN, 3-hydrochianthranilic acid, 3-hydroxyquinurenin, quinolinic acid, and KYN/TRP ratios correlated with improved mood scores after ECT in a subgroup of patients with MDD (42). Aarsland et al. found that ECT treatment increased the levels of neopterin, an inflammatory marker, and 3-hydrochianthranilic acid and picolinic acid, putative neuroprotective KYNs in patients with MDD (43).

There have been no metabolomic studies of TRP metabolites for rTMS treatment in patients with TRD. We demonstrated that plasma TRP levels were significantly increased and plasma serotonin levels were significantly decreased in TRD patients after 6 weeks of rTMS treatment, but plasma KYN and kynurenic acid levels and KYN/TRP ratios were not altered (31). The increase in melatonin levels and improvement in categories achieved of the WCST and deterioration in part 1 of the CST scores showed significant correlations (31). Although rTMS increased serum TRP levels, the extent to which rTMS improves cognitive function through changes in TRP metabolites requires further study with an increased sample size.

Elevated peripheral pro-inflammatory cytokines, such as IL-1β, are observed in some subgroups of patients with MDD (44, 45). Inflammatory cytokines such as IL-1β can ultimately increase KYN formation through the activation of indoleamine 2,3-dioxygenase, a TRP-degrading enzyme (46). Kynurenines play an important role not only in immunomodulation but also in the pathology of various diseases including depression (47, 48). The effect of rTMS on IL-1β may also affect KP. In future studies, inflammatory cytokines involved in KP should be investigated.

rTMS AND WHITE MATTER INTEGRITY

The relationship between peripheral pro-inflammatory markers, such as IL-1β, IL-6, TNF-α, and CRP, in patients with MDD and functional and structural neuroimaging markers in magnetic resonance imaging (MRI) is being increasingly investigated (49). Functional MRI studies have shown a correlation between blood levels of pro-inflammatory markers and abnormal activation

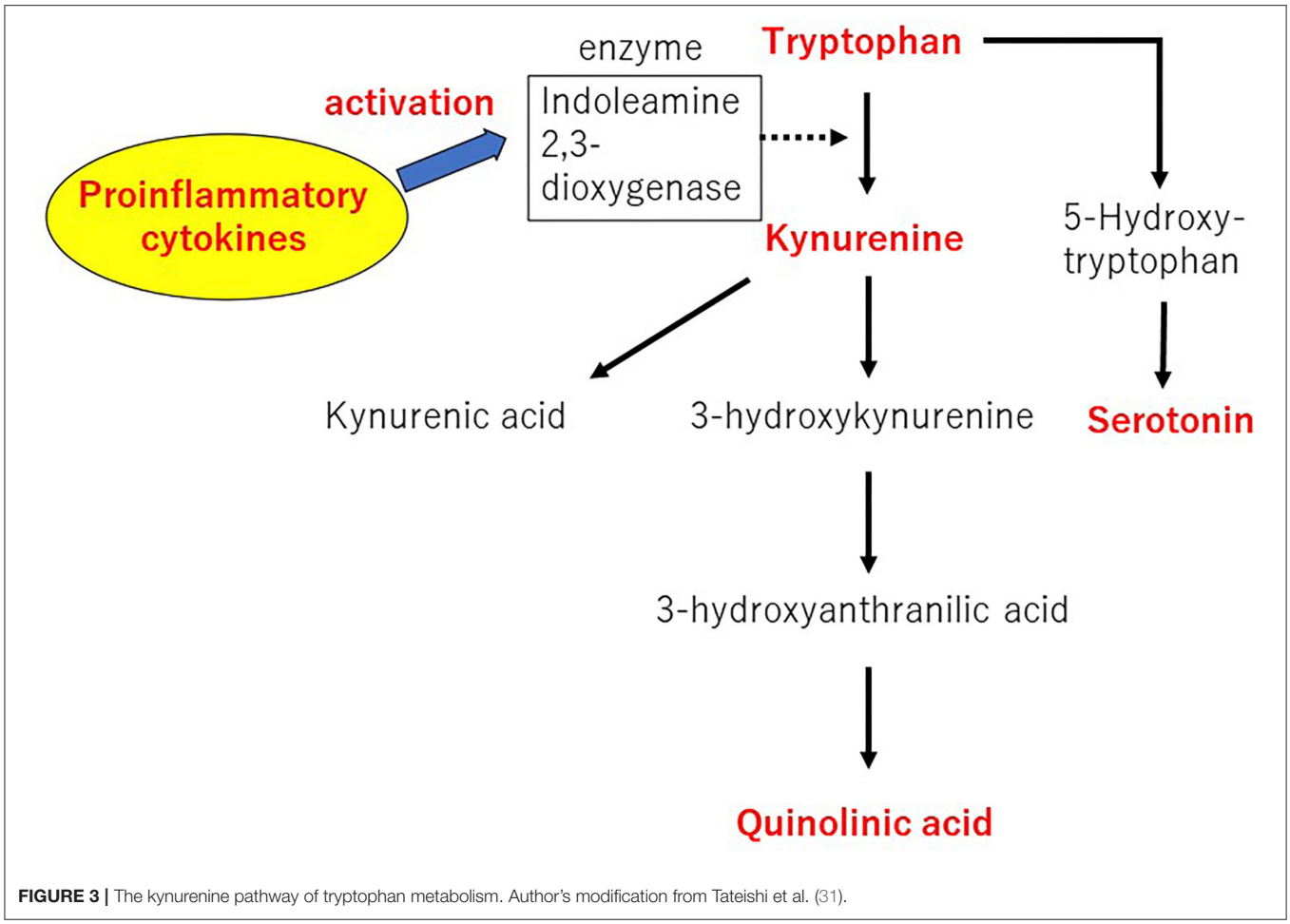

FIGURE 3 | The kynurenine pathway of tryptophan metabolism. Author's modification from Tateishi et al. (31).

patterns and functional connectivity changes in neural circuits involved in cognitive control, emotional regulation, and reward processing in depression (49). Structural MRI studies have shown correlations between blood levels of pro-inflammatory markers and cortical thinning, decreased cortical gray matter and subcortical volume, and decreased integrity of the white matter tract in neural circuits associated with patients with MDD (49).

To date, only a few studies have investigated the association between rTMS-induced changes in the white matter microstructure and therapeutic response (50, 51). Kozel et al. reported that active stimulation of rTMS did not cause harmful damage to the white matter compared to the sham stimulation group (50). Peng et al. reported that rTMS significantly reduced fractional anisotropy (FA) in the left middle frontal gyrus in patients with TRD using a voxel-based analytical method (51). Active rTMS treatment significantly improved this reduction of FA, but sham rTMS treatment did not.

We suggest that rTMS significantly improves depressive symptoms and some cognitive dysfunction (category in WFT and part 3 of CST scores) in patients with TRD (52). Repetitive transcranial magnetic stimulation treatment improved the category in WFT and part 3 of the CST scores independently of the improvement of depressive symptoms (52). We demonstrated that the amelioration of cognitive dysfunction induced by rTMS is not associated with increased white matter integrity (FA value) induced by rTMS in patients with TRD (52). Although rTMS affects the FA values of the cerebral white matter in patients with depression, further research is needed on how it is related to the improvement of cognitive function with a larger sample size.

Inflammatory cytokines, such as IL-1β, are histologically damaging to oligodendrocytes and can lead to histological changes such as white matter lesions found in patients with depression (53, 54). The effect of rTMS on IL-1β may affect white matter integrity, and further studies are needed to examine the association between inflammatory cytokines, including IL-1β, and cerebral white matter integrity. Cognitive dysfunction in MDD may be associated with aberrant functional connectivity in default mode network and executive control network using resting-state fMRI (55). Meta-analysis revealed that clinical response to rTMS treatment, ECT, transcutaneous vagus nerve stimulation, cognitive behavioral therapy, and pharmacotherapy could be predicted by baseline default mode network connectivity in patients with depression (56). The

rTMS treatment had larger effect size compared to other treatment strategies. It is possible that the cognitive function improving effect of rTMS directly or indirectly affects the brain network.

CONCLUSIONS AND FUTURE PERSPECTIVES

Our latest research results and relevant literature suggest that the therapeutic mechanism of rTMS in cognitive dysfunction of depression could be related to neuroinflammatory processes mainly mediated by the pro-inflammatory cytokine IL-1β. We make assertions based on correlational data more than clearly defined causative interactions, which we believe warrants further study. Electroconvulsive therapy alters various cytokines in patients with MDD and is associated with changes of affective states such as depressed mood (57). Comparison of rTMS and ECT with respect to the therapeutic mechanism is necessary for future research. Whether the therapeutic mechanism of rTMS based on the neuroinflammatory hypothesis is essential not only for MDD but also for other psychiatric disorders such as obsessive-compulsive disorder and schizophrenia, further research is needed. Future studies on the synergistic effects of neuromodulation such as rTMS and pharmacologic approaches such as SSRIs and anti-cytokine drugs are useful.

AUTHOR CONTRIBUTIONS

HT and AM drafted and revised the manuscript, respectively. YM conducted a literature review under the supervision of HT and AM. All authors have approved the final submitted manuscript.

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Differential Sphingosine-1-Phosphate Receptor-1 Protein Expression in the Dorsolateral Prefrontal Cortex Between Schizophrenia Type 1 and Type 2

Ganesh B. Chand[1†], Hao Jiang[1†], J. Philip Miller[2], C. Harker Rhodes[3‡], Zhude Tu[1‡] and Dean Foster Wong[1,4‡]*

[1] Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, MO, United States, [2] Division of Biostatistics, Washington University School of Medicine, St. Louis, MO, United States, [3] NeuroDex Inc., Natick, MA, United States, [4] Department of Psychiatry, Neuroscience, and Neurology, Washington University School of Medicine, St. Louis, MO, United States

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***Correspondence:**
Ganesh B. Chand
gchand@wustl.edu;
ganeshchand64@gmail.com

†These authors share first authorship
‡These authors have contributed equally to this work

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Understanding the etiology and treatment approaches in schizophrenia is challenged in part by the heterogeneity of this disorder. One encouraging progress is the growing evidence that there are subtypes of schizophrenia. Recent *in vitro* findings of messenger ribonucleic acid (mRNA) gene expression on postmortem dorsolateral prefrontal cortex (DLPFC) showed that schizophrenia has two subtypes, those with a relatively normal DLPFC transcriptome (Type 1) and those with differentially expressed genes (Type 2). Sphingosine-1-phosphate receptor-1 (S1PR1) is one of the genes that was highly upregulated in Type 2 compared to Type 1 and controls. The impact of that finding is limited because it only can be confirmed through analysis of autopsy tissue, and the clinical characteristics such as symptoms severity or illness duration except for cause of death was not available from that Medical Examiner based autopsy study. However, S1PR1 has great potential because it is a target gene that can be accessed *via* positron emission tomography (PET) *in vivo* using specific radioligands (starting with [^{11}C]CS1P1) successfully developed at our center in human brain imaging. As a preliminary study to validate this PET target in schizophrenia, S1PR1 protein expression was assessed by receptor autoradiography (ARG) using [^{3}H]CS1P1 and immunohistochemistry (IHC) in the DLPFC from patients with schizophrenia classified as Type 1 or Type 2 based on their DLPFC transcriptomes and from controls. Our analyses demonstrate that ARG S1PR1 protein expression is significantly higher in Type 2 compared to Type 1 ($p < 0.05$) and controls ($p < 0.05$), which was consistent with previous mRNA S1PR1. These findings support the possibility that PET S1PR1 can be used as a future imaging biomarker to distinguish these subgroups of schizophrenic patients during life with obvious implications for both patient management and the design of clinical trials to validate novel pharmacologic therapies.

Keywords: autoradiography, schizophrenia, sphingosine-1-phosphate receptor-1 (S1PR1), postmortem brain tissues, molecular imaging, neuroimaging

INTRODUCTION

Schizophrenia is a neuropsychiatric condition that currently affects ∼3 million people in the United States and ∼7.8 billion people worldwide (1–3). Individuals with schizophrenia exhibit highly heterogeneous genetic profiles (4), clinical symptoms (5), illness course (6, 7), treatment response (8, 9), and neuroimaging markers (10–12). Despite extensive efforts, understanding schizophrenia mechanisms remains challenging. Even though typically in mRNA studies one generally looks at gene expressions in context and examine a large number of dysregulated mRNAs that are involved in mitochondrial and proteasome functions, the direct link between these functions and schizophrenia mechanisms/features remain unclear (13, 14). While decreases in Cannabinoid (CB) mRNA that targets GABA interneurons were previously reported in schizophrenia (15), OMAR CB1 PET radioligand showed elevations in the same schizophrenia tissues (16) suggesting that this strategy is not as fruitful in developing schizophrenia treatment. Recent findings by Bowen et al. (17) suggest that the mRNA expression can be used to divide schizophrenic patients into two types, Type 1 schizophrenia patients with an essentially normal transcriptome in their dorsolateral prefrontal cortex (DLPFC) and Type 2 schizophrenia patients with hundreds of differentially expressed genes in their DLPFC. Although there are many highly upregulated interesting genes including S1PR1 ($p < 10^{-15}$; after multiple comparisons) in Type 2 (17), S1PR1 is only one that has currently been developed and the radioligands for the other target genes are not available yet. Additionally, S1PR1 radioligand has gained significant interest for *in vivo* targeted imaging of inflammation in brain diseases, with the recent FDA-approved S1PR1-based treatments such as Fingolimod, Siponimod, and Ozanimod (18) for multiple sclerosis.

However, a serious limitation of mRNA expression studies like that of Bowen et al. (17) is that they require brain tissue which is generally not available except at autopsy. Fortunately, PET ligands for S1PR1 have been recently developed at Washington University School of Medicine (19–24). The present study examines the differential expression of S1PR1 in the DLPFC of Type 1 and Type 2 schizophrenic patients at the protein level as a preliminary step toward the use of PET to distinguish Type 1 from Type 2 schizophrenia during life. We performed ARG and IHC analyses in DLPFC tissues of controls, Type 1 and Type 2 schizophrenic patients. Since ARG is a more accurate quantitative method compared to IHC (25, 26), ARG was used for S1PR1 quantitation while IHC was used only to confirm ARG signals. We hypothesized that S1PR1 protein expression will show elevations in Type 2 schizophrenia compared to Type 1 schizophrenia and controls consistent with Bowen's S1PR1 mRNA findings (17).

MATERIALS AND METHODS

Human Brain Tissues

Human brain tissues were obtained from the Human Brain Collection Core at the National Institute of Mental Health. These tissues corresponded to the same tissues studied by Bowen et al. (17) because any other tissues from different sources would be difficult to distinguish Type 1 and Type 2 schizophrenia. Tissues were used in accordance with the guidelines of Washington University in St. Louis. All samples were stored at −80°C at the Washington University's Radiology labs until used.

In vitro Immunohistochemistry Staining Study

In vitro IHC staining of S1PR1 was carried out in frozen sections from human DLPFC. All sections were pre-warmed at room temperature (RT) for 5 min and then fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min at RT, washed 3 times in PBS, and then heated in boiling water bath in antigen retrieval buffer for 30 min. Sections were then rinsed with PBS and blocked with 5% horse serum for 1 h at RT. After that, all sections were stained with anti-S1PR1 antibody (Alomone, Jerusalem, Israel) overnight at 4°C, washed and followed by incubation with ImmPRESS HRP Horse anti-rabbit polymer for 1 h at RT, and developed using ImmPACT DAB (Vector Laboratories, Burlingame, CA). Hematoxylin and eosin (H&E) staining was also performed in adjacent slides to identify gray and white matters in the brain.

In vitro Autoradiography Study

In vitro ARG study was carried out in frozen sections from human DLPFC using [^{3}H]CS1P1 for S1PR1 receptor protein. Sections were pre-warmed to RT, and then incubated with Hank's balanced salt solution (HBSS) buffer containing 10 mM HEPES, 5 mM $MgCl_2$, 0.2% BSA, and 0.1 mM EDTA at pH 7.4 for 5 min at RT in a coplin jar. All sections were then incubated with 0.5 nM [^{3}H]CS1P1 for 30 min in a coplin jar with gentle shaking at RT. After that, all sections were washed with a buffer for 3 min for three times, and then rinsed in ice-cold H_2O for 1 min and air dried overnight. Slides were incubated with Carestream BioMax Maximum Sensitivity ARG film (Carestream, Rochester, NY) in a Hypercassette ARG cassette (Cytiva, Amersham, UK) for 30 days along with an ART-123 Tritium Standards (American Radiolabeled Chemicals, St Louis, MO). The film was processed using a Kodak film developer (Kodak, Rochester, NY). To determine the non-specific binding, 10 μM of S1PR1 specific antagonist NIBR-0213 (Cayman, Ann Arbor, MI) was introduced and incubated with samples as described above. The image was processed and analyzed using Fiji ImageJ. Brain regions of interest (ROIs) were selected from the ARG images according to the hematoxylin staining in the adjacent slide. ROIs were randomly selected from different regions of the DLPFC gray matter, and the intensity was measured and calculated in fmol/mg.

Statistical Analysis

In statistical analysis, we fitted a mixed repeated measure model to take into account the triplicate measure variability as well as the interpersonal variability (within group). In mixed model analysis of variance (ANOVA), the groups were used as a fixed effect. The *F*-test for the null that all three groups have the same mean was tested. To compare between groups, a one-tailed *t*-test was used. A $p \leq 0.05$ was considered statistically

TABLE 1 | Human DLPFC tissues used in this study from normal controls, schizophrenia Type 1 and Type 2 patients.

Normal				Schizophrenia Type 1				Schizophrenia Type 2			
Subject	**Sex**	**Race**	**Age**	**Subject**	**Sex**	**Race**	**Age**	**Subject**	**Sex**	**Race**	**Age**
1	M	CAUC	54	1	M	AA	60	1	F	AA	63
2	F	AA	64	2	M	AA	53	2	M	CAUC	48
3	M	AA	57	3	M	CAUC	59	3	M	AA	53
4	M	AA	64	4	M	CAUC	66	4	M	AA	63
5	M	CAUC	49	5	M	CAUC	44	5	M	CAUC	39
6	M	CAUC	51								
7	M	AA	58								
8	M	CAUC	32								
9	M	AA	60								
10	M	CAUC	63								

F, Female; M, Male; AA, African American; CAUC, Caucasian; Age in years.

***Normal subjects: Cause of death (Manner of death)**.*

Subject 1: Hypertensive and arteriosclerotic cardiovascular disease (Natural).

Subject 2: Cardiac tamponade due to acute dissecting aneurysm of aortic arch due to atherosclerotic hypertensive cardiovascular disease (Natural).

Subject 3: Heat exposure associated with pulmonary emphysema (Accident).

Subject 4: Acute pulmonary thromboembolism due to deep venous thrombosis due to immobility following right inguinal herniorrhaphy (Natural).

Subject 5: Cardiomyopathy (Natural).

Subject 6: Acute myocardial infarction due to atherosclerotic cardiovascular disease (Natural).

Subject 7: Poorly differentiated pulmonary adenocarcinoma (Natural).

Subject 8: Cardiac arrest of undetermined etiology during repair of paraesophageal hernia (Natural).

Subject 9: Acute myocardial infarction, secondary to severe atherosclerotic heart disease (Natural).

Subject 10: Massive spinal cord injury (Accident).

***Schizophrenia Type 1 subjects: Cause of death (Manner of death)**.*

Subject 1: Hypertensive and atherosclerotic cardiovascular disease (Natural).

Subject 2: Non-small cell carcinoma of lung with erosion of primary branch of pulmonary artery (Natural).

Subject 3: Multiple blunt force injuries (Suicide).

Subject 4: Atherosclerotic cardiovascular disease (Natural).

Subject 5: Myocardial infarction (Natural).

***Schizophrenia Type 2 subjects: Cause of death (Manner of death)**.*

Subject 1: Hypertensive cardiovascular disease (Natural).

Subject 2: Drowning (Accident).

Subject 3: Acute bronchopneumonia due to hypertensive and atherosclerotic cardiovascular disease (Natural).

Subject 4: Atherosclerotic hypertensive cardiovascular disease (Natural).

Subject 5: Combined drug poisoning (clozapine and sertraline) (Accident).

significant. Statistical calculations were performed with PROC MIXED in SAS 9.4.

RESULTS

Postmortem Human Subjects

DLPFC tissues from 20 human subjects including 10 neurologically normal controls, five Type 1 schizophrenia subjects, and five Type 2 schizophrenia subjects were used in this study (**Table 1**). Mean age (standard deviation) was 55.20 years (9.69) of normal controls, 56.40 years (8.32) of Type 1 schizophrenia, and 53.20 years (10.26) of Type 2 schizophrenia subjects. There was one female in controls, none in Type 1 schizophrenia, and one in Type 2 schizophrenia. There were five Caucasians and five African-Americans in controls, three Caucasians and two African-Americans in Type 1 schizophrenia, and two Caucasians and three African-Americans in Type 2 schizophrenia. Age, sex, and race were not significantly different among groups. Each subject's cause of death and manner of death are also included in the footnote of **Table 1**.

Immunostaining of S1PR1

Immunostaining of S1PR1 was performed in control and schizophrenia samples (**Figures 1**, **2**). In general, S1PR1 was mainly expressed in the gray matter of the DLPFC. In particular, the expression of S1PR1 was relatively high in the outer granular layer, outer pyramidal layer, inner granular layer, inner pyramidal layer, and multiform layer with very low to no amount in the molecular layer of gray matter and white matter.

Autoradiography of S1PR1 Specific [^{3}H]CS1P1

ARG analysis of S1PR1 was performed in control and schizophrenia DLPFC samples.

The distribution pattern of [^{3}H]CS1P1 matched well with immunostaining analysis using S1PR1 specific antibody, indicating [^{3}H]CS1P1 is specific to S1PR1 in postmortem human tissues (**Figures 2**, **3**). Similar to S1PR1 immunostaining analysis, S1PR1 specific [^{3}H]CS1P1 was mainly distributed in the gray matter of DLPFC, with no to very low amount of [^{3}H]CS1P1

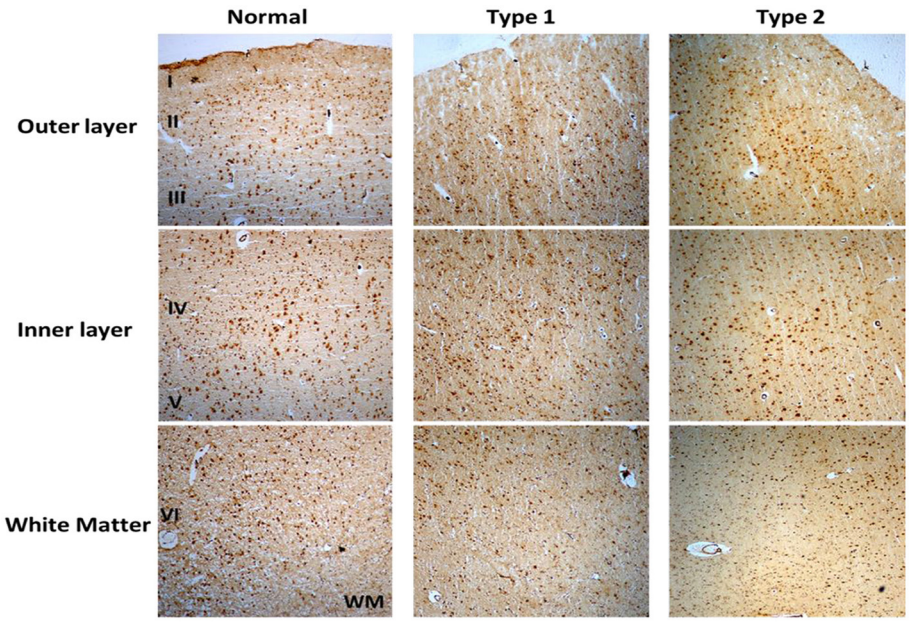

FIGURE 1 | Immunohistochemistry (IHC) of S1PR1 in postmortem DLPFC tissues from the representative normal control and schizophrenia Type 1 and Type 2.

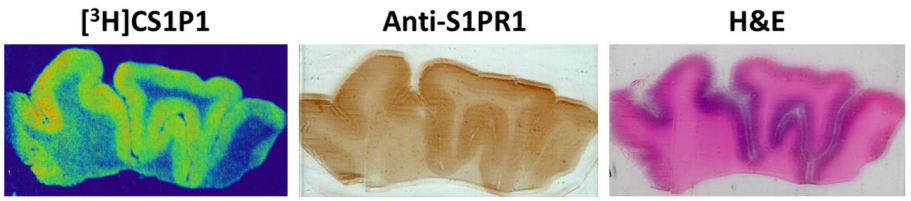

FIGURE 2 | Representative images of [^{3}H]CS1P1 autoradiograph, S1PR1 immunostaining, and Hematoxylin and eosin (H&E) staining in postmortem human DLPFC tissues. The distribution of [^{3}H]CS1P1 matched well with anti-S1PR1 antibody, and was mainly located in the gray matter regions as indicated in the H&E staining.

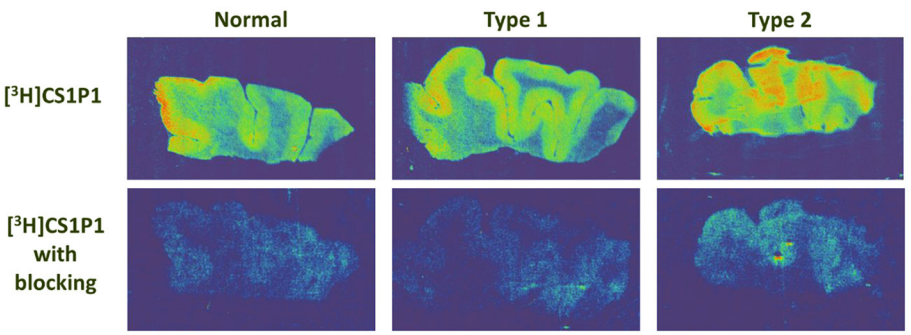

FIGURE 3 | Autoradiography images of S1PR1 using [^{3}H]CS1P1 in postmortem DLPFC tissues from representative normal control, schizophrenia Type 1, and schizophrenia Type 2. In general, [^{3}H]CS1P1 was higher in Type 2 schizophrenia subjects compared with normal control and Type 1 schizophrenia subjects.

distributed in the white matter region. In addition, blocking study using S1PR1 antagonist NIBR-0213 showed significant reduction of [^{3}H]CS1P1 indicating the [^{3}H]CS1P1 is specific to S1PR1 (**Figure 3**). Compared with IHC analysis, ARG provides both quantification and localization of radioligand at the same time in distinct anatomical structures, and enables us to quantify the absolute amount of [^{3}H]CS1P1 in control and different types of schizophrenia subjects.

ARG S1PR1 intensity in the DLPFC was compared between the groups. In general, the intensity of [^{3}H]CS1P1 was higher in

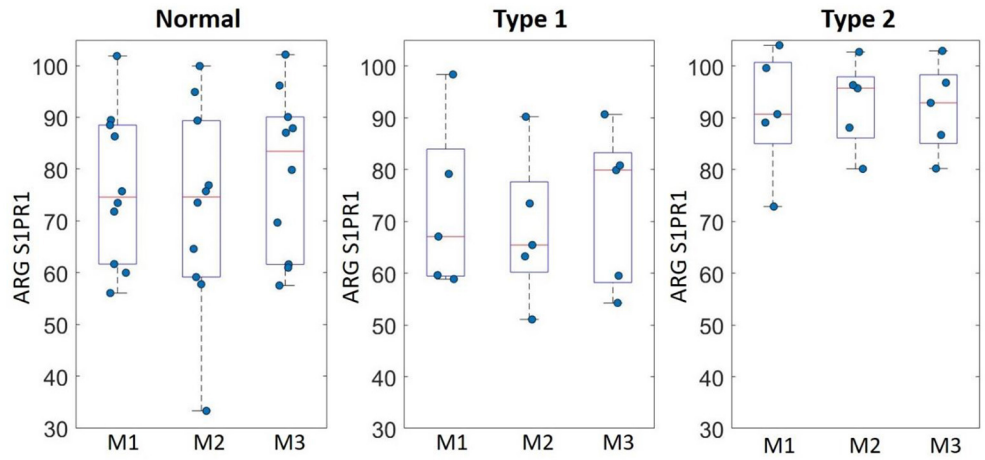

FIGURE 4 | ARG S1PR1 intensity expression (fmol/mg) triplicate measures (M1, M2, and M3) in the DLPFC from normal controls, schizophrenia Type 1, and schizophrenia Type 2.

Type 2 schizophrenia subjects compared with normal control and Type 1 schizophrenia subjects (**Figure 3**).

ARG S1PR1 intensity expressions were measured at three randomly selected ROIs within the DLPFC in all subjects. These ARG S1PR1 triplicate measures from normal controls, Type 1 schizophrenia, and Type 2 schizophrenia are shown in **Figure 4**. AGR S1PR1 is highly expressed in Type 2 schizophrenia in all three measures compared to Type 1 schizophrenia and controls. The *F*-test for the null that all three groups have the same mean was $F_{(2,17)} = 3.49$, $p = 0.05$. Overall ARG S1PR1 intensity mean (standard error) was 76.10 (4.66) for controls, 71.44 (5.87) for Type 1 schizophrenia, and 91.91 (5.87) for Type 2 schizophrenia (**Figure 5**). ARG S1PR1 expression was significantly higher in Type 2 schizophrenia compared to controls ($t = 2.20$, $p = 0.021$) and Type 1 schizophrenia ($t = 2.47$, $p = 0.012$), but there was no difference between controls and Type 1 schizophrenia ($t = 0.65$, $p = 0.525$).

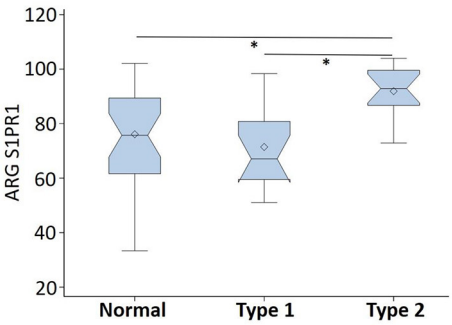

FIGURE 5 | ARG S1PR1 intensity expression (in fmol/mg) comparison between normal controls, schizophrenia Type 1, and schizophrenia Type 2 (*$p < 0.05$).

DISCUSSION

In this study, we evaluated the expression of S1PR1 and distribution of S1PR1 specific tracer [^{3}H]CS1P1 in human DLPFC tissues from normal control, Type 1 and Type 2 schizophrenia subjects. Our data showed, in the DLPFC of humans, S1PR1 is highly expressed in the gray matter region with much lower expression in the white matter regions. Similar to the immunostaining study, ARG analysis using [^{3}H]CS1P1 showed a relatively high tracer uptake in the gray matter of DLPFC whereas no to very low amount of [^{3}H]CS1P1 was identified in the white matter region. Tracer uptake in the Type 2 schizophrenia samples was significantly higher than the Type 1 schizophrenia samples and normal controls.

The present ARG findings are consistent with a previous study in other regions of the human brain (27) that suggests S1PR1 is mainly localized in gray matter and confirmed the specificity of our S1PR1 specific radioligand [^{3}H]CS1P1. This study extends the previous findings (17) by demonstrating that S1PR1 protein as well as mRNA is differentially expressed in the DLPFC of Type 2 schizophrenic patients. The trend of tracer uptake between control and all schizophrenia subjects was similar to previous findings using RT-PCR (28). For the two types of schizophrenia patients, it appeared that only Type 2 schizophrenia has significantly upregulated S1PR1 compared to Type 1 schizophrenia and controls, respectively. Thus, the present ARG findings and previous reports taken together indicate that both S1PR1 protein and S1PR1 mRNA upregulate in DLPFC only in a subset of schizophrenia.

S1PR1 is localized in astrocytes (27), the most abundant glial cells in the brain. Prior study from our group (19) shows S1PR1 localization in astrocytes (interlaminar) and microglia. S1PR1 involvements have been suggested in astrocyte morphogenesis

and in bi-directional communications between astrocytes and neurons (29). Our results using both immunostaining and ARG indicated that S1PR1 is highly expressed in the gray matter compared to white matter, which was consistent with previous studies in other brain regions (27). Since frozen DLPFC tissues were used, the immunostaining-based morphology will not be well-suited compared to fixed tissues. Present immunostaining results indicate S1PR1 looks like in astrocytes, but fixed tissues will need to be studied in future for S1PR1 cellular localization. Both IHC and ARG confirmed S1PR1 signals at macro level, but only ARG was used for S1PR1 quantitation as suggested previously (25, 26). The present results will be important for the future schizophrenia subtype-based studies with *in vivo* PET [^{11}C]CS1P1. The [^{11}C]CS1P1 has recently emerged as a promising radiotracer for *in vivo* PET imaging of neuro-inflammation (19, 21–23). Neuro-inflammation has been associated with schizophrenia throughout the literature (30, 31). This has included elevated cytokines (32, 33) that may be due to activated microglia. The neuro-inflammatory observation seems to be most associated with early illness stages and patients with acute psychosis symptom exacerbations (34). S1PR1 PET will potentially complement for investigating neuro-inflammation in schizophrenia with other currently used ligands, such as Translocator protein (18 kDa) (TSPO) PET (35–38). Future studies will be needed for the direct comparison of S1PR1 and TSPO. Previous literature and our present findings taken together thus suggest that there is great potential for studying neuro-inflammation (or related mechanisms) in schizophrenia with *in vivo* S1PR1 PET. If S1PR1 PET reveals biologically distinct subtypes of schizophrenia, the clinical relevance is that PET study can be used to stratify patient cohorts in therapeutic trials of new drugs. The implications for neurogenetic and epidemiologic studies are equally obvious. The only limitation is the cost of PET, especially for bigger sample studies. Modulators targeting S1PR1 are already FDA-approved therapeutics of treating multiple sclerosis (18, 39), which could further accelerate its important role for treatment development in schizophrenia.

Limitations

One limitation is that present study as well as previous S1PR1 mRNA study (17) cannot be used to identify schizophrenia Type 1 and Type 2 *in vivo* without PET and S1PR1 specific radiotracer. However, there is mounting evidence that schizophrenia has two neuroanatomical types *in vivo* (40). This recent study by Chand et al. (40) can potentially be used to divide schizophrenia patients into types *in vivo* using structural MRI. To evaluate whether volumetric MRI-based schizophrenia subtypes (40) and S1PR1-based schizophrenia subtypes map each other, the combined *in vivo* MRI and S1PR1 PET studies will be needed in future. We also acknowledge that the sample size is relatively small for this study, and the future studies should focus on replicating these findings in larger samples. However, we were limited by the available tissues in the HBCC NIMH brain bank that corresponded to the same tissues studied by Bowen et al. (17), and any other tissues would be difficult to distinguish Type 1 and Type 2 schizophrenia. Another limitation of our study is that clinical characteristics such as symptoms severity and illness duration were not available and the relationships between these variables and subtypes remain unknown. Lastly, it remains to be investigated S1PR1 protein and S1PR1 mRNA expressions in other brain regions besides DLPFC of controls and Type 1 and Type 2 schizophrenia patients.

Conclusions

The present study evaluated DLPFC postmortem tissues from controls, schizophrenia Type 1 and Type 2, demonstrated S1PR1 protein is highly expressed in gray matter region, and most importantly showed that only Type 2 schizophrenia has upregulated S1PR1 protein expression in line with previous S1PR1 mRNA results. Overall, these findings strongly suggest S1PR1 might serve as a candidate target in schizophrenia subtypes with PET where protein is the target.

DATA AVAILABILITY STATEMENT

Human brain tissue data used in this study are publicly available from the Human Brain Collection Core (HBCC) at the National Institute of Mental Health (NIMH) following the data request procedure. The original data presented in the study are included in the article, and further inquiries can be directed to the corresponding author (GC).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Brain Collection Core (HBCC) at the National Institute of Mental Health (NIMH). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GC, CR, and DW conceived the project. HJ and ZT performed autoradiography and immunohistochemistry experiments. JM and GC performed data analyses. GC wrote the initial manuscript draft. GC, HJ, JM, CR, ZT, and DW critically reviewed and revised the manuscript draft, and approved the final version. All authors contributed to the article and approved the submitted version.

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Microglial Inflammatory-Metabolic Pathways and Their Potential Therapeutic Implication in Major Depressive Disorder

Reza Rahimian[1†], Claudia Belliveau[1,2†], Rebecca Chen[1,2] and Naguib Mechawar[1,2,3]*

[1] Douglas Mental Health University Institute, McGill Group for Suicide Studies, Verdun, QC, Canada, [2] Integrated Program in Neuroscience, McGill University, Montreal, QC, Canada, [3] Department of Psychiatry, McGill University, Montreal, QC, Canada

Increasing evidence supports the notion that neuroinflammation plays a critical role in the etiology of major depressive disorder (MDD), at least in a subset of patients. By virtue of their capacity to transform into reactive states in response to inflammatory insults, microglia, the brain's resident immune cells, play a pivotal role in the induction of neuroinflammation. Experimental studies have demonstrated the ability of microglia to recognize pathogens or damaged cells, leading to the activation of a cytotoxic response that exacerbates damage to brain cells. However, microglia display a wide range of responses to injury and may also promote resolution stages of inflammation and tissue regeneration. MDD has been associated with chronic priming of microglia. Recent studies suggest that altered microglial morphology and function, caused either by intense inflammatory activation or by senescence, may contribute to depression and associated impairments in neuroplasticity. In this context, modifying microglia phenotype by tuning inflammatory pathways might have important translational relevance to harness neuroinflammation in MDD. Interestingly, it was recently shown that different microglial phenotypes are associated with distinct metabolic pathways and analysis of the underlying molecular mechanisms points to an instrumental role for energy metabolism in shaping microglial functions. Here, we review various canonical pro-inflammatory, anti-inflammatory and metabolic pathways in microglia that may provide new therapeutic opportunities to control neuroinflammation in brain disorders, with a strong focus on MDD.

Keywords: microglia, neuroinflammation, metabolic pathway, major depressive disorder, anti-inflammatory pathway, pro-inflammatory pathway, microglial pathways as therapeutic targets

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***Correspondence:**
Naguib Mechawar
naguib.mechawar@mcgill.ca

[†]These authors share first authorship

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INTRODUCTION

An association between inflammation and major depressive disorder (MDD) has long been hypothesized based on investigations using various approaches. Studies have reported elevated levels of both peripheral (1) and central (2–7) pro-inflammatory cytokines in depressed patients, supporting the hypothesis of an immune-mediated etiology of MDD (8–10). Indeed, subsets of

MDD patients have increased concentrations of circulating cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 (11) and increased expression of innate immune-related genes in the blood. Also, bidirectional relationships between depression and inflammatory or autoimmune disorders exist. Namely, there is a high incidence of co-morbid inflammatory disease and rheumatoid arthritis (RA) in MDD patients (12).

One of the most important cell types involved in regulating neuroinflammation are microglia, which can modulate immunological responses and play a fundamental role in maintaining homeostatic brain functions. The implication of microglia in normal brain physiology includes, but is not limited to, synaptic pruning, phagocytosis, oligodendrocyte maturation and neurogenesis (10, 13). Functionally, microglia are one of the most diverse cell types in the central nervous system (CNS), as they dynamically adapt, at both the cellular and molecular levels, to their ever-changing environment (10, 14). The heterogeneous nature of microglia has been highlighted by high-throughput approaches like single cell RNA-sequencing. Indeed, factors such as brain region, sex, age and type of pathology can significantly affect microglial phenotype, including gene expression signatures and secretory profiles (15, 16). Under physiological and pathological conditions, microglia display spatial heterogeneity in density, morphology, turnover rate, pruning, metabolism and molecular signature (17–19).

Microglial activation occurs through inflammatory insult or slight alterations in brain homeostasis. This activation is dependent on the context and the type of stressor or pathology. Microglia determine the pathological outcome of stressors through secretion of cytokines, chemokines and growth factors and psychopathologies have repeatedly been associated with long-lasting priming and sensitization of cerebral microglia (10, 20, 21). Microglia also modulate communication between the nervous and the immune system in response to different physiological, psychological and immunological stressors. They are in fact considered to be responsible for the decreased neuroplasticity observed in depression (22) and recent findings have associated microglial abnormalities with neuropsychiatric disorders such as MDD, which have been termed microgliopathies by some (23–25).

Manipulating the microglial phenotype is an intriguing strategy for developing new therapeutics for MDD. Particular attention is currently given to exploiting alternative microglial polarization as a potential therapeutic option in a wide range of neurological and neuropsychiatric disorders (15). To achieve this, canonical pathways that govern tuning of the microglial phenotype have been investigated including transforming growth factor β (TGF-β), IL-4 receptor and peroxisome proliferator-activated receptors-gamma (PPAR-γ) (26, 27). We and others previously published reviews on the roles played by microglia in psychiatric disorders (10, 13, 21, 28). However, a review focused on different canonical microglial pro-inflammatory, anti-inflammatory and metabolic pathways and their translational value in drug discovery for MDD is lacking. We aim to fill the gap.

ROLE OF NEUROINFLAMMATION IN PSYCHOPATHOLOGY

The role(s) played by the innate immune response in MDD has been the object of several experimental and clinical studies in which microglia have increasingly become the focus of investigation. Microglia have been studied in different psychiatric disorders using various approaches, including postmortem investigations in humans as well as experimental studies in animal models (21, 29, 30). The term neuroinflammation, denoting inflammatory processes in the CNS, is a rather general notion that could include both peripheral and central components of inflammation. Interestingly, a number of studies have suggested the involvement of peripheral inflammation in the pathogenesis of depression. Menard et al. associated a reduced expression of the endothelial cell tight junction protein claudin-5 with abnormal blood vessel morphology in the nucleus accumbens of stress-susceptible but not resilient mice (31). In a more recent study, the same group showed that epigenetic regulation of claudin-5 is associated with stress resilience. Indeed, they identified nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signaling pathway and histone deacetylase 1 as mediators of stress susceptibility. Pharmacological inhibition of histone deacetylase 1 rescued claudin-5 expression in the nucleus accumbens and promoted resilience (32). It is to be noted, however, that blood–brain barrier (BBB) disruption is not a common phenomenon in animal models of depression (31) and that the mechanism underlying this region-specific BBB abnormality remains to be clarified.

Recently, neuroinflammation has been used to explain functional microglial abnormalities observed in psychopathologies. A developed mouse model of obsessive-compulsive disorder serves as an interesting example of the link between microglial abnormalities and mental illness.

Abbreviations: MDD, major depressive disorder; TNF-α, tumor necrosis factor-alpha; IL, interleukin; RA, rheumatoid arthritis; TGF-β, transforming growth factor β; PPAR-γ, peroxisome proliferator-activated receptors-gamma; CNS, central nervous system; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; BBB, blood–brain barrier; CUS, chronic unpredictable stress; TNF-α, tumor necrosis factor-alpha; ACC, anterior cingulate cortex; LPS, lipopolysaccharide; CSD, chronic social defeat; ROS, reactive oxygen species; CUMS, chronic unpredictable mild stress; TLR, toll-like receptor; TNFR1, TNF-α Receptor 1; TNFR2, TNF-α Receptor 2; MAPK, mitogen-activated protein kinases; CSF, cerebrospinal fluid; HPA, hypothalamic-pituitary-adrenal; gp130, glycoprotein 130; RSD, repeated social defeat; IL-6R, IL-6 receptor; DAMPs, danger-associated molecular patterns; MyD88, myeloid differentiation primary response 88; AD, Alzheimer's disease; PFC, prefrontal cortex; IFN-γ, interferon-gamma; JAK, janus kinase; STAT, signal transducer and activator of transcription; ERK, extracellular-signal-regulated-kinase; SSRIs, selective serotonin reuptake inhibitors; ChP, choroid plexus; α7 nAChR, Alpha-7 nicotinic acetylcholine receptor; Nrf2, nuclear factor-erythroid factor 2-related factor 2; CRS, chronic restraint stress; Gal-3, galectin-3; PG, prostaglandin; PPARs, peroxisome proliferator-activated receptors; NMDA, *N*-methyl-D-aspartate; IGF-1, insulin-like growth factor 1; DAM, disease-associated microglia; BDNF, brain-derived neurotrophic factor; endocannabinoid, endogenous cannabinoid; AEA, anandamide; 2-AG, 2-arachidonoylglycerol; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; GPR55, G-protein-coupled receptor 55; FAAH, fatty acid amide hydrolase; COX, cyclooxygenase; MAGL, monoacylglycerol lipase; ATP, adenosine triphosphate; ACEA, arachidonyl-2′-chloroethylamide; mTOR, mammalian target of rapamycin; ADP, adenosine di-phosphate; BBG, Brilliant Blue G; Tf-Fe, transferrin bound iron; Fp1, ferroportin 1; Cp, ceruloplasmin; HepC, hepcidin.

Mutant *HOXB8* mice display unexpected behavior manifested by compulsive grooming and hair removal (33). These actions directly mirror trichotillomania seen in humans with obsessive-compulsive spectrum disorder (33). Chen et al. reported that, in the brain, the *HOXB8* cell lineage exclusively labels bone marrow-derived microglia. This finding strongly fosters the theory that the excessive grooming behavior observed in *HOXB8* mutant mice is a consequence of defective microglia, thus relating hematopoietic function to mouse behavior (33). Another interesting example is the effect of CX3CR1 deficiency on behavior relevant to post-traumatic stress disorder. Neuronal CX3CL1 and its microglial target CX3CR1 play an essential role in synaptic plasticity and a correlation between CX3CR1 deficiency and increased fear behavior as well as an anxiolytic-like phenotype have been reported by Schubert et al. (34). However, Milior et al. observed a contradicting finding in which microglial CX3CR1 knock-out mice were resilient to chronic unpredictable stress (CUS) suggesting microglia-neuron communication may be at the interplay of resilience or susceptibility to a depression-like phenotype (35). Colony-stimulating factor 1 receptor signaling was also shown to control cerebellar microglia and to be essential for motor function and social interaction (36).

IMPORTANCE OF MICROGLIA IN MAJOR DEPRESSIVE DISORDER

Despite a strong correlation between microglial activation and depression in pre-clinical and clinical studies, it remains unclear whether microglial abnormities play a causal role in depression (37). Clinical findings indicate a strong correlation between disease etiology and inflammation (37). Subsets of MDD patients consistently display increased levels of pro-inflammatory cytokines such as TNF-α and IL-6 (11, 30). A previous postmortem investigation by our group has shown that the percentage of primed microglia is increased in the dorsal anterior cingulate cortex (ACC) of depressed suicides compared to matched controls (38). This observation is consistent with independent reports of microglial activation in the ACC of MDD patients (39).

There is a growing body of literature showing increased microglial activation in inflammatory and non-inflammatory rodent models of depression. The most studied inflammatory model is that of lipopolysaccharide (LPS) injection. Systematic LPS administration not only triggers peripheral immune responses but also activates microglia in the brain (37, 40). Following LPS challenge, pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 are upregulated in different brain areas (41, 42). These inflammatory changes are accompanied by decreased sucrose preference and increased immobility in the forced swim test (43). Moreover, animals that receive acute or chronic non-inflammatory stress also show microglial activation along with morphological changes and increased levels of pro-inflammatory cytokine release in different brain regions including the hippocampus, thalamus and prefrontal cortex. For example, mice subjected to chronic social defeat (CSD) exhibit increased numbers of CD68-expressing microglia that have increased phagocytic capacity. Several other groups have reported microglial dysregulation following CSD stress (44–49). Lehmann et al. have reported that depressive-like behavior throughout and following CSD involves microglia-derived reactive oxygen species (ROS). Using colony stimulating factor receptor antagonist PLX5622 to deplete microglia before and during the 14-day CSD procedure, mice were protected from the effects of stress as measured by light/dark and social interaction paradigms (10, 50). More evidence indicating the involvement of microglial activation in depression comes from minocycline studies. Minocycline is an anti-inflammatory tetracycline that inhibits microglial activation and subsequent neuroinflammation (37, 51). Minocycline treatment does not have anti-depressive behavioral effects in naïve mice (52), however, it elicits significant anti-depressive effects in the rat model of chronic unpredictable mild stress (CUMS) (53). Intriguingly, combinatorial therapy of minocycline with antidepressants provides better clinical outcomes in some MDD patients, implying the contribution of neuroinflammation and microglial activation in a subset of patients afflicted with this psychopathology (54).

In the following sections, we aim to highlight the potential roles played by microglia in the pathogenesis of MDD, with a focus on the roles played by the canonical pro-inflammatory, anti-inflammatory and metabolic pathways.

PRO-INFLAMMATORY PATHWAYS IN MICROGLIA

Tumor Necrosis Factor-α Mediated Pathway

TNF-α is a trimeric cytokine that is expressed either in a 27 kDa transmembrane form or a 17 kDa soluble form processed by TNF-α converting enzyme (55–57). TNF-α exerts its pleiotropic effects by binding to two primary receptors: TNF-α Receptor 1 (TNFR1) and TNF-α Receptor 2 (TNFR2). Soluble TNF-α typically binds TNFR1 after clustering at the cell membrane (58, 59). Transmembrane TNF-α preferentially binds TNFR2 as a ligand and can serve as a receptor for cell-to-cell contact (56, 59). Regardless of form, after a TNF-α ligand binds, TNFR1 and TNFR2 from homodimers to induce downstream signaling for cellular processes such as defense from foreign pathogens, enhancing inflammation and promoting cell survival or apoptosis (57, 60, 61).

TNF-α is produced by different cell types in the CNS, including neurons, astrocytes, microglia and endothelial cells (62, 63). However, monocytic immune cells like microglia are the dominant secretors and targets of TNF-α (57). TNF-α is now well understood as a critical pro-inflammatory cytokine that has an instrumental roles in the CNS, including innate immunity, sleep regulation, neuronal activity and necrotic and apoptotic cell death (58, 64). Under physiological conditions TNF expression is induced by basal activity in microglia, neurons and astrocytes. This cytokine is essential for regulating neuronal function including synaptic activity. Neurons constitutively

express TNF-α receptors, which are important for mediating neuroprotection against neurotoxic stimuli (65).

Microglia-derived TNF-α is critical in innate immune responses within the CNS (66). Previous studies have documented the ability of TNF-α to influence microglial function in response to neuroinflammatory insults. For instance, to achieve a swifter recognition of foreign pathogens, TNF-α binds to its microglial receptors and upregulates the expression of toll-like receptor (TLR) 2, a pattern recognition receptor specialized for bacteria, enhancing microglia's overall immune response (67). Aside from priming microglia for enhanced pathogen detection, this cytokine also enhances natural killer cells' and macrophages' ability to kill cells and phagocytose, respectively (58, 67).

TNFR1 and TNFR2 notably have varying functions within the brain (**Table 1**), some of which are region-specific. It was previously observed that the reparative function of TNF-α on neurons in the striatum is reliant upon TNFR1 (68). At the same time, similar capabilities of TNF-α in the hippocampus depend upon TNFR2 but the receptors' expressions were equal in both regions (68). A more recent study found similar effects when investigating TNFR1 and TNFR2 single nucleotide polymorphisms (69). These results indicated that TNF-α regulation of striatal morphology was predominated by TNFR1 signaling while regulation of hippocampal morphology was shown to rely primarily on TNFR2 (69). It is thought that this region-specificity may be due to the two receptors' differential impacts on cell survival since TNFR1's downstream processes promote apoptosis and TNFR2's pathways are more anti-apoptotic (68).

The distinct effects of these receptors on cell survival and inflammation can be understood through their different recruitment of signaling complexes after a ligand binds (**Figure 1**). Active TNFR1 homodimers allow four different complexes to form to engage various cellular processes (70). Complexes I, IIa, and IIb similarly activate NF-κB and mitogen-activated protein kinases (MAPK) to promote cell survival, cell proliferation, immune defense and inflammation (70). Complexes IIa and IIb are additionally responsible for activating the caspase apoptotic pathway (70). The last complex, complex IIc, also plays a role in inflammation but is most notable for its role in necroptosis (70). Unlike TNFR1 pathways, the current understanding of TNFR2 is not as comprehensive (71). However, previous studies with TNFR2 knockout mice have shown its importance in anti-inflammatory and cell-protective processes (71). Furthermore, it is theorized that active TNFR2 homodimers recruit adapter protein TNF receptor-associated factor 2 to activate NF-κB pathways (71). Despite TNFRs sharing similar structures and ligands there is a clear heterogeneity in their downstream effects.

TNF-α has long been implicated in several peripheral and central inflammatory conditions (58, 72, 73). Meanwhile, research on the role of TNF-α in psychiatric disorders is evolving. In this context, TNF-α's pro-inflammatory functions may exacerbate or contribute to depressive symptoms (74). Both TNFR1 and TNFR2 pathways can modulate inflammatory pathways through downstream NFkB signaling (60). Additionally, TNF-α can induce glutamate-mediated excitotoxicity. This cytokine facilitates crosstalk between microglia and astrocytes to promote the release of astrocytic glutamate, the formation of excitatory synapses and the release of more TNF-α from microglia (75). Therefore, TNF-α mediated pathways can lead to extraneous inflammation and cell death contributing to the worsening of MDD.

TABLE 1 | Overview of studies on TNFR1 and TNFR2's differential processes within the brain.

Study	Study performed	TNFR type	Cell type	Observed effects
(68)	TNFR1 and TNFR2 levels measured in encephalitis mouse model	TNFR1	Striatal Neurons	TNFR1 carries out reparative function of TNF-α on striatum neurons
		TNFR2	Hippocampal neurons	TNFR2 carries out reparative function of TNF-α in hippocampus
(69)	Single nucleotide polymorphisms and associated brain morphology changes	TNFR1	Striatal neurons	TNFR1 responsible for TNF-α regulation of striatal morphology through apoptosis
		TNFR2	Hippocampal neurons	TNFR2 responsible for TNF-α regulation of hippocampal morphology through anti-apoptotic processes
(87)	Anti-TNF-α therapy and its effects on cell survival	TNFR1	Cholinergic neurons	When TNFR1 receptor blocked by anti-TNF-α, reduced apoptosis
		TNFR2	Cholinergic neurons	When TNFR2 blocked by anti-TNF-α, reduced cell protective processes
(88)	TNFR2 KO mice	TNFR2	Microglia	KO mice displayed early onset experimental autoimmune encephalitis
(89)	Activating TNFR2 in cultured mouse microglia	TNFR2	Microglia	TNFR2 regulates production of pro-regenerative and neuroprotective factors such as granulocyte colony-stimulating factor and IL-10

TNF-α, tumor necrosis factor-alpha; TNFR1, TNF-α receptor 1; TNFR2, TNF-α receptor 2; KO, knockout; IL-10, interleukin-10.

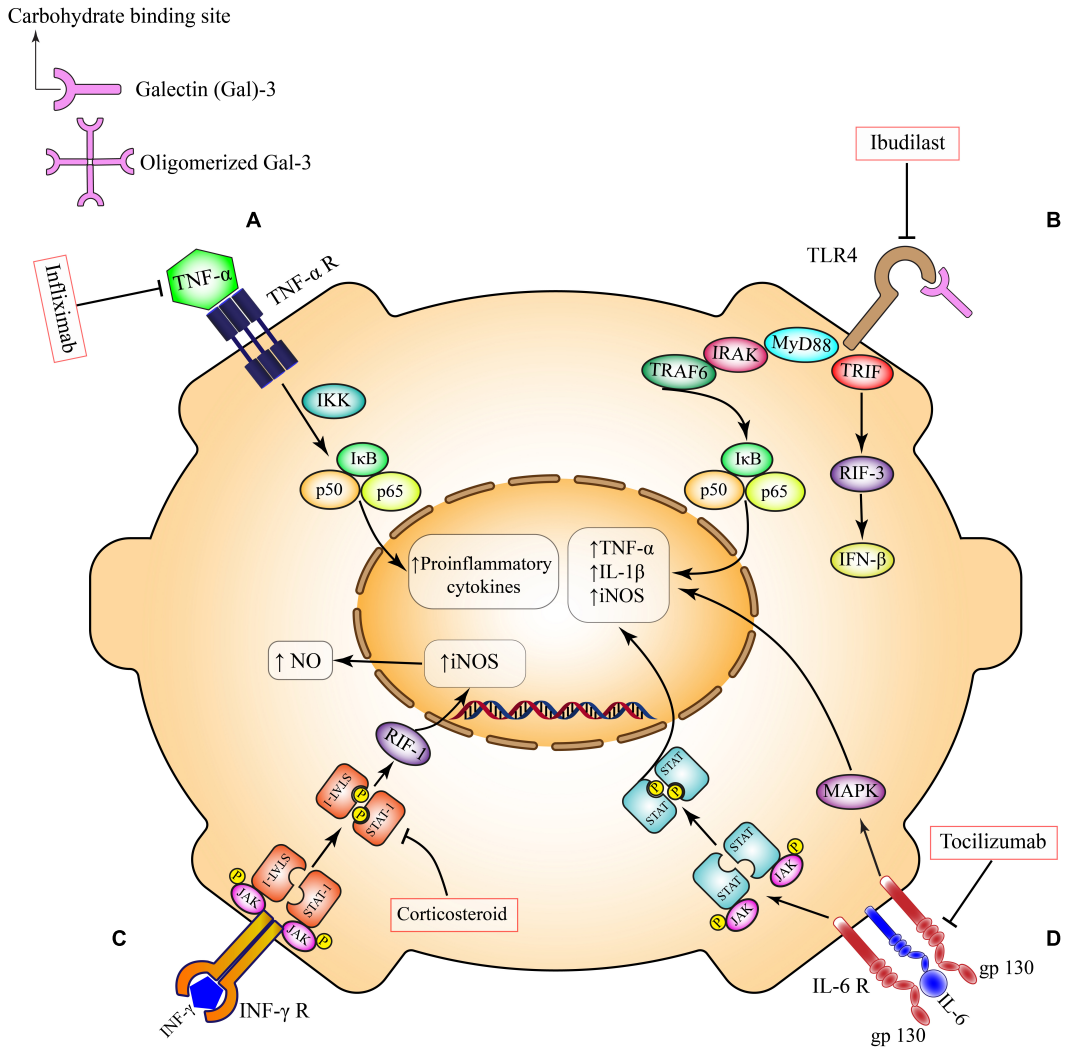

FIGURE 1 | Pro-inflammatory pathways in microglia. **(A)** TNF-α receptor activation, induces the canonical pro-inflammatory transcriptional factors such as NFκB and subsequent production of inflammatory mediators. This pathway can be inhibited by Infliximab. **(B)** TLR4 ligands and secreted Gal-3 directly bind to TLR4 on the microglial surface and exacerbates inflammatory responses through induction of different cytokines and chemokines. This pathway can be inhibited by Ibudilast. **(C)** Activation of INF-γ receptor on microglia triggers the overexpression of inducible nitric oxide synthase (iNOS) and overproduction of nitric oxide via Janus kinase (JAK)/signal transducer and activator of transcription (STAT)/RIF-1 pathway. Corticosteroids can suppress this pathway at the level of STAT factors. **(D)** IL-6 *trans*-signaling occurs in brain cell types that have membrane bound gp130, including microglia. IL-6 bound to soluble IL-6R activates signaling through membrane bound gp130. This *trans*-signaling is thought to be pro-inflammatory via the induction of JAK/STAT and MAPK signaling pathways. Tocilizumab inhibits this pro-inflammatory pathway. TNF-α, tumor necrosis factor-α; IKK, the IκB kinase; NO, nitric oxide; iNOS, inducible nitric oxide synthase; IL-1β, interleukin 1 beta; IL-6, interleukin 6; JAK, Janus kinase; STAT, signal transducer and activator of transcription; RIF-1, replication timing regulatory factor 1; MAPK, a mitogen-activated protein kinase; TLR4, toll-like receptor 4; MYD88, myeloid differentiation primary response 88; TRIF, TIR-domain-containing adapter-inducing interferon-β; IRAK, interleukin 1 receptor associated kinase; TRAF, TNF receptor associated factor; IFN-β, interferon-β; IFN-γ, interferon gamma.

In accordance, several studies have seen higher levels of serum TNF-α associated with depressive symptoms and in MDD patients compared to matched controls (76–78). A few studies measuring cytokine profiles in cerebrospinal fluid (CSF) have not found differences in TNF-α levels between depressed patients and healthy controls (79). However, there are only few such studies of CSF cytokine levels and the results seem rather inconsistent as compared to studies of peripheral (plasma) cytokines (79). In addition, plasma cytokine profiles do not necessarily represent cytokine levels within the CNS (80).

In a pioneer study, Ohgidani et al. showed that acute stress induces TNF-α secretion from hippocampal microglia resulting in mouse working memory deficits. These authors observed that morphological changes in hippocampal microglia did not occur (81). Furthermore, etanercept, a TNF-α inhibitor, rescued the working memory impairment accompanied by a reduction in hippocampal TNF-α (81). Indeed, maladaptive microglial activation may be linked to MDD and modulating microglial activation seems a promising therapeutic target for depression.

A previous study with monoclonal antibody against TNF-α revealed that this intervention quelled symptoms of anhedonia but did not affect depression scores significantly compared to placebo groups (82). Current data seem to support the effectiveness of anti-inflammatory agents as antidepressants only in patients with increased peripheral inflammation (83). This subgroup may include MDD patients with increased inflammatory markers or those with medical conditions characterized by increased levels of peripheral inflammation (84). Specifically, anti-TNF-α therapy was found not to be effective in all treatment-resistant depression patients, but it did improve depressive symptoms in those with higher baselines of inflammatory markers (85). One major reason for such failure is the double-edged role of microglial TNF-α in fundamental physiological processes such as the neuroinflammatory response to tissue damage, neuronal circuit formation, synaptic plasticity and myelin degeneration and repair (86). Indeed, any positive impact from blocking TNFR1 receptors would be nullified by blocking TNFR2 activation, adding another complex layer upon possible therapies for MDD (87). It has been shown that the TNF-α mediated activation of microglial TNFR2 is instrumental for the protective functions of these cells (86). For instance, microglia-specific TNFR2 knockout mice display early onset of experimental autoimmune encephalitis (88). In this context, it has been shown that TNFR2 regulates the production of pro-regenerative and neuroprotective factors including granulocyte colony-stimulating factor and IL-10 in microglia (89). It is noteworthy that protective aspects of TNF-α signaling in microglia have been greatly overlooked with respect to the pro-inflammatory ones. Thus, more investigations are needed to decipher the mechanisms regulating the balance between TNFR1 and TNFR2 pathways in microglia in order to limit the detrimental immune responses without blocking the protective ones (86).

IL-6 Mediated Pathway

Over the years, the cytokine IL-6 has been linked to stress-related disorders such as depression and anxiety (90). This cytokine is a small multifunctional protein (91) that can be produced by several cell types including endothelial cells, epithelial cells, astrocytes, microglia and neurons (92, 93). IL-6 belongs to a family of proteins that utilize glycoprotein 130 (gp130) as a signal transducer (**Figure 1**). Depending on the presence of IL-6 receptor (IL-6R) or membrane bound gp130 which are expressed differently in different cell types, IL-6 has pro- or anti-inflammatory properties (93) resulting in either inflammatory or anti-inflammatory cascades (90).

According to a few major meta-analyses, IL-6 is one of the most consistently elevated cytokines in the blood of patients with MDD (11, 94, 95). Remarkably, IL-6 blood levels might have a predictive value as a biomarker. Moreover, peripheral levels of IL-6 correlate with symptom severity of antidepressant non-responders (96). In addition, increased levels of IL-6 have been reported in the CSF of patients with MDD as well as in suicide attempters (3, 97). Unfortunately, research addressing the role of microglial derived IL-6 are lacking in human postmortem studies. Only one pioneer study indicates a non-inflammatory phenotype of microglia in MDD following single-cell mass cytometry of microglia (98). The authors performed single-cell analysis of microglia from four different postmortem brain regions including frontal lobe, temporal lobe, thalamus and subventricular zone of medicated individuals with MDD and they found no evidence for the induction of canonical pro-inflammatory (IL-1β, IL-6, and TNF-α) and anti-inflammatory cytokines such as IL-10 (98).

Various pre-clinical studies have investigated the role of microglial IL-6 in the context of stress. For instance, increased IL-6 mRNA is found in microglia isolated directly from the brains of mice that have undergone repeated social defeat (RSD) stress (99) and treatment with the antidepressant imipramine inhibits social avoidance behavior and diminished microglia IL-6 in mice exposed to stress (90, 99). In another study, Aniszewska et al. found that stress induced a significant increase in the number of IL-6-immunoreactive microglia in the hippocampus, cortex and brain stem (100).

Blocking IL-6R-mediated pathways (e.g., tocilizumab) or neutralizing IL-6 function (e.g., sirukumab) might have clinical value in a subset of MDD patients, especially in treatment-resistant cases or in patients with peripheral inflammatory diseases (101). Interestingly, the efficacy of interleukin-6 neutralizing antibodies on symptoms of MDD patients with RA has been reported (102). However, BBB penetration and adverse effects may limit their use in MDD patients without the history of peripheral inflammatory diseases such as RA (103). In addition, there are two types of IL-6 signaling: a classical anti-inflammatory signaling and a *trans*-signaling proinflammatory signaling. It means that general targeting of IL-6 pathways in MDD either with IL-6R inhibitors or IL-6 blocker is not an optimal choice (101, 103). A more selective intervention seems more promising for future drug discovery targeting IL-6 signaling (101).

Toll-Like Receptor 4 and Nuclear Factor-Kappa B Mediated Pathways

TLR4 is one of nine members in the TLR family of pathogen-specific pattern recognition receptors dedicated to responding to unique structural components of foreign microbial agents to trigger immune responses (104, 105). As such, this receptor is highly involved in regulating brain innate immune responses in pathophysiological conditions. TLR4 is notably the most researched receptor within the TLR family. It is well known to be primarily responsible in the reaction against Gram-negative bacteria by binding to LPS, its pathogen-associated molecular pattern (106). However, TLR4 has also been shown to recognize damage-associated molecular patterns (DAMPs) and xenobiotics (107). Additionally, TLR4 can be activated by other non-bacterial TLR4 agonists naturally present within the body such as saturated fatty acids (108). TLR4s are generally expressed in myeloid lineage cells like macrophages and other non-immune cells like endothelial cells (106). Within the CNS, TLR4 is expressed primarily by microglia but can also be found in astrocytes, oligodendrocytes and neurons (107).

NF-κB refers to a group of transcription factors that serve as significant regulators of pro-inflammatory genes and has been

of particular interest as a target for pharmacological treatments in inflammatory diseases (109). They exist as two subfamilies of inducible dimers, made up of either DNA binding proteins from the 'Nκ-kB' or 'Rel' family, which can then form homodimers or heterodimers (110). Alternatively, the term NF-κB can also describe p50-RelA heterodimer, the predominant NF-κB dimer present in many cells (111). Regardless, all NF-κB dimers are constitutively inhibited by IkB proteins that are degraded to activate and translocate the transcription factor into the nucleus (109). NF-κB activity is rapidly induced in microglia following inflammatory insults (112).

During activation with LPS, the pathogen-associated molecular pattern forms a multimolecular complex with TLR4 and its accessory molecules which readies the receptor for dimerization (113). The formed dimer then induces downstream effects (**Figure 1**) either through the myeloid differentiation primary response 88 (MyD88)-dependent pathway or the MyD88-independent pathway (106, 114). The MyD88-dependent pathway leads to activation of transcription factors that induce the expression of pro-inflammatory cytokine genes (114). The MyD88-independent pathway, also known as the TIR-domain-containing adapter-inducing interferon-β dependent pathway, also activates transcription factors but mediates the induction of type 1 interferon-inducible genes (114). In both cases NF-κB is activated (115).

TLR4 and NF-κB are of particular interest in pathophysiological conditions due to their significant roles in innate immunity. Accordingly, many of the following studies have implicated their dysregulation within various neurodegenerative diseases and psychiatric disorders (116–118). Elevated TLR4 expression and associated overactive microglia were observed within a transgenic mouse model of Alzheimer's Disease (AD) leading to cognitive impairment (119, 120). Furthermore, previous investigations have also characterized an increase in NF-κB activation within the CNS of animal models and patients with neurodegenerative disorders (117). Interestingly, a study of postmortem brains from patients with schizophrenia revealed that TLR4 levels are increased within the cerebellum but decreased within the prefrontal cortex (PFC) suggesting that general dysregulation, rather than upregulation, could lead to harmful effects (118). In the same study, NF-κB levels were notably altered inversely to TLR4 levels, with increased levels within the PFC and decreases in the cerebellum (118). Another investigation demonstrated increases in NF-κB levels, especially in microglia, as a neuroinflammatory mechanism in autism spectrum conditions (116). The studies above support the idea that TLR4, NF-κB and their combined pathway likely play significant roles in neuroinflammatory response in MDD. More specifically, the dysregulation of TLR4 and NF-κB inflammatory processes has been suggested to be involved in MDD. TLR4 single gene polymorphisms were associated with suicide and anxiety scores in MDD patients, while methylation levels of TLR4-associated CpGs were related to the severity of depressive symptoms (121, 122). In general, increased TLR4 expression and decreased expression of its inhibitor TNFAIP3 were associated with depressive symptoms (107). Similarly, increased NF-κB activity also appears to play a role in depression. In a rat early-life stress model, the depression-susceptible animals generally displayed more activated NF-κB. Inversely, the subgroup showing resilient phenotype had more inactivated NF-κB (123). Another notable study supported this idea by demonstrating improved depressive-like behaviors in mice when increased NF-κB expression was inhibited (124). Other studies have specifically investigated TLR4-NF-κB pathways in depression. A supporting study indicated that suppressing the TLR4-NFκB signaling pathway inhibits depression-like behavior in mice (125). These studies sparked further interest in investigating these pathways to develop new antidepressant candidates for MDD patients (**Table 2**).

The current literature strongly supports the existence and importance of TLR4, NF-κB and their conjoined roles in MDD. Although TLR4 activation seems to play a role in MDD, the underlying mechanisms are unclear. Much of the previous work implicating TLR4 in MDD have primarily focused on the role of TLR4 following activation by LPS in bacterial infections (114). However, as noted previously, TLR4 can be triggered by many different ligands; thus, current perspectives are limiting and require further investigation from novel angles (106–108). More specifically, it may be useful to consider the immune signaling that coincides with other events aside from infection, such as during the critical period of development or epigenetic modifications (126, 127). These are important time points that may impart predisposing factors on individuals if TLR4 signaling is altered (126, 127). Furthermore, these deleterious inflammatory states associated with altered TLR4 pathways may also be attributed to other mechanisms such as neuroendocrine signaling and dysregulated gut microbiota (127). Therefore, to improve the efficacy of future drugs targeting TLR4 in MDD, research into more diverse mechanistic facets should be conducted. A recent study used this approach to propose polyphenols as a potential group of drug candidates for future consideration (128).

Interferon-Gamma Mediated Pathway

Interferon-gamma (IFN-γ) is a pleiotropic soluble cytokine that is produced by different immune cell types including lymphocytes, B cells and antigen-presenting cells. In the CNS, different cells such as neurons, microglia and astrocytes produce this cytokine and express its receptors. The activation of the IFN-γ receptor induces several canonical downstream pathways (**Figure 1**) such as the janus kinase (JAK) 1 and 2, signal transducer and activator of transcription (STAT) 1 and the extracellular-signal-regulated-kinase (ERK) 1/2. Many genes, as well as micro RNAs and long non-coding RNAs, are activated following IFN-γ receptor stimulation (129). Neuroinflammation mediated by IFN-γ has been reported in neurological disorders. However, the effects of IFN-γ on behavior in the context of stress are mostly unknown (129). Intriguingly, IFN-γ knockout mice show decreased anxiety- and depressive-like behaviors (129). These effects are accompanied by elevation of serotonergic and noradrenergic activity in the central amygdaloid nucleus, together with increased baseline plasma corticosterone, decreased neurogenesis in the hippocampus and decreased levels of nerve growth factors in the PFC, indicating

that IFN-γ modulates anxiety and depressive states and is involved in CNS plasticity (130–132). In an intriguing report, Zhang et al. showed that intracerebroventricular injection of IFN-γ in mice causes impairment of adult hippocampal neurogenesis, behavioral despair, anhedonia and cognitive loss. Furthermore, IFN-γ induces microglial activation that is associated with morphological changes and upregulation of phagocytic marker CD68 and pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6) (133). Inhibition of the JAK/STAT1 pathway, downstream of IFN-γ receptor, suppresses microglial-mediated neuroinflammation, diminishes depressive-like behaviors and improves memory (133).

An array of studies has demonstrated the possible effects of antidepressants, particularly selective serotonin reuptake inhibitors (SSRIs), on inflammatory responses and microglial function (10, 134). Besides their known therapeutic mechanism involving the modulation of the serotonergic system, SSRIs can regulate the activation state and secretory profile of microglia (10). In this context, Horikawa et al. reported that paroxetine and sertraline prevent microglial activation by inhibiting IFN-γ-induced elevation of intracellular calcium (135). Interestingly, Alboni et al. found that quality of the environment effects the mechanism of action of Fluoxetine. Enriched environments coincident with Fluoxetine administration induced pro-inflammatory microglial profiles while a stressful environment resulted in anti-inflammatory secretory profiles (134).

It is established that microglia adopt reactive states in response to an inflammatory insult. However, at both transcriptional and functional levels, microglia appear to be more complex and dynamic than anticipated. This might explain why engagement of microglia can be either neuroprotective or neurotoxic, leading to attenuation or exacerbation of disease progression (10, 15, 136) depending on the context. According to the traditional classification of macrophages/microglia, during microglial activation following an inflammatory insult, cell morphology is altered either to M1, the typically activated phenotype, or to M2, an alternative activated phenotype; and this phenotypic switch depends on the type of insult. M1 microglia are considered proinflammatory and produce mediators such as TNF-α and IL-1β. It has been shown that INF is a canonical cytokine that can polarize microglia toward M1. It is noteworthy that the classification of the M1 and M2 phenotypes have been challenged (10, 136, 137). The reason is that such classification has been defined mainly based on *in vitro* studies of peripheral macrophages and that M1 and M2 states fail to emerge in brain resident microglia. It is now accepted that activated microglia co-express canonical gene products associated with both M1 and M2 states. Indeed, following brain injury, microglia do not simply switch to a polarized "M1-only" or "M2-only" phenotype but rather display a mixed phenotype due to the complex signaling cascades surrounding them (27, 136, 137). However, in the context of this review, we will continue to use the broad categories of activated pro-inflammatory and anti-inflammatory unless the study mentioned investigated more dynamic phenotypes.

The choroid plexus (ChP), a highly vascularized tissue that produces CSF and lacks a BBB, is an interface between peripheral and central immune responses (138). Our group previously investigated the cellular and molecular inflammatory profile of the ChP of the lateral ventricle in depressed suicides and healthy controls (138). We measured the content of several pro- and anti-inflammatory transcripts as well as the density of $Iba1^+$ macrophages associated with the ChP epithelial cell layer. The levels of pro-inflammatory markers, ICAM1 (a protein implicated in immune cell trafficking) and Iba1, were measured to be significantly downregulated in depressed suicides as compared to controls (138). Intriguingly IFN-γ signaling has been shown as a selective key regulator of immune cell trafficking across the ChP epithelium under physiological conditions of CNS immune surveillance and following neuroinflammatory insult (139). This unique mechanism could be harnessed to adjust the interplay between the peripheral immune system and microglia in affective disorders.

TABLE 2 | Overview of studies performed to investigate TLR4 and NF-κB's roles in neuropsychiatric disorders.

Study	Method	Condition of Brain/Behavior	Finding
(118)	Measure TLR4 and NF-κB levels in human postmortem brains	Schizophrenia	PFC: (1) ↓ TLR4, (2) ↑ NF-κB Cerebellum: (1) ↑ TLR4, (2) ↓ NF-κB
(116)	Measure NF-κB levels in postmortem human brains	Autism spectrum conditions	↑ NF-κB levels especially in microglia
(121)	Associative study on TLR4 single gene polymorphisms and MDD	Individuals with higher suicide and anxiety scores	↑ TLR4 single gene polymorphisms
(122)	Associative Study of epigenetic effects of TLR4 on severity of symptoms	Higher severity of depressive symptoms	↑ methylation levels of TLR4-associated CpGs
(123)	Measure levels of active NF-κB in an early-life stress rat model	Depression susceptible phenotype	↑ activated NF-κB
		Depression resilient phenotype	↑ inactivated NF-κB
(124)	NF-κB inhibited in mice model to see behavioral changes	Improved depressive-like behaviors	↓ overactivated NF-κB
(125)	Suppressing TLR4-NF-κB pathway in mice	Inhibits depression-like behavior	↓ signaling of TLR4-NF-κB pathway

↑, increased; ↓, decreased; TLR4, toll-like receptor 4; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; PFC, prefrontal cortex.

ANTI-INFLAMMATORY PATHWAYS IN MICROGLIA

Alpha-Seven Nicotinic Receptor Mediated Pathway

The alpha-7 nicotinic acetylcholine receptor (α7 nAChR) is a ligand-gated ion channel expressed by macrophage/microglia (140, 141) and has proved to be a promising target in pharmacotherapy of psychiatric disorders. α7 nAChR agonists or partial agonists are known to improve cognitive dysfunction by regulating microglial activation through inhibition of canonical pro-inflammatory transcriptional factors such as NFkB and induction of anti-inflammatory signaling pathways such as nuclear factor-erythroid factor 2-related factor 2 (Nrf2) (142–144). In fact, there is ample evidence that the cholinergic system plays a fundamental role in regulating central inflammation and glial activation via homomeric α7 nAChRs (145–147). The α7 nAChRs consist of five α subunits and are expressed by neuronal and glial cells (148, 149). These ligand-gated ion channels allow for calcium influx and subsequent ultra-rapid desensitization (150, 151). α7 nAChRs are widely expressed in the brain, including in regions such as the PFC, hippocampus and other limbic areas (150). Microglial α7 nAChRs play important roles in regulating inflammatory processes in the CNS (148, 150). Stimulation of α7 nAChR leads to a reduction in glial activation and decreases in proinflammatory cytokine levels in different brain regions (152–154).

The microglial α7 nAChRs have dual ionotropic/metabotropic properties and their intracellular signaling pathways that modulate inflammation do not only depend on transient ion influx (145, 155, 156). Indeed, neuronal α7 nAChRs mainly have an ionotropic function (145). The downstream metabotropic signaling pathways of microglial α7 nAChRs are different from neuronal α7 nAChRs (157). Activation of microglial α7 nAChRs induces phospholipase C and enhanced calcium release from intercellular stores which are sensitive to inositol trisphosphate (153). This process results in the inhibition of NF-κB transcriptional activity (**Figure 2**) (158). As a result of this inhibition the levels of pro-inflammatory cytokines are decreased (154).

It has been shown that chronic restraint stress (CRS) alters central cholinergic signaling in brain regions that have been implicated in MDD (159). Namely, CRS induces hippocampal choline acetyltransferase protein expression and decreases nuclear STAT3 signaling. CRS also augments signaling activity, IL-1β and TNF-α expression and microglial activation. Intriguingly, cholinergic stimulation with a selective α7 nAChR agonist significantly diminishes CRS-induced depressive-like behavior, neuroinflammation and neuronal damage. Moreover, activation of α7 nAChRs restores central cholinergic signaling function, inhibits TLR4-mediated inflammatory signaling and microglial activity and increases the number of regulatory T-cells in the hippocampus following stress (159).

α7 nAChR activation induces the transcriptional activity of Nrf2 (143). Previous studies especially by the Lopez group indicate that α7 nAChR mediated activation of Nrf2 elicits anti-inflammatory mechanisms in microglia (160–165). This anti-inflammatory axis might play an instrumental role in antidepressant aspects of α7 nAChR modulators. In this context, the efficacy of selective or promiscuous ligands that can activate α7 nAChR-Nrf2 pathway have been shown in depressive disorders (144, 164). One interesting example for the promiscuous ligand is Tropisetron. This ligand is a 5-HT_3 receptor antagonist and α7 nAChR partial agonist. This serotonergic ligand has shown a great efficacy in a wide range of psychiatric disorders including MDD and schizophrenia in both experimental models and clinical trials (144, 166). The other example is RG3487 ($C_{15}H_{19}ClN_4O$), the novel 5-HT_3 antagonist with α7 nAChR partial agonist properties. This ligand significantly improves attentional performance in experimental models and has shown promising results in clinical trials for cognitive impairment associated with schizophrenia (142, 167).

IL-4 Receptor Mediated Pathway

IL-4 is a multifunctional cytokine secreted by Th2 cells, mast cells, eosinophils and basophils (168, 169). IL-4 is a crucial molecule for microglia and macrophage polarization and it plays pivotal roles in brain function following neuroinflammatory insult (169). The effects of IL-4 are mediated through the IL-4 receptor α-chain. Following binding to its ligand, IL-4 receptor α-chain dimerizes either with the common γ-chain to produce the type-1 signaling complex located mainly on hematopoietic cells, or with the IL-13 receptor α 1 to produce the type-2 complex, which is expressed also on non-hematopoietic cells. The type-1 signaling complex (**Figure 2**) is pivotal for alternatively activated macrophages (168). Upon activation, the type-1 complex signals through JAK1 and JAK3, which phosphorylate and create docking sites for the transcription factor STAT6. This transcriptional factor then dimerizes and translocates to the cell nucleus to regulate the expression of several genes (168).

IL-4 might be protective against depression due to its ability to harness inflammation and to inhibit serotonin transporter activity. Wachholz et al. demonstrated that a decreased IL-4 responsiveness of microglia is specifically related to the development of depressive-like behavior. IL-4 deficient mice show notable augmentation of depressive-like behavior in the forced swim and tail suspension test (170). In experimental models of stress, the decline in IL-4 levels in the locus coeruleus may be involved in anxiety-like behavior and an inverse relationship between IL-4 secretion and hypothalamic-pituitary-adrenal (HPA)/sympathetic-adrenal-medullary-axes activation has been reported (171). These findings suggest that modulation of the IL-4 receptor signaling pathway is required to adapt to homeostatic mechanisms in response to stressful events (171). In addition, it has been shown that microglial IL-4 receptor pathway modulates cognitive function following neuroinflammation (172).

It is well established that adult neurogenesis in the dentate gyrus of the hippocampus is regulated by specific microglia population and potentially implicated in MDD (10, 173). Very recently, Zhang et al. showed in rodents that IL-4 driven microglia modulate stress resilience through BDNF-dependent neurogenesis (173). Their findings indicated

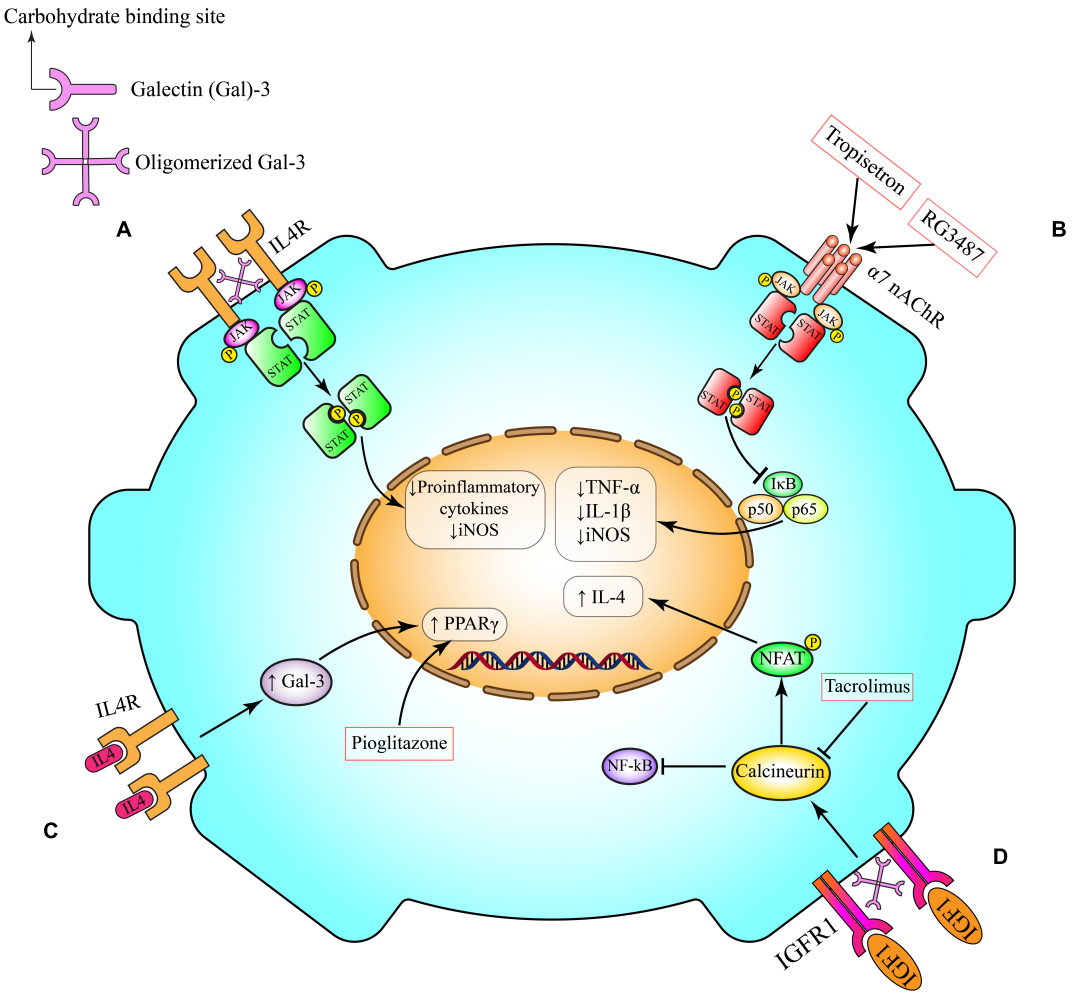

FIGURE 2 | Anti-inflammatory pathways in microglia. **(A)** Gal-3 induces alternative microglia activation through interaction with IL-4 receptor (IL4R). Following Gal-3 lattice formation the carbohydrate-binding site of Gal-3 molecules interacts with glycosylated IL4R and prevents their endocytosis and also over activation of IL4R and its anti-inflammatory signaling. **(B)** Activation of α7 nAChR on microglia triggers anti-inflammatory cascades, including Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and PI3K/Akt, which potentiate the activity of transcriptional factor Nrf2 and its downstream pathways (HO-1 and CAT), and inhibits the canonical proinflammatory protein NFκB, which governs the production of proinflammatory cytokines (e.g., TNF-α) and enzymes (e.g., iNOS and COX-2) involved in neuroinflammation. This pathway can be induced by tropisetron and RG3487. **(C)** IL-4 can interact with its tyrosine kinase IL4R on microglia cell surface. This interaction might activate one of the canonical transcriptional factors that are involved in microglia polarization such as PPAR-γ. Pioglitazone activates PPAR-γ. **(D)** By crosslinking insulin-like growth factor 1 receptor (IGFR-1), secreted Gal-3 will prevent early endocytosis and over-activate the Janus kinase (JAK)/signal transducer and activator of transcription STAT pathway and the transcription of genes needed for production of anti-inflammatory cytokines such as IL-4. IL-4, interleukin 4; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor-α; JAK, Janus kinase; STAT, signal transducer and activator of transcription; NFAT, nuclear factor of activated T-cells; NF-κB, nuclear factor kappa B; α7 nAChR, α7 nicotinic acetylcholine receptors; Gal-3, galectin-3; IGFR, insulin-like growth factor 1 (IGF-1) receptor; PPAR-γ, peroxisome proliferator- activated receptor-gamma.

that IL-4 driven microglia are characterized by a high expression of Arg1 which is critical in maintaining hippocampal neurogenesis and stress resistance. Decreasing $Arg1^+$ microglia in the hippocampus by knocking down the microglial IL-4 receptor inhibited hippocampal neurogenesis and enhanced stress vulnerability. Indeed, Increasing $Arg1^+$ microglia in the hippocampus by enhancing IL4 signaling restored hippocampal neurogenesis and the resilience to stress-induced depression (173).

Following an inflammatory insult, endogenous IL-4 can interact with its tyrosine kinase IL-4 receptor on the microglia cell surface (**Figure 2**). This interaction induces the production of Galectin-3 (Gal-3), prostaglandin (PG) J2 and activates STAT6. Production or activation of these molecules ultimately leads to activation of canonical transcriptional factors for microglia polarization such as PPAR-γ. The transcriptional activity of PPAR-γ can induce microglia alternative activation by decreasing the production of ROS, pro-inflammatory cytokines and suppressing the activity of NF-κB (15, 27, 174). PPAR-γ is an important and canonical transcriptional factor in the induction of anti-inflammatory signaling pathways (discussed in detail in the next section).

Peroxisome Proliferator-Activated Receptor Gamma Mediated Pathway

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors of the nuclear hormone receptor superfamily. PPARs exist as three isoforms (α, γ, and δ/β). PPARs have a ligand binding domain and a DNA binding domain. When their endogenous or exogenous ligands bind to PPARs, they create a heterodimeric complex which recruits other co-activators including PPAR coactivator-1, PPAR-interacting protein, PPAR-binding protein, steroid receptor co-activator-1 and CREB binding protein. This complex binds to the promoter regions of specific genes that contain a regulatory element known as the peroxisome proliferator response element which either activates or transrepresses the target genes (175). In the mammalian body, PPARs control glucose metabolism, cell proliferation and differentiation (176). The fact that PPAR-γ is involved in the modulation of macrophage differentiation and activation in peripheral tissues led to studying of the role of PPAR-γ in CNS resident microglia. Several investigations indicate that PPAR-γ endogenous ligand and synthetic agonists might influence brain inflammation by inhibiting different functions related to microglial activation, such as production of inflammatory cytokines, chemokines, nitric oxide and prostaglandins (PGs) (177).

PPARγ ligands elicit anti-inflammatory and neuroprotective actions in various experimental models of neurodegenerative diseases (178). Indeed, PPARγ activation inhibits the activity of transcription factors including NF-κB, AP1, and STAT. Some studies indicate that IL-4 receptor signaling increases the endogenous level of PPAR-γ ligands such as PGJ2 and subsequently amplifies transcriptional activity of PPAR-γ which might polarize microglia phenotype toward the anti-inflammatory one (179).

The PPARγ-mediated pathway has been the subject of several pre-clinical and human studies of MDD. A low PPARγ level in the hippocampus and PFC has been associated with depressive-like behavior in mice (180). Selective agonists of PPAR-γ already have FDA approval for the treatment of type 2 diabetes and their potential antidepressant effects, through modulation of metabolism and inflammation, have been investigated in different models (181). Interestingly, the efficacy of PPAR-γ ligands has been shown in metabolic disorder to be associated with depressive-like behavior in rodents. Namely, obesity in rats results in downregulation of PPARγ in the PFC (182), meanwhile chronic treatment with pioglitazone reversed depressive-like behaviors associated with obesity in CUMS mouse model (183). There is an increased risk for obese patients with chronic low-grade inflammation to develop depression (184, 185). Also, obesity induces microglial activation and neuroinflammation that play crucial roles in the pathogenesis of depression (186).

In an interesting report Qin et al. demonstrated that CUMS can induce severe depressive-like behaviors, neuroinflammation and reduced expression of PPARγ in leptin-deficient (ob/ob) mice as compared to wild type mice. Administration of a selective PPARγ agonist, pioglitazone rectified the behavioral abnormalities and alleviated microglial pro-inflammatory cytokine levels and NF-κB activation in PFC and hippocampus (187). Other studies also investigated anti-inflammatory and antidepressant effects of PPAR-γ agonists. Li et al. aimed to explore the effects of pioglitazone on depressive-like behaviors of mice treated with LPS and elucidated the underlying mechanisms. Their findings indicated that PPAR-γ activation induces PI3K/AKT/JNK/p38 signaling pathway and counteract LPS mediated apoptosis in mice PFC (188).

Studies have provided evidence that the antidepressant-like effect of pioglitazone in the forced swim test is mediated partly through *N*-methyl-D-aspartate (NMDA) receptor signaling and nitric oxide pathway (189, 190). Cognitive impairment is a feature of both AD and psychiatric disorders. The PPARγ agonist, rosiglitazone improves hippocampus-dependent cognitive deficits. Its cognitive enhancement partly occurs through the induction of ERK cascade, a critical mediator of memory consolidation. Jahrling et al. showed that PPARγ agonism facilitated recruitment of PPARγ to pERK during memory consolidation (191). Other investigations have pointed to the involvement of NMDA receptor and nitric oxide pathway in the memory improving effects of PPARγ ligands (192, 193). These findings highlight the fact that PPARγ ligands might have therapeutic implication in MDD specifically in the patients that have memory impairments. Namely, Sepanjnia et al. showed that pioglitazone, a selective PPAR-γ agonist, is an effective and safe short-term add-on therapy to Citalopram in non-diabetic patients with MDD and was associated with a high rate of early improvement and remission (194). Taken together, these findings showcase the potential for developing new interventions that target the brain's innate immune responses in different psychiatric disorders. However, much is still unknown about role of microglia in psychiatric disorders and why neuroinflammation is not a common phenomenon in all MDD patients (21).

Galectin-3 Mediated Pathway

Galectins are a family of soluble β-galactoside-binding proteins found in all multicellular organisms. They act as both DAMPs in innate immunity and/or as pattern-recognition receptors that bind to pathogen-associated molecular patterns. Gal-3 has recently been implicated in studies of neuroinflammatory diseases (195). This lectin is involved in cell-cell adhesion, modulation of the brain's innate immune response and microglial activation patterns in both physiological and pathophysiological settings. Gal-3 also mediates cell proliferation and migration (196, 197). Several studies using different approaches and methods have demonstrated both protective and deleterious effects of Gal-3 in neuroinflammatory diseases making Gal-3 an attractive target in drug discovery. Among different galectin family members, Gal-3 is unique in that in addition to the carbohydrate recognition domain, it possesses a proline and glycine-rich N-terminal domain through which it forms oligomers (195, 198). Gal-3 is expressed in epithelial cells, endothelial cells, neurons and immune cells where it is synthesized as a cytosolic protein. It can be released or secreted into the extracellular space where several bind to cell surface glycoproteins (199). Originally identified as a marker of activated

macrophages, there is increasing evidence suggesting its role as a modulator of microglial phenotypes in neuroinflammation (200).

Gal-3 plays important extracellular physiological roles. It uses IL-4 dependent mechanisms to mediate microglial arborization (195). *In vitro* studies highlight the importance of the carbohydrate-binding site of extracellular Gal-3 in microglia motility and ramification. Microglia pruning of axons and synaptic terminals might involve Gal-3 (18). Intracellular Gal-3 also holds distinct roles in physiological and pathological conditions. Following inflammatory insult, endogenous IL-4 interacts with microglial IL-4 receptors, thereby increasing the production of Gal-3 and subsequently inducing the canonical transcriptional factor PPAR-γ leading to anti-inflammatory signaling (**Figure 2**), as described in Section "Peroxisome Proliferator-Activated Receptor Gamma Mediated Pathway" (15, 201). In neuroinflammatory events Gal-3 elicits time-dependent protective actions. For instance, a Gal-3 feedback loop is critical for IL-4-mediated alternative polarization of peripheral macrophages (200, 202). It was also shown that following neuroinflammatory insult induction of Gal-3 in proliferating resident microglia is neuroprotective (27). Additionally, Gal-3 positive proliferating microglia are the major contributing cells of neurotrophic molecules such as insulin-like growth factor 1 (IGF-1) (200) which can also enhance the effects of trophic factors such as IGF-1 through inhibition of IGF-1 endocytosis (**Figure 2**) (195, 200). In 2019, Rahimian et al. studied time- and context-dependent effects of Gal-3 as a neuroprotective mediator following neuroinflammation (27). We showed that Gal-3 induces an anti-inflammatory microglial phenotype through IL-4 receptor pathway. It is likely that the polarization following neuroinflammatory insults is influenced by Gal-3 binding to glycans attached to IL-4 receptors (27).

In addition to its protective actions, Gal-3 also plays a pro-inflammatory role as reported in different animal and human studies especially in neurodegenerative disorders (195). Literature suggests that microglia-derived Gal-3 is detrimental in certain neuroinflammatory conditions. Indeed, comprehensive single-cell RNA analyses of CNS immune cells in neurodegenerative conditions including AD have led to the discovery of disease-associated microglia (DAM). This subpopulation of microglia displays a distinct transcriptional and functional signature (17). Boza-Serrano et al. showed that expression of Gal-3 in DAM in a mouse model of familial AD (5xFAD). They demonstrated that in 5xFAD mice Gal-3 is expressed solely in microglia associated with amyloid-β plaques and its deletion both decreases amyloid-β burden and improves memory function. Moreover, Gal-3 was found to be a TREM2 endogenous ligand binding through its carbohydrate-binding domain (203). Gal-3 direct interaction with TLR4 receptor may be an additional mechanism by which it regulates the severity of inflammation. Burguillos et al. showed that interaction of Gal-3 with TLR4 receptors in acute phase of neuroinflammation exacerbates neural cell death and prolongs inflammation, while its ablation elicits anti-inflammatory and neuroprotective effects (204).

Emerging experimental and clinical evidence indicates that Gal-3 may also play a role in MDD (205). Recently, Stajic et al. investigated the role of Gal-3 in modulation of anxiety levels in mice (206). The finding of this study revealed contradictory effects of Gal-3 on anxiety levels in the physiological condition and following acute inflammatory challenge with LPS. Gal-3 deficiency showed clear anxiogenic effect in basal conditions that is accompanied with lower expression of brain-derived neurotrophic factor (BDNF) and $GABA_A$ receptors. Gal-3 deficiency was also associated with anxiolytic response following acute administration of LPS (206). Intriguingly, the relationship between the novel inflammatory aspect of Gal-3 and depression symptom severity has been studied. In a large sample size, King et al. demonstrated higher Gal-3 levels were associated with higher levels of depressive symptoms. Their findings suggest that Gal-3 may be a new and useful inflammatory biomarker associated with depression (207). Another interesting clinical investigation showed that depression in type 1 diabetes is associated with high levels of circulating Gal-3 (208).

METABOLIC PATHWAYS IN MICROGLIA

Cannabinoid Signaling in Microglia

The endogenous cannabinoid (endocannabinoid) system has been implicated in synaptic communication and influences anxiety and cognition, metabolism, growth and development and response to internal and external immune insults via an array of actions mediated by their receptors (209, 210). In the CNS, endocannabinoids such as anandamide (AEA) and 2-arachidonoylglycerol (2-AG) regulate several physiological functions via two main G-protein-coupled cannabinoid receptors 1 and 2 (CB1 and CB2) (211). Endocannabinoids can also interact with several extracellular and intracellular targets such as G-protein-coupled receptor 55 (GPR55), PPARs and transient receptor potential vanilloid 1 (212). CB1 receptors are expressed in the cortex, hippocampus, cerebellum, basal ganglia and brainstem, usually at presynaptic terminals or on axons (210). They have also been reportedly found on glial cells (213, 214). CB2 receptors are much less expressed in the CNS compared to CB1 receptors, however, they have been found in the brainstem, cerebellum and hippocampus among other areas (212) and are primarily found on immune cells, astrocytes and less commonly in neurons (215). Endocannabinoids are generally synthesized post-synaptically after Ca^{2+} influx or activation of Gq/11-linked G-protein-coupled receptors; they act in a retrograde fashion influencing presynaptic cell firing (210, 215).

AEA is synthesized by phospholipase D catalyzed hydrolysis of *N*-acylphosphatidylethanolamine (216), meanwhile, 2-AG synthesis from membrane phospholipids is catalyzed by phospholipase C and diacylglycerol lipase (217). AEA degradation occurs predominantly by the enzyme fatty acid amide hydrolase (FAAH) or by cyclooxygenase (COX)-2 oxidation creating PGs (210) while 2-AG degradation occurs mostly through monoacylglycerol lipase (MAGL) but it can sometimes be oxidized by COX-2 or hydrolyzed by FAAH (210, 218). Although some processes occur due to crosstalk between cell types (219), microglia contain the complete machinery required for a functional endocannabinoid system. Rodent

microglia are known to express both CB1 and CB2 (220, 221). The presence of CB1 in human microglia is controversial (211, 222), however, a few studies describe CB1 microglial expression in active multiple sclerosis plaques of postmortem human brain samples (223, 224). Microglia also produce the enzymes responsible for hydrolysis and inactivation of AEA and 2-AG.

The endocannabinoid system has recently been implicated as a regulator of microglial migration and activity which points to cannabinoids being a useful target for modifying microglia in pathological conditions. Reusch et al. showed that the CB2 receptor is necessary for TLR-mediated microglia activation through p38 MAPK signaling (225). Other studies have revealed that the CB2 receptor is instrumental to induce the anti-inflammatory phenotype in microglia (226, 227). Tao et al. (227) found that JWH133, a selective CB2 receptor agonist promotes the anti-inflammatory phenotype in microglia through CB2 receptor stimulated cAMP/PKA pathway (227). We and others have showed that CB2 activation can trigger the activity of canonical anti-inflammatory transcriptional factors such as PPAR-γ (**Figure 3**) (228–230). As discussed in previous sections, the transcriptional activity of PPAR-γ is pivotal for microglia alternative activation by diminishing the production of pro-inflammatory cytokines and inhibiting the activity of NF-κB. Following neuroinflammation, CB2 receptors are upregulated (212) which has been shown to trigger microglia migration to the site of injury/lesion (231, 232). Experimental studies elucidate that neuroinflammation produces adenosine triphosphate (ATP) (discussed in the Purinergic Signaling section), which causes 2–AG production, commencing microglia migration through activation of the CB2 receptors at the microglial leading edge (233).

The human endocannabinoid system has been implicated in MDD (234, 235). A 2019 meta-analysis revealed a very strong association of CB2rs2501432 polymorphism with depressive disorder, but not CB1rs1049353 polymorphism (236). Moreover, peripheral serum levels of AEA and 2-AG are significantly reduced in women diagnosed with MDD (237). Few studies have investigated the endocannabinoid system in postmortem human brain of psychiatric cases. Hungund et al. (238) found that CB1 receptor protein is increased in the dorsolateral PFC of depressed suicides. Moreover, using [35S]GTPgammaS binding assays which assesses coupling of G-proteins to G-protein-coupled receptors in postmortem brain, the authors found that CB1 cannabinoid signaling was increased in the same region when compared to healthy controls (238) implicating cannabinoid signaling in both depression and suicide.

The role of the endocannabinoid system has also been studied in different stress paradigms (**Table 3**) (239, 240). RSD of mice has been used to model depression and anxiety. This paradigm not only has stress related behavioral outcomes and impaired fear extinction but also an increase in inflammation both peripherally and in the brain. Lisboa et al. showed that stimulating CB1/2 by injecting WIN55,212-2, a non-selective agonist, daily before the RSD paradigm, reduced IL-1β mRNA in the brain but specifically in $CD68^{+}$ activated microglia. Moreover, activation of the CB1/2 receptors before RSD repaired fear extinction and stress-related behavioral deficits (240). Although interesting, this study begs the question of whether these protective effects were mediated through the CB1 or CB2 receptor. In 2011, Zoppi et al. showed that daily pre-stress administration of arachidonyl-2′-chloroethylamide (ACEA), a selective CB1 receptor agonist, prevented upregulation of pro-inflammatory markers in the PFC of wild type mice; but not in $CB1^{-/-}$ knockouts (239). In another study by García-Gutiérrez et al. overexpression of CB2 receptor in mice had a protective effect, providing resilience to chronic mild stress and decreased depressive-like behaviors measured by the forced swim test and novelty-suppressed feeding test. Interestingly, chronic (4-week) administration of the CB2 receptor antagonist AM630 had anti-depressant like effects in wild type mice but not those that overexpressed CB2 receptor (241). More recently, a CDS stress paradigm was used in both $CB1^{-/-}$ knockouts and wild type littermates. Beins et al. found that CB1 knockouts mice were much more susceptible to CSD stress and mild CSD showing significant stress behaviors. Moreover, these stressed $CB1^{-/-}$ mice had dysregulated HPA axes with insufficient glucocorticoid signaling and hyper-activated microglia (242). Interestingly, at baseline, $CB1^{-/-}$ mice have increased expression of *Fkbp5*, a negative regulator of glucocorticoid signaling and a gene already implicated in depression (243). Overall, it seems that the endocannabinoid system serves a protective role in counteracting neuroinflammation by induction of anti-inflammatory profiles in microglia.

Rescuing cellular function as a treatment for MDD has been mainly considered for neurons but not for glial cells. However, many investigations have demonstrated the functional impairment in glia cells as mentioned throughout this review. Microglial malfunctions have been studied in many different neuroinflammatory settings (244). Research has shown that different aspects of microglia such as phagocytic activity, secretory profile and metabolic pathways can be affected by neuroinflammation. Due to this complexity, selective modulators rather than general anti-inflammatory agents might be needed to rescue microglial functions following different types of inflammatory insults (10). One interesting and promising target could be endocannabinoid system. Fine tuning of this system in microglia can open a new avenue of research in pharmacotherapy of depression although several experimental and clinical studies should be performed long before being able to design cell-specific interventions for treating MDD.

Prostaglandin Signaling in Microglia

The PGs are a class of eicosanoids that are formed by the liberation of arachidonic acid from phospholipids and a 2-step conversion by COX, the rate-limiting enzymes (245). Two main isoforms of COX exist, COX-1 and COX-2. COX-1 is traditionally considered as a constitutive enzyme while COX-2 is inducible. However, such classification is not very precise especially in the brain where constitutive expression of COX-2 has been reported (245). One of the canonical PGs in the CNS is PGE2. It interacts with different G-protein-coupled receptors including EP1, EP2, EP3, and EP4 (**Figure 3**) (246). Intriguingly, elevated PGE2 in the saliva, serum and CSF of depressed patients has previously been reported (247–250).

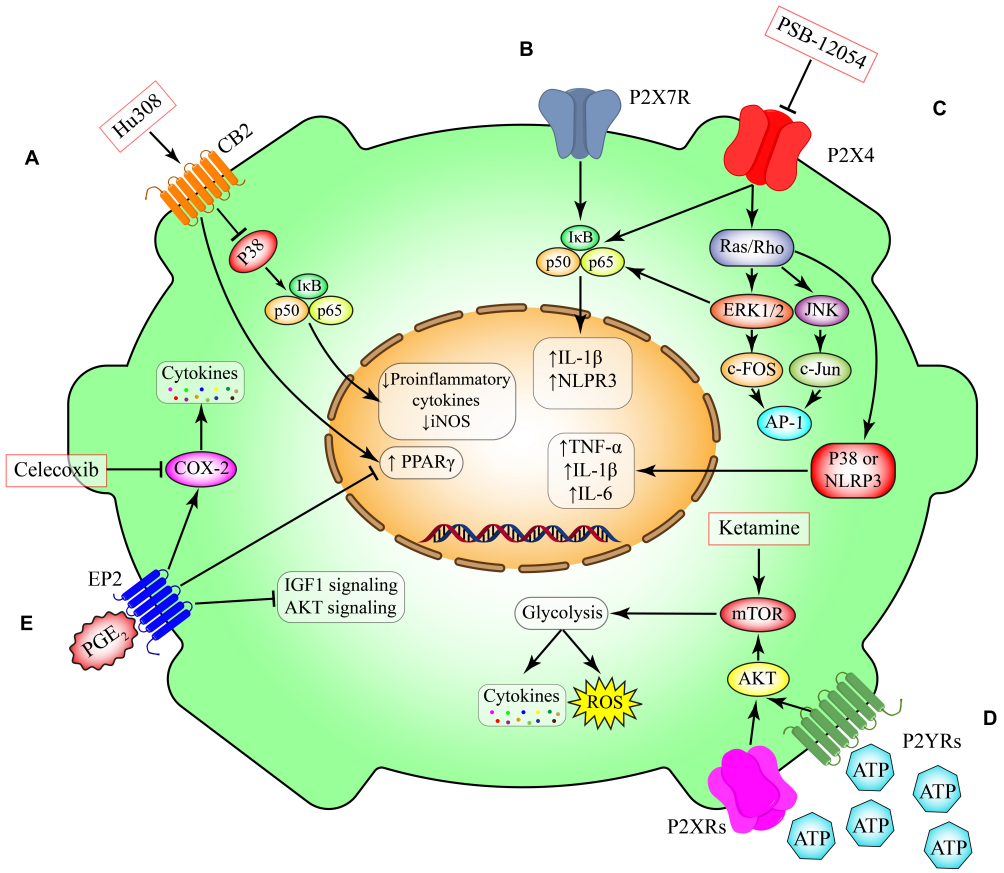

FIGURE 3 | Metabolic pathways in microglia. **(A)** Activation of CB2 receptors that are expressed in non-neural cells including microglia promotes anti-inflammatory cascades through inhibition of NFκB or induction of anti-inflammatory transcriptional factor such as peroxisome proliferator-activated receptor gamma (PPAR-γ). This pathway can be induced by Hu308. **(B)** P2X7 receptor activation, induces the canonical pro-inflammatory transcriptional factors such as NFκB and subsequent production of inflammatory mediators such as IL-1 beta and NLPR3. **(C)** Activation of ligand gated ion channel P2X4 triggers the switching on two canonical pathways including NFκB and Ras/ERK/JNK. These proteins induce the production of several cytokines such as TNF-α, IL-beta and IL-6. This pathway can be inhibited by PSB-12054. **(D)** Induction of the G-protein coupled receptors P2YRs have essential roles in modulating the expression of metabolic pathways such as mTOR and their downstream glucose metabolism. The induction of glycolysis through mTOR has been implicated in production of several cytokines and chemokines. Ketamine triggers the mTOR pathways leading to induction of glycolysis. **(E)** Prostaglandin E2 (PGE 2) is a lipid mediator derived from the fatty acid arachidonic acid. Its interaction with the microglial G-protein coupled receptor EP2 induces the activity of cyclooxygenase-2 (COX-2) and inhibits several intracellular pathways including PPAR-γ, AKT and IGF1. Celecoxib is s selective COX-2 inhibitor. CB2, cannabinoid type 2 (CB2) receptor; P2X4, P2X purinoceptor 4; P2X7, P2X purinoceptor 7; P2YR, purinergic receptor P2Y; EP2, prostaglandin E2 receptor 2; PGE2, prostaglandin E2; IGF1, insulin-like growth factor 1; AKT, RAC(Rho family)-alpha serine/threonine-protein kinase; COX-2, cyclooxygenase-2; TNF-α, tumor necrosis factor-α; iNOS, inducible nitric oxide synthase; IL-1β, interleukin 1 beta; IL-6, interleukin 6; PPAR-γ, peroxisome proliferator- activated receptor-gamma; mTOR, mammalian target of rapamycin; AP-1, activator protein 1; JNK, c-Jun N-terminal kinases; NLRP3, NLR family pyrin domain containing 3; ERK, extracellular-signal-regulated-kinase; IκB, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; ROS, reactive oxygen species.

Clinical investigations also revealed that adjunctive therapy with non-steroidal anti-inflammatory drugs, known as COX inhibitors, might have therapeutic effects in a subset of MDD patients (251, 252).

Human studies have shown decreased dopamine metabolites in the CSF of MDD patients. Furthermore, acute treatment with antidepressants induces dopamine release in the medial PFC (253, 254). These findings imply that activation of the mesocortical dopaminergic pathway has anti-depressive properties. In this context, the role of PGE2 has been studied in several experimental paradigms. It has been shown that EP1-deficient mice showed hyperdopaminergic activity, leading to impulsive behaviors under acute social and environmental stress (255). It is noteworthy that EP1 is located on GABAergic terminals on midbrain dopamine neurons and electrophysiological recording indicates that EP1 stimulation potentiates inhibitory synaptic inputs to these neurons (256). These findings suggest that PGE2-EP1 signaling suppresses midbrain dopamine neurons and regulates impulsive behaviors under acute stress (257). However, the mechanisms underlying involvement of the mesocortical dopaminergic pathway in vulnerability to repeated stress is unknown. Indeed, PGE2-EP1 signaling attenuates mesocortical dopaminergic pathway, leading to susceptibility of mice to RSD (254). Analyses of c-Fos

expression of ventral tegmental area dopamine neurons and dopamine turnover in medial PFC showed that the mesocortical dopaminergic pathway is activated upon social defeat and attenuated with repetition of social defeat in wild-type mice. EP1 deficiency abolished such repeated stress-induced attenuation of mesocortical dopaminergic pathway (254).

Intriguingly, PGE2 acting on striatal medium spiny neurons has been suggested to elicit a negative affective state in response to inflammatory or social stress (254, 258). Our knowledge about the source of PGE2 was limited until recently. Klawonn et al. revealed that microglial PG signaling as critical for inflammation-induced aversion and is a potential mechanism by which different types of stressors may converge to produce a negative affective state and potentially depression (259). Indeed, these findings demonstrated that striatal microglial activation induces negative affect and both IL-6 and PG dependent signaling in microglia is critical for inflammation induced aversion. Chemogenetic activation of striatal microglia induces an aversive affective state while chemogenetic inhibition of microglia blocks inflammation induced aversion. Microglial IL-6 signaling and PG synthesis regulate affective state and finally PGE2 from activated microglia reduces the excitability of striatal neurons (259). In agreement with these findings, different investigations indicate that low-dose aspirin, which primarily inhibits COX-1 and consequently PG production, reduces the risk of depression (260). The inducible form of COX, COX2, might also be involved in the MDD pathology, since the COX-2 selective inhibitor celecoxib has beneficial effects in subset of depressed patients (83, 261). Since strong microglial COX-1 expression is complemented by COX-2 in response to chronic inflammation and stress (262, 263), both enzymes could contribute to depressive symptoms at different stages of the disease or in distinct patient groups (259).

Mammalian Target of Rapamycin Signaling in Microglia

The mammalian target of rapamycin (mTOR), the evolutionarily conserved serine/threonine protein kinase, may be activated by phosphorylation in response to growth factors (such as BDNF), mitogens and stress (264, 265). The mTOR signaling pathway plays a fundamental role in the regulation of protein synthesis, energy metabolism, lipid metabolism, cell growth and autophagy (266). In the CNS, mTOR is also involved in axonal sprouting, axonal regeneration and myelination, ionic and receptor channel expression, dendritic spine growth, as well as astrocyte migration and proliferation. mTOR-regulated processes in the brain influence neuronal excitability, neuronal survival, synaptic and behavioral plasticity, cognition, feeding, and control of circadian rhythm (265). In recent years, special attention has been given to the role of mTOR signaling in MDD. Several investigations have reported decreased brain mTOR activation in animal models of depression (267). One of the most studied models is CUS, which mimics several behavioral and neurochemical alterations that occur in depressed individuals (268). Rodents exposed to CUS exhibit depressive-like behaviors associated with a reduction in phosphorylation levels of mTOR and its downstream signaling components, such as phosphor-p70S6K, in the PFC, hippocampus and amygdala (268, 269). Regarding the anti-depressive role of the mTOR pathway, an elegant study by Li et al. revealed a single dose of ketamine can activate mTOR, resulting in increased PFC synaptic

TABLE 3 | Summary of cannabinoid receptors involvement in stress response.

Study	Stress paradigm	Animal model	Treatment	Major findings
(239)	Immobilization/acoustic stress (2 h/day for 4 days)	Male Swiss ICR mice (WT and $CB1^{-/-}$ KO)	CB1 agonist: arachidonyl-2′-chloroethylamide (ACEA) (2.5 mg/kg, daily before stress, intraperitoneally)	WT: stress (1) ↑ CB1 mRNA and protein, (2) ↑ TNF-a mRNA (3) ↑ MCP-1, (4) ↑ NOS-2. ACEA pretreatment is protective against neuroinflammatory response to stress. KO: stress (1) dysregulates HPA axis, (2) ↑ TNF-a, (3) ↑ MCP-1. In sum: pretreatment with CB1 agonist is protective and $CB1^{-/-}$ KO aggravates neuroinflammation after stress.
(240)	Repeated social defeat (2 h/night for 6 nights)	Male C57BL/6 and aggressor CD-1 mice	CB1/2 agonist WIN55,212-2 (WIN) (1 mg/kg, daily 30 min before stress, intraperitoneally)	WIN (1) ↓ stress-induced anxiety, (2) ↓ IL-1 beta in $CD68^{+}$ microglia (3) prevented stress-induced prolonged fear response and repaired fear extinction
(241)	Chronic unpredictable mild stress (several times a day for 7–8 weeks)	Male Swiss ICR mice (WT and CB2 overexpressors)	CB2 antagonist (6-iodo-2-methyl-1-(2-morpholinoethyl)-1H-indol-3-yl) (4-methoxyphenyl)methanone (AM630). (1 mg/kg, twice daily, post-stress, for 4 weeks, intraperitoneally)	WT: stress (1) induced depression, (2) ↓ BDNF in hippocampus. CB2 overexpressor: (1) ↓ susceptibility to depression (2) no change in BDNF. Chronic AM630 treatment acted as an anti-depressant in stressed mice, protected against BDNF reduction.
(242)	Mild CSD stress (1–2 min/day for 10 days)	Male WT and B6.cg $Cnr1^{tm1Zim}$ *Cnr1*$^{-/-}$ KO mice Male CD-1 aggressor mice	–	KO: (1) ↑ susceptibility to mild CSD stress, (2) ↑ CD11b on microglia (3) ↑ percentage of $CD11b^{+}$ microglia (4) ↓ microglial complexity.

WT, wild-type; KO, knockout; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; ↑, increased; ↓, decreased/reduced; HPA, hypothalamic-pituitary-adrenal; MCP-1, monocyte chemoattractant protein-1; NOS-2, nitric oxide synthase-2; BDNF, brain derived neurotrophic factor.

protein expression within 2 h and increased dendritic spine density and synaptic activity within 24 h (267). Importantly, clinical evidence also confirms the role of mTOR signaling in MDD pathology (270). It has been shown that mTOR, p70S6K, eIF4B, and p-eIF4B protein expression in PFC of deceased MDD subjects were reduced when compared with controls, indicating a deficit in mTOR-dependent signaling leading to impairment in its downstream targets that control translation of synaptic proteins (270).

Intriguingly, mTOR signaling can regulate several aspects of microglial function such as phagocytosis and cell survival (271). For instance, inhibition of mTOR diminishes the viability of primary cultured microglia (272), whereas induction of mTOR activity by inhibiting its upstream suppressor, tuberous sclerosis 1, enhanced phagocytosis in microglia (271, 273). Furthermore, microglial-specific inhibition of mTOR pathway decreases proinflammatory cytokines and chemokines (274). More recently, it has been shown that mTOR-mediated metabolic reprogramming shapes distinct microglial functions in response to LPS and ATP (271). Hu et al. showed that both LPS and ATP induced rapid activation of mTOR and glycolysis in microglia. Blocking either glucose metabolism or mTOR activity inhibits glycolysis significantly and mitigates LPS-induced production of proinflammatory cytokines, indicating that mTOR-driven glycolysis is required for the proinflammatory responses of LPS-primed microglia (271). Additionally, blocking mTOR activity not only inhibits glycolysis but also suppresses BDNF and TNF-α production in ATP-activated microglia, suggesting the critical role of mTOR in tuning microglia function (271). Better understanding of the metabolic regulation of microglia help us to manipulate and control the activity of microglia following different neuroinflammatory insults. The distinct metabolic adaptation in microglia in response to different stressors may provide diverse approaches to target microglia at different states and restrain microglia-triggered neuroinflammation in neuropsychiatric disorders (271).

Our group provided the first evidence of increased microglial activation in dorsal ACC white matter of depressed suicides. Although total density of Iba1$^+$ microglia remained unchanged between depressed suicides and matched controls, the ratio of primed to ramified microglia was significantly increased in depressed suicides (10, 38). The mechanisms underlying microglial priming are unknown. However, it has been shown that the mTOR signaling pathway plays a crucial role in microglial priming during aging. Keane et al. were the first to show that microglia from aged mice have upregulated mTOR complex 1 signaling controlling translation and protein levels of inflammatory mediators (275). Genetic ablation of mTOR signaling in mouse microglia caused an NF-κB–dependent upregulation of priming genes at the mRNA level. However, mice displayed reduced cytokine protein levels, lessened microglial activation and milder sickness behavior. Similar changes were present in aged human microglia revealing that upregulation of mTOR-dependent translation is an essential aspect of microglia priming in aging (275). It is possible that abnormalities in microglial mTOR signaling are involved in the emergence of the primed microglial phenotype in MDD.

Purinergic Signaling in Microglia

ATP, adenosine di-phosphate (ADP) and adenosine are molecules that are involved in purinergic signaling through P1, P2X, and P2Y receptors. G-protein-coupled P1 receptors are selective for adenosine and there are four subtypes A_1, A_{2A}, A_{2B}, and A_3. Meanwhile P2 receptors are activated by both ATP and ADP. There are two types of P2 receptors, namely P2X ionotropic channels and P2Y G-protein-coupled receptors (**Figure 3**). P2X receptors have seven subtypes while P2Y have eight subtypes. Microglia express several purinergic receptors (276) including: P2X1, P2X4, P2X7 (277), P2Y4, P2Y7, P2Y6, P2Y11 P2Y12, P2Y13, A_1, A_{2A} and A_{2B} (278). Notably, adenosine binding to A_{2A} has been shown to mediate microglial process retraction (279). Besides microglial motility, purinergic signaling is important in many other processes such as neurodevelopment and neuron-glial crosstalk and inflammation (280). Interestingly, many aspects of the purinergic signaling system have been implicated in depression (281) and here, we will focus on the receptors expressed by microglia (**Table 4**).

ATP is released by injured cells into the extracellular space acting as DAMPs and a chemoattractant for microglial processes (233, 282, 283). Experimentally injected ATP causes microglia chemotaxis and process extension toward the site of injection (284). This process occurs through the G-protein-coupled P2Y (P2Y1R and P2Y12R) receptors (285, 286). P2Y12R is involved in long-range communication between neurons and microglia (287, 288), as well as microglia chemotaxis preceding phagocytosis (288). Additionally, P2Y12R acts as a marker for healthy microglia and is downregulated in the active pro-inflammatory phenotype in mice (289). There seems to be a species difference in the expression of P2Y12R as human brain resection from epileptic temporal lobe shows PY212R in both ramified and amoeboid microglia (290). Furthermore, the same tissue shows ADP stimulating process retraction (291) conversely to process extension in mouse microglia (284, 289). Although similar methods of comparative investigation have not yet been applied to all purinergic signaling, these findings alone suggest that they may differ greatly between species, providing necessary context to rodent studies. Loss of P2Y12R is associated with alteration in recognition and social memory as well as anxiety-like behavior in adult mice (292). Also, microglial P2Y12R regulates neural excitability and fear behaviors in developing and adult mice (293). Intriguingly, a human postmortem study found increased levels of P2Y12R protein in MDD microglia from four different brain regions, suggesting more resting state microglia in MDD and contradicting the neuroinflammation hypothesis (98).

Activation of microglial P2X4R by ATP (**Figure 3**) leads to a phenotype switch to pro-inflammatory state (276). Interestingly, in an ischemic stroke model, deletion of P2X4R from myeloid cells was shown to be protective in mice by inhibition of excessive pro-inflammatory cytokine release. However, after 30 days, the mice developed depressive-like phenotypes possibly due to missing P2X4R's crucial role in BDNF release (294).

Once microglia are activated, cytokine secretion is mediated by P2Y6R (295) and P2X7R. Activation of P2X7R by ATP acting as DAMPs signal leads to oligomerization of NLR3P

TABLE 4 | Summary of microglial purinergic signal transduction.

Receptor	Type	Ligand	Role
A_{2A}	G-protein coupled receptor	Adenosine	Process retraction (279)
P2Y1	G protein coupled receptor	ADP	Migration and chemotaxis (285, 286)
P2Y6	G-protein coupled receptor	Uridine	Mediates cytokine secretion (295)
P2Y12	G-protein coupled receptor	ADP	Specifically expressed in microglia (288). Migration and chemotaxis (285, 286, 289). Neuron-microglia interactions (287, 288, 293). Regulates innate fear behaviors (293)
P2X4	Ligand gated ion channel	ATP	Phenotype switch to pro-inflammatory (276) Drives release of BDNF (294)
P2X7	Ligand gated ion channel	ATP	Mediates cytokine secretion (295) Oligomerization of NLR3P inflammasome (296)

BDNF, brain derived neurotrophic factor; NLR3P, NLR family pyrin domain containing 3.

inflammasomes (**Figure 3**) (296). NLR3P inflammasome cleavage of pro-caspase 1 into caspase 1 results in the production of IL1-β from these activated microglia in situations of neuroinflammation such as in depression (297–299). In fact, a 2017 study showed that a 3-week CUS paradigm induced a depressive phenotype, increased extracellular ATP leading to P2X7R activation; this was inhibited by chronic treatment with P2X7R antagonists Brilliant Blue G (BBG) and A438079. Furthermore, P2X7R null mice that underwent the CUS did not develop a depressive phenotype irrespective of sex (300). In fact, the antidepressant phenotype of P2X7R deletion (301, 302) might be mediated by increased neurogenesis and serotonin bioavailability in the hippocampus (303). In humans, studies have suggested that polymorphisms Gln460Arg (rs2230912) (304) and His155Tyr (rs208294) in the *P2RX7* gene are associated with depression and other mood disorders (305, 306). However, a 2014 meta-analysis has contradicted these findings (307).

In the healthy brain, P2X7R is not activated by physiological ATP levels, in fact, P2X7R activation only occurs when ATP acts as DAMPs (308). Intriguingly, P2X7R antagonists that cross the BBB are in clinical trials with the hopes of using them as adjunct or monotherapy for depression caused by neuroinflammation (309, 310). Interestingly, cholesterol and other lipids like phosphatidylglycerol and sphingomyelin modulate the function of P2X7R (311). Our lab and others have found altered brain lipids in psychopathology (312) and depression (313–315) suggesting lipid control of P2X7R should be further investigated. Generally, lipids in the cell membrane organize position and function of proteins. They can be also released from the membrane acting as messengers transmitting signals (316). Lipid composition of the brain affects cognition, perception and mood most likely by influencing neuroinflammation and neurogenesis (317). Moreover, decreased polyunsaturated fatty acids seem to be the most frequently implicated lipid change in depression (313, 317) and they are known in regulating CB1 receptor (317) and P2X7R (311, 318). Interestingly, dietary supplementation with fish oil rich in polyunsaturated fats decreases depressive-like behavior in LPS treated mice and suppresses activation of the NLRP3 inflammasome and P2X7R (318). Meanwhile, sphingolipids have been implicated in both the etiology of MDD and the beneficial effects of antidepressants (316, 319). In addition to diet (320), lipid composition in the brain can be altered by environmental factors including exercise (321) and medications (322), making them promising targets for treatment (319).

To our knowledge, there is a paucity of studies on the role of microglial adenosine receptors in depression. However, A1 receptor activation generally inhibits serotonin release in the hippocampus, while A2 receptor activation promotes the release of this neurotransmitter (323). Moreover, A2 receptors have been found to be involved in the dysregulation of the HPA axis observed in depression (324). Furthermore, a 2021 study showed that 14 days of restraint stress induced depression and anxiety in the mice with a neuroinflammatory phenotype. The authors discovered increased expression of P2X7R and A_{2A} receptor in the hippocampus and prefrontal cortex. Next, they showed that BBG a P2X7R-selective antagonist and caffeine an A_{2A} receptor antagonist attenuated the depressive phenotype (325).

Iron Metabolism in Microglia

Iron metabolism is crucial for normal functioning of the brain, including myelination by oligodendrocytes and neurotransmitter synthesis (serotonin, dopamine, and norepinephrine) (326). Transferrin, a glycoprotein is mainly responsible for the movement of iron through the body and has high affinity to Fe^{3+} (ferric iron) (327). Movement of iron from the periphery into the brain occurs through the BBB. Transferrin receptor 1 (TfR1) on endothelial cells mediates the uptake of transferrin bound iron (Tf-Fe) into the brain, while also regulating the return of iron-depleted transferrin back to the blood (326, 328). Tf-Fe is packaged into endosomes and then released into the brain through ferroportin 1 (Fp1) as Fe^{2+} where it is oxidized to Fe^{3+} through ferroxidases hephaestin or ceruloplasmin (Cp) (326, 329). Fp1 levels are controlled by hepcidin (HepC) which binds to Fp1 leading to its internalization and subsequent degradation (330). The metabolism of iron in the brain is highly controlled as it is extremely important for proper cell functioning, however, iron overload can be extremely toxic and cause cell death through oxidative stress (329). In the brain, iron is distributed heterogeneously with concentration varying by region (331, 332) and cell type (333). Iron is mainly distributed in the hippocampus (331) and the basal ganglia stored in

ferritin (334) or neuromelanin (335). Presumably all cell types can uptake iron through either Tf-Fe or non-transferrin bound pathways. Neurons and astrocytes uptake Tf-Fe through TfR1 and divalent metal transporter 1 (DMT1). Oligodendrocytes uptake iron through the TfR1/DMT1 pathway only in immature states; once they mature, iron enters through heavy (H-) chain ferritin binding to H-ferritin receptor on the cell membrane (336). Oligodendrocytes have very high iron necessity for their normal functioning and proper myelination (333, 336). Microglia have two different ways of regulating iron influx; the first being through Tf-Fe uptake via the TfR1/DMT1 pathway while the second is uptake of non-Tf-Fe through transferrin-independent mechanisms (326). In healthy brain, iron usually travels bound to transferrin, which is produced by oligodendrocytes and ChP, however, it is only secreted by the latter (337).

Pathogens such as bacteria utilize iron from the host to replicate. To protect the host, immune cells are programmed to reduce iron availability to prevent further infiltration by the invader (338). This happens through increased production of HepC in hepatocytes, stimulated by IL-6 release from immune cells (339) which reduces the export of iron into the blood by negative regulation of ferroportin (340). It has been shown that different activation status in microglia prefer one iron intake pathway over the other (341). In neuroinflammatory conditions, iron is sequestered in pro-inflammatory microglia and neurons through non-transferrin bound pathways (342). *In vitro* LPS stimulation of microglia results in downregulation of TfR1 (338, 343) and ferroportin (338) and upregulation of DMT1 and ferritin (344). Under this condition, iron accumulates intracellularly and secretion of inflammatory cytokines and metalloproteinases is increased (343). Conversely, when microglia are stimulated with IL-4, an anti-inflammatory phenotype of microglia prefers Tf-Fe uptake and increases cellular transferrin receptor levels (344).

The WHO estimates that 37% of females are iron deficient globally, while males are rarely diagnosed (345). Both iron deficiency and depression were in the top disorders for most years lived with disability globally (346). Interestingly, females are twice as more likely to be diagnosed with MDD. Recent literature has implicated iron dysregulation, specifically iron deficiency, in the pathology of depression (347–351). Indeed, a recent study found that depressed individuals are 3 times more likely than healthy individuals to have hypoferritinemia (low iron storage) and acquired hypotransferrinemia (decreased levels of protein transferrin) (351). The fact that iron is a co-factor for rate limiting steps of serotonin, dopamine and norepinephrine synthesis and neuronal uptake further implicates involvement of iron metabolism in the etiology of depression (351, 352). Whether iron deficiency detected in the periphery is adequately representative of iron deficiency in the brain is still under speculation due to the tight control of iron metabolism in the brain (352). However, a 2007 study by Vostrikov et al., reported significantly reduced numbers of oligodendrocytes in layer 3 of BA9 in the PFC of postmortem samples from MDD patients (353). Given that oligodendrocytes hold the most iron in the brain, it is reasonable to infer decreased iron levels in postmortem MDD brain. Moreover, research from our lab has implicated oligodendrocyte precursor cell dysregulation with MDD (354). Iron deficiency has been shown to lead to hypomyelination in both animals and humans (355). A 2018 study revealed that developmental iron deficiency kept not only oligodendrocyte lineage cells in immature states but also other glia cells. Interestingly, iron deficiency inhibits microglial inflammatory cytokine secretion after LPS stimulation (356). Additionally, it has been shown that microglia constitute the primary source of iron to oligodendrocyte precursor cells during myelination (357).

Considering that microglia are involved in oligodendrocyte differentiation and myelin repair (358), it is a logical next step to investigate the role of iron metabolism in microglia and the relationship to depression. Few studies have investigated the role of microglia iron metabolism and depression and the ones that examined this relationship in animals found a different trend than the aforementioned human studies. Gao et al. showed that CSD stress resulted in depressive phenotype, increased microglial activation, increased iron, decreased Fp1 and increased ferritin, DMT1 and HepC in the hippocampus (359). Similarly, Jiao et al. found that mice with CUMS-induced behavioral despair were found to have increased brain iron, increased inflammation and Fe^{2+} levels in the hippocampus. Genes involved in ferroptosis were differentially regulated; specifically, glutathione peroxidase (GPX4, necessary for repairing oxidative damage) was downregulated after CUMS suggesting a role of ferroptosis in the phenotype of depression. In addition, this study also showed that fluoxetine not only resolved depressive behaviors but also restored normal ferroptosis signaling (360). Experimental models also display an increase in iron load in the brain which results in anxiety symptoms. A study by Texel et al. shows that Cp knockout mice have iron overload in the periphery, but lower levels of iron in the hippocampus accompanied by decreased levels of 5-HT, norepinephrine and BDNF (361). In another study, Pellegrino et al. showed that knockout of transferrin receptor 2 leads to iron overload in the brain, dysregulation of microglia activation status and increased anxiety levels in the animals (362). Overall, regulation of iron metabolism by microglia is an important factor in brain physiology and pathophysiology. Future studies are needed to investigate the postmortem brain of depressed individuals to better understand the link between iron metabolism abnormalities in brain innate immune system and MDD pathology.

SEXUAL DIMORPHISM IN MICROGLIAL RESPONSE IN HEALTHY BRAIN AND FOLLOWING STRESS

In recent years, special attention has also been given to the sexually dimorphic role of microglia in both healthy neurodevelopmental and diseased neurodegenerative brains (15). Sex differences have been reported in distribution, structure, function, transcriptomic and proteomic profiles of microglia in both physiological and neuroinflammatory conditions (10). Evidence demonstrates that microglia play an instrumental role in the sexually dimorphic differentiation of the developing brain (363). Interestingly, the number and phenotype of microglia

differ in many regions of the brain when comparing male and female rodents (364). Brain areas such as the preoptic area, cerebral cortex and the amygdala and hippocampus have been reported to have microglial sexual dissimilarity (363–366). Importantly, the postnatal sexual dimorphism persists into the adult brain. These marked differences may lead to development of distinct, sex-dependent microglia inflammatory responses in pathological conditions. In fact, sex differences in microglial activation patterns following neuroinflammation have been reported by different groups (367, 368). However, whether the observed differences are exclusively hormone-dependent and/or stem from distinct developmental mechanisms remains to be elucidated. The analyses of the different anti- and pro-inflammatory signaling events reveal that they differ in males and females.

Several observations suggest marked sex differences in the microglial activation patterns following stress. In a pioneer study, Bollinger et al. revealed differential effects of stress on microglial activation in female and male medial PFC (369). Importantly, dysfunction of this brain area is implicated in MDD pathology. It has been shown that chronic stress affects the medial PFC in a sex-dependent manner and impairs prefrontal mediated behaviors in males and females (369). Unstressed female rats show a greater proportion of primed to ramified microglia relative to males, alongside increased CX3CL1-CX3CR1 levels. In addition, acute stress and CRS diminished the ratio of primed to ramified microglia and microglial CD40 expression in females but not in males (369). Another intriguing study revealed that the expression of genes related to cellular stress, neuroimmune state and neuron-microglia communication varied between unstressed male and female rats in a region-specific manner (370). Namely, in the dorsal hippocampus, chronic stress increased immune markers expression in males but not females. It is noteworthy that the type of stress can also mediate sex-specific innate immune response. For instance, acute stress increased microglia-associated transcripts in basolateral amygdala in males, whereas chronic stress altered immune factor expression in basolateral amygdala more broadly in females (370). It was recently shown that chronic stress induces different neurobiological adaptations in the PFC of male and female mice. CUS causes behavioral changes and microglia-mediated neuronal remodeling only in the frontal cortex of male mice (371). Fluorescence-activated cell sorting and gene expression analyses by Woodburn et al. indicate that CUS increased expression of markers of phagocytosis only in male PFC microglia (371). Overall, these findings suggest that sex differences might impact microglia pro- and anti-inflammatory signaling pathways, leading to different outcomes.

CONCLUSION

Cytokine research in MDD faces several difficulties, such as conflicting results and high variability of cytokine levels within samples. Several studies have only reported serum or plasma cytokine levels without taking into account potential confounding factors such as age, body weight, smoking, alcohol consumption and medication, which can all influence the plasma content of cytokines (372, 373). These limitations imply that serum levels of cytokines may not reflect their levels in the brain and, therefore, may not reflect pathology (373, 374). Direction of causality is another important factor that is not clear in the cytokine research in psychiatric disorders. Although there is strong evidence for the involvement of cytokines in the pathology of MDD, the direction of this relationship has not yet been clarified with certainty. For instance, the alteration of cytokine levels might also be the consequence of a psychiatric disorder. Factors such as antidepressants or body mass index (that could change as a result of the disorder) might cause fluctuation in cytokine levels (373, 375).

An association between neuroinflammation and MDD has been reported by multiple studies (10) and it is well-established that inflammatory markers are increased in a subset of MDD patients (84, 85, 376) but not all (10, 21). Although microglial abnormalities have been reported in MDD, no link has been made with specific diagnostic categories. It is noteworthy that recent attempts at targeting inflammation as a new therapy for MDD have not been very promising. In fact, it appears that anti-inflammatory treatment options may only be effective in patients who show signs of increased peripheral inflammation along with depressive behaviors (83, 84). Namely, it has been shown that antagonizing TNF-α as a therapy may only prove useful in patients with high baseline inflammatory biomarkers (85). With similar uncertainty, general immunosuppressants and anti-inflammatory agents such as minocycline and non-steroidal anti-inflammatory drugs that were promising in animal studies (10) have failed in treating neuropsychiatric disorders probably due to their non-selective dampening of the immune response. Targeting of pro- or anti-inflammatory brain cytokines for developing new pharmacotherapeutics is not easy. As discussed in detail in this review, these cytokines have essential physiological actions and blockade of their signaling might influence several crucial processes such as cell survival and neural plasticity. Furthermore, the diversity of brain cell types that produce cytokines makes it difficult to target their signaling in a neuroinflammatory context. Finally, different receptor subtypes have different roles following neuroinflammation (e.g., TNF-α and IL-6 receptors).

It is becoming increasingly obvious that microglia act as a double-edged sword in the etiopathology of MDD. On one hand, some experimental studies both *in vitro* and *in vivo* have linked neuronal damage with the inflammatory phenotype of microglia releasing neurotoxic mediators and ROS (377). On the other hand, other investigations have reported the beneficial roles of microglial anti-inflammatory phenotype in neuronal regeneration and neurogenesis. Whether microglia play a positive or negative role depends on several factors including type of neuroinflammatory insult, brain region, time, sex and age. Indeed, heterogeneity of microglia and their context-dependent properties warrant more research to gain a clearer understanding

of this dynamic cell type before new therapeutics can be successfully developed for psychiatric disorders such as MDD.

AUTHOR CONTRIBUTIONS

RR, CB, and NM conceived the review. RR, CB, and RC wrote the manuscript. NM supervised the project and revised the manuscript. All authors read and agreed with the final version of the manuscript.

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Brenda Penninx,
Academic Medical Center, Netherlands

REVIEWED BY
Patrizia Risé,
University of Milan, Italy
Maria Bove,
University of Foggia, Italy

*CORRESPONDENCE
Hanna C. Gustafsson
gustafha@ohsu.edu

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The association between heightened ADHD symptoms and cytokine and fatty acid concentrations during pregnancy

Hanna C. Gustafsson[1]*, Geoffrey A. Dunn[2], A. J. Mitchell[3], Kathleen F. Holton[4], Jennifer M. Loftis[1,3,5], Joel T. Nigg[1] and Elinor L. Sullivan[1,3,6]

[1]Department of Psychiatry, Oregon Health & Science University, Portland, OR, United States, [2]Department of Human Physiology, University of Oregon, Eugene, OR, United States, [3]Department of Behavioral Neuroscience, Oregon Health & Science University, Portland, OR, United States, [4]Departments of Health Studies and Neuroscience, American University, Washington, DC, United States, [5]VA Portland Health Care System, Portland, OR, United States, [6]Division of Neuroscience, Oregon National Primate Research Center, Beaverton, OR, United States

Objective: Previous research conducted with samples of children suggest that individuals with attention-deficit/hyperactivity disorder (ADHD) have altered fatty acid concentrations and may have increased systemic inflammation. Whether these differences are also apparent in other populations of individuals with heightened ADHD symptoms (e.g., pregnant adults) is unknown. The goal of the current study was to examine whether there are ADHD-associated differences in polyunsaturated fatty acid concentrations or pro-inflammatory cytokine concentrations during pregnancy, a developmental period when fatty acid concentrations and systemic inflammation have implications for the health of both the pregnant person and the developing child. We hypothesized that plasma levels of the ratio of omega-6s to omega-3s (n-6:n-3) and plasma inflammatory cytokine levels would be higher in individuals with heightened ADHD symptoms, consistent with previous findings in children with ADHD.

Methods: Data ($N = 68$) came from a prospective study of pregnant community volunteers who were oversampled for ADHD symptoms. During the 3rd trimester, plasma concentrations of fatty acids and the pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) were assessed. Dietary intake was examined in the 3rd trimester using three 24-h recalls conducted by trained dietitians and by examining plasma levels of conjugated linoleic acid (n-6) and α-linolenic acid (n-3), essential fatty acids that must come from dietary intake.

Results: The group with heightened ADHD symptoms had higher n-6:n-3s ($\beta = 0.30$, $p < 0.01$) and higher TNF-α concentrations ($\beta = 0.35$, $p < 0.001$) relative to controls. There were no group differences in dietary variables, as assessed by self-report and *via* plasma concentrations of essential fatty acids. IL-6 was not reliably associated with ADHD status in this sample.

Conclusion: Pregnant individuals with ADHD, on average, had higher plasma n-6:n-3s and higher TNF-α concentrations relative to controls. A difference was not detected in their dietary intake of fatty acids or other relevant nutrients. Though these null findings are inconclusive, they are consistent with the hypothesis that ADHD-associated differences in plasma fatty acid concentrations are the result of ADHD-associated differences in fatty acid metabolism, rather than simply differences in dietary intake.

KEYWORDS

ADHD, pregnancy, pro-inflammatory cytokines, tumor necrosis factor-α, interleukin-6 (IL-6), omega-3 fatty acids (n-3), omega-6 fatty acids (n-6)

Introduction

Interest in the nutritional correlates of attention-deficit/hyperactivity disorder (ADHD) has grown over the last two decades, with substantial attention paid to the role of polyunsaturated fatty acids (PUFAs). Most of this research has focused on whether omega(n)-3 fatty acid levels, either consumed in the diet or measured in circulation, differ between individuals with and without ADHD. Results generally suggest that ADHD is associated with lower plasma n-3 fatty acid concentrations (1–3) however differences in fatty acid concentrations between ADHD and control groups often are small in magnitude (1) and n-3 supplement trials aimed at treating ADHD have reported mixed results (1, 3–5). One proposed reason for the small observed differences between ADHD and control groups (1, 6) is that consideration of circulating levels of n-3 fatty acids alone is not a sufficiently comprehensive indicator of fatty acid levels, as it does not take into account concentrations of n-6 fatty acids, which compete for the same enzymes for elongation and desaturation (7). Thus, absolute levels of n-3 or n-6 fatty acid concentrations may not be as meaningful of a metric as the ratio of n-6 to n-3 fatty acids (n-6:n-3). A greater n-6:n-3 has been linked with increased systemic inflammation and with increased risk for diseases with inflammatory underpinnings (8–10), though few studies have empirically examined blood levels of fatty acids and inflammatory markers together in the context of psychiatric disorders [see (11, 12) for exceptions], and none to our knowledge have investigated these associations among individuals with heightened symptoms of ADHD.

Research looking at the n-6:n-3 in individuals with and without ADHD is more limited than studies of n-3s alone (there have been fewer than 10 studies to date), but these studies also support a picture of altered fatty acid status in ADHD (6, 13, 14). As is true for the n-3 studies, there is some heterogeneity in the sample types used (e.g., plasma vs. serum vs. red blood cells) as well as in which fatty acids were used to compute the n-6:n-3. Most studies report the ratio of all measured n-6s to n-3s (the specific fatty acids considered varies from study to study), while some also report the ratio of arachidonic acid (AA; an n-6) to eicosapentaenoic acid (EPA; an n-3; AA:EPA), selected because they are precursors to important inflammatory mediators (15) and because they are important biologically active n-6 and n-3 fatty acids in the brain (6). With these study design differences aside, the extant studies typically report that the n-6:n-3 is increased in individuals with ADHD, even in the absence of differences in absolute values of n-3s or n-6s [for exceptions see (4, 16)]. For example, Parletta et al. (8), using a sample of children from Melbourne, Australia, found that the AA:EPA and the n-6:n-3 in red blood cells were elevated in children with ADHD relative to controls (mean age = 9.10 years; 80% male). Utilizing data from a sample of children living in Taiwan, Wang et al. (13) found that the n-6:n-3 was elevated in the serum of children with ADHD relative to controls (mean age = 9.20 years; 86% male). In another study, Stevens et al. (17), using a US sample (mean age = 9.10 years; 100% male), found that males with ADHD had greater plasma (but not red blood cell) n-6:n-3s, even though they did not differ in their absolute levels of n-6 fatty acids. Though these studies offer important preliminary information about the n-6:n-3 and ADHD, a number of important questions remain.

First, while the previous studies on this topic are informative, they have been conducted almost exclusively with samples of children or adolescents and have utilized data from samples that are predominantly male. While this focus on male youth is consistent with the known demographics of ADHD [which is more common in males (18) and is typically diagnosed in childhood], these demographics are not representative of all individuals with ADHD, and thus it is unclear if these results generalize to other populations with ADHD (e.g., adult females). ADHD is a neurodevelopmental disorder that is commonly diagnosed in childhood, however in about two-thirds of cases, clinically significant or impairing symptoms persist into adulthood (19, 20), making this an important population to study. Evaluating whether other subgroups of individuals with ADHD also show evidence of altered fatty acid concentrations

is important, as this information may give further insight into the etiology and pathophysiology of ADHD, and because this information may help with the design of treatments that have potential to help a wider range of individuals.

Arguably, if there were to be a group of individuals with ADHD that these results would be least likely to extend to, it would be pregnant individuals. Previous research shows that there are sex differences in fatty acid synthesis that are the result of sex hormone-induced alterations in the expression of desaturase and elongase enzymes (21, 22); these differences, however, have typically been studied in non-pregnant populations. They are likely to be exaggerated during pregnancy given the normative and marked increase in sex-hormones that accompanies the pregnant state (23). Indeed, pregnancy is typically characterized by hyperlipidemia (24, 25), which appears to intensify in correspondence with pregnancy-related hormonal changes, and may be responsible for the depletion in long-chain fatty acid concentrations that appears to occur starting in the second trimester (26–28). These pregnancy-specific changes in fatty acid metabolism make our primary research question (i.e., whether the n-6:n-3 differs in pregnant individuals with and without ADHD) a non-trivial extension of previous studies in this area.

Second, there exists ambiguity as to whether ADHD-associated differences in the n-6:n-3 in plasma reflect differences in fatty acid metabolism or if they are due to ADHD-associated differences in dietary intake (either in the form of diet or n-3 supplementation). One hypothesis is that these disparities are the result of metabolic differences (29); this hypothesis is supported by research that suggests that there are ADHD-associated differences in fatty acid desaturase genes and phospholipid metabolism genes (30, 31). However, few studies have examined the n-6:n-3 in ADHD alongside measures of dietary intake, and the existing studies have yielded somewhat mixed results. While two studies (4, 32) suggest that youth with and without ADHD do not differ in their dietary intake of relevant fatty acids, at least one study reported that children with ADHD consumed less healthful foods, including more frequent consumption of high-fat and high-sugar foods, assessed using a food frequency questionnaire (13). This question has not yet been addressed during pregnancy, a time when dietary patterns may change to accommodate the nutritional requirements needed to support the developing child, and that may change in response to physiologic changes associated with food aversions, cravings, nausea, vomiting, tiredness, and heartburn (33). Importantly, the circulating fatty acid concentrations of the pregnant individual has implications for the health of both the pregnant person and the developing fetus (26–28).

To examine dietary-intake differences in individuals with and without ADHD, the gold standard is 24-h recall data conducted by trained dietitians. A second source of information about dietary intake comes from examining blood levels of essential fatty acids (those that cannot be synthesized by the human body, and thus must come from diet). Most relevant here are the essential fatty acids linoleic acid (n-6) and α-linolenic acid (n-3) (34). If n-6:n-3 differences in plasma are observed in the absence of differences in dietary intake (assessed either *via* recalled dietary intake or by concentrations of essential fatty acids), this would be consistent with the hypothesis that there are ADHD-associated differences in the metabolism rather than the consumption of fatty acids.

The third remaining question revolves around the relations among fatty acid concentrations, systemic inflammation, and ADHD. Increased systemic inflammation has been implicated in the etiology and pathophysiology of ADHD (35–37), yet limited empirical attention has been paid to the association between ADHD and inflammation in humans. The evidence that does exist generally suggests that, relative to controls, children (38–40) with ADHD have higher circulating levels of pro-inflammatory cytokines, including interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α). Whether these differences in cytokines are also apparent in pregnant individuals with ADHD remains untested, despite evidence that these cytokines are elevated in the context of other psychiatric symptoms during pregnancy (e.g., depression) (41, 42). There are normative changes in immune system functioning that occur during pregnancy to support healthy fetal growth and development (43, 44)), which again makes it challenging to extend the findings of previous research to this population. Additionally, although the n-6:n-3 has been shown to be related to greater inflammation in non-pregnant adults (45–47), its association with inflammation in pregnancy, and in the context of ADHD, has not yet been explored. This is important because it would have implications for both intervention and even for theories of mechanisms of intergenerational transmission.

To address these outstanding questions, the goal of the current study was to test:

1. Does the ratio of n-6s to n-3s in plasma differ in pregnant individuals with and without heightened symptoms of ADHD?
2. Are there differences in dietary intake of fatty acids (assessed using 24-h dietary recall and/or essential fatty acid concentrations) between individuals with and without heightened symptoms of ADHD?
3. Do pregnant individuals with heightened symptoms of ADHD have higher plasma cytokine concentrations (IL-6 and TNF-α) than controls? Is the ratio of n-6s to n-3s in plasma related to greater cytokine concentrations?

We hypothesized that, on average, individuals with heightened symptoms of ADHD would have higher plasma n-6:n3s and higher plasma concentrations of IL-6 and TNF-α. We also hypothesized that a higher n-6:n-3 would be positively

correlated with inflammation. Consistent with the hypothesis that there are fatty acid conversion or metabolic differences in ADHD, we hypothesized that there would be no ADHD-associated differences in dietary intake or in essential fatty acid concentrations.

In testing our hypotheses, we controlled for whether the pregnant individual reported that they were currently consuming a fish oil or fatty acid supplement. In models that considered inflammation, we also controlled for pre-pregnancy adiposity. These variables were selected as covariates *a priori*, given presumed effects of fatty acid supplementation on plasma fatty acid concentrations, and given the large literature linking adiposity and pro-inflammatory cytokine concentrations (48, 49).

Methods

Participants and procedures

Data came from a prospective longitudinal study described previously (50, 51). The overarching goal of this study was to investigate prenatal and early life influences on offspring risk for psychopathology. Pregnant individuals ($n = 62$) were recruited through an urban hospital-based outpatient prenatal clinic in the second trimester of pregnancy and were followed into the postpartum period. After enrolling in the study with the first target child, six participants conceived a second child and completed all of the same assessments for their second pregnancy, resulting in data on six sibling pairs and yielding a final analytic sample of $N = 68$). This nesting of children within families was handled statistically (see Analytic Strategy for details).

To serve the goals of the overarching project, an effort was made to oversample for individuals who endorsed a current or childhood history of ADHD or high levels of current ADHD symptoms (see full description of ADHD status definition below). Exclusion criteria included high-risk or medically complicated pregnancy, extreme life circumstances (specifically, homelessness), being <18 years old, and active substance use (including alcohol, tobacco, marijuana, opioids, and cocaine). Data used in the current analyses came from laboratory visits when participants were 37 weeks pregnant. At this visit, participants provided a blood sample and reported on demographic information *via* surveys. Participants also reported on their dietary intake within 2 weeks of their laboratory visit. Participant medical records were reviewed for information about their health. Oregon Health & Science University's (OHSU) Institutional Review Board approved all procedures, and written informed consent was obtained from all participants.

Measures

Demographics

Pregnant person age (years) and highest completed education (1 = Grade School, 2 = Some High School, 3 = High School Equivalent, 4 = High School Degree, 5 = Some College but No Degree, 6 = Associates Degree, 7 = Bachelor's Degree, 8 = Masters, Law, 2–3 years degree, 9 = Doctorate, PhD, Medical Degree) were self-reported.

ADHD status

Pregnant person ADHD status was defined as a current or childhood history of ADHD or current elevated symptoms of ADHD, defined as >80th percentile on the Barkley Adult ADHD Rating scale (BAARS-IV) Quick Screen (52). Fifty-three percent of participants ($n = 36$) met this criterion. In this manuscript, we will refer to these individuals as the heightened ADHD symptoms (vs. control) group, given their history of or current levels of significant ADHD symptomatology, though we recognize that this shorthand does not adequately capture all of the nuance of our measurement.

Plasma fatty acids

Plasma fatty acids were assessed using blood samples collected in the 3rd trimester (34–37 weeks gestation). Blood was drawn by venipuncture into K_2 EDTA tubes (BD Vacutainer Systems, Franklin Lakes, NJ), centrifuged, and plasma was separated, aliquoted, and frozen at −80°C until assay. Plasma fatty acids were analyzed by direct transesterification using a Trace GC coupled to a DSQ mass spectrometer (ThermoElectron) as described previously (53). N-6 fatty acids included in this study were conjugated linoleic acid, γ-linolenic acid (C18:3), dihomo-γ-linolenic acid (C20:3), and arachidonic acid (AA; C20:4). N-3 fatty acids included in this study were α-linolenic acid (C18:3), eicosapentaenoic acid (EPA; C20:5), and docosahexaenoic acid (C22:6). The n-6:n-3 was captured two ways: (1) by summing all of the n-6 fatty acid concentrations and dividing that total by the sum of all of the n-3 fatty acid concentrations (n-6:n-3), and (2) the AA:EPA, calculated by dividing arachidonic acid concentrations by eicosapentaenoic acid concentrations.

Pre-pregnancy adiposity

Participants' medical records were reviewed for pre-pregnancy body mass index (BMI; kg/m^2).

Inflammation

Pregnant person inflammation was assessed using 3rd trimester plasma concentrations of IL-6 and TNF-α. Blood

used for these assays was collected at the same time as the blood used in the fatty acid assay (described above); blood was drawn, processed, and stored in the same manner. Blood draws were scheduled around participant prenatal care appointments. While this helped to minimize participant burden, it precluded our ability to completely standardized the exact time of day that the blood draw occurred. While most study visits were collected in the early to mid-morning, not all were.

Plasma concentrations of IL-6 were measured by enzyme-linked immunosorbent assays (Human IL-6 quantikine HS ELISA kits (HS600B; assay range: 0.2–10 pg/ml, sensitivity: 0.11 pg/ml), R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions and as described previously (41). All standards and samples were run in duplicate. Briefly, plasma samples were initially diluted 1:2 using Assay Diluent RD1-75 and incubated for 2 h at room temperature on a horizontal orbital microplate shaker. Following standard wash procedures, human IL-6 HS Conjugate was added to each well, and plates were incubated as described above. Plates were then washed and incubated with the Substrate Solution (60 min), Amplifier Solution (30 min), and Stop Solution. Plates were read within 30 min of adding the Stop Solution using a microplate spectrophotometer (Benchmark Plus microplate, Bio-Rad Laboratories, Inc., Hercules, CA).

Plasma concentrations of TNF-α (assay range: 5.3–3,900 pg/ml, sensitivity: 1.5 pg/ml) were assayed using Luminex polystyrene bead-based multiplex immunoassays (customized Luminex Performance Human Obesity Panel, FCST08-05; R&D Systems) according to the manufacturer's instructions and as described previously (41). This panel also assessed concentrations of monocyte chemoattractant protein-1 (MCP-1), however these values were not considered in this study because this cytokine has not been implicated in previous research comparing children with and without ADHD. All standards and samples were run in duplicate. Plasma samples were initially diluted 1:2 using the matrix solution provided, and samples were incubated overnight at 4°C with color-coded beads that were pre-coated with cytokine-specific capture antibodies. The plates were then washed by vacuum filtration, incubated with biotinylated detection antibodies (1 h, room temperature), washed, and incubated with phycoerythrin-conjugated streptavidin (30 min, room temperature). Plates were read on the dual-laser, flow-based Luminex 100 Analyzer (Luminex, Austin, TX). For both the ELISAs and multiplex assays, sample values were determined based on standard curves calculated using computer software to generate four- and five-parameter curve-fits, respectively (Prism 7 for Windows, GraphPad Software, Inc., La Jolla, CA).

Dietary intake

Diet during pregnancy was measured by three, non-consecutive 24-h diet recalls that were conducted over the phone by trained dietitians in the 3rd trimester of pregnancy. Trained staff, overseen by a dietitian certified in the procedure, used the multi-pass method (54) and Nutrition Data System for Research Software, developed by Nutrition Coordinating Center (University of Minnesota, Minneapolis, Minnesota) to record the previous day's food and drink intake and supplement use over the previous 30 days. The software facilitates the collection of recalls in a standardized fashion in a multiple-pass interview approach. The recalls were unscheduled and unannounced, including 1 weekend day and 2 weekdays, and took place over a 2-week period. At each time point, the dietitian inquired about food intake the day prior. All interviewers completed a training program and met qualification standards established in the Oregon Clinical and Translational Research Institute Bionutrition Unit using the Nutrition Data System for Research (NDSR) software (Nutrition Coordinating Center (NCC), University of Minnesota). Nutrient values were averaged across the three recalls to create average dietary intake values. In the current study, we examined total calories (kcal), total fat (g), percent of calories from fat (%), total polyunsaturated fatty acids (g), total n-3 fatty acids (g), saturated fatty acids (SFA) (g), monounsaturated fatty acids (MUFA) (g), and the PUFA-to-SFA ratio (PUFA:SFA).

Whether the participant reported taking a fish oil or fatty acid supplement during the previous 30 days (0 = not taking a supplement, 1 = taking a supplement) was also recorded. Individuals who endorsed taking a fatty acid supplement typically reported taking a supplement that contained n-3 fatty acids (e.g., DHA, EPA), though the specific ingredients varied between participants. Forty-one percent of participants reported taking a fish oil or fatty acid supplement in the previous 30 days.

Analytic strategy

Research questions were tested using M*plus* 8.5 (Muthén and Muthén, 1998–2017) using the robust maximum likelihood estimator. This estimator can accommodate non-normally distributed data. Missing data were handled using full information maximum likelihood (55). Non-independence of observations (i.e., the nesting of children within families) was handled using the M*plus Cluster* command.

Descriptive statistics (means, standard deviations, and percentages) for all study variables are presented in Tables 1, 2. In Table 2, we present raw cytokine concentrations for ease of interpretation, however these values were log-transformed prior to all analyses. In preliminary analyses, we compared the ADHD and control groups on all study variables using independent samples *t*-tests. These preliminary analyses were followed by formal hypothesis testing, which involved examining these same group differences while controlling for relevant confounds and accounting for the nesting of children within families.

TABLE 1 Sample demographic information ($N = 68$).

	Mean (SD)	Mean (SD)	Mean (SD)
	Total ($N = 68$)	**Control ($n = 32$)**	**ADHD ($n = 36$)**
Pregnant person age (Years)	30.49 (5.00)	31.77 (5.07)	29.35 (4.82)$^{+}$
Pre-pregnancy body mass index (BMI)	26.85 (6.83)	23.58 (2.73)	29.12 (8.04)**
Highest completed education[a]	6.72 (1.20)	7.41 (0.97)	6.15 (1.06)**
Average BAARS-IV score	9.98 (4.21)	6.41 (1.45)	13.03 (3.29)**

The ADHD and Control groups were compared to one another using t-tests. Significant and marginally significant groups differences based on these analyses are denoted in the table: $^{+}p < 0.10$, $^{**}p < 0.01$. [a]Education was reported as follows: 1 = Grade School, 2 = Some High School, 3 = High School Equivalent, 4 = High School degree, 5 = Some College but no degree, 6 = Associates degree, 7 = Bachelor's degree, 8 = Masters, Law, 2–3 years degree, 9 = Doctorate, PhD, Medical degree. BAARS-IV, Barkley Adult ADHD Rating scale Quick Screen.

Our hypotheses were tested using a series of linear regressions. To examine whether there were ADHD group differences in the n-6:n3 (Hypothesis 1), we ran two regression models. In the first model, the plasma n-6:n-3 was regressed on ADHD status and on a variable that captured whether the participant was taking a fatty acid supplement or not. In the second model, the plasma AA:EPA was regressed on ADHD status and fatty acid supplement status. Two regressions were used to test Hypothesis 2 (whether there are ADHD-associated differences in cytokine levels), one where TNF-α was regressed on ADHD status and on fatty acid supplementation status and pre-pregnancy BMI, and a second where IL-6 was regressed on the same variables. In four additional models, we examined the association between the n-6:n-3 and cytokine levels, where (a) TNF-α was regressed on the n-6:n-3, (b) TNF-α was regressed on the AA:EPA, (c) IL-6 was regressed on the n-6:n-3, and (d) IL-6 was regressed on the AA:EPA. Given the dearth of research relating these metrics in pregnant individuals (particularly in the context of ADHD), these models were run once without covariates and a second time, controlling for fatty acid supplementation and pre-pregnancy BMI to provide the full context for these results. To address Hypothesis 3 (whether there are ADHD-associated dietary differences), each 24-h dietary intake variable was regressed on the ADHD status variable and on fatty acid supplementation status. Additionally, concentrations of the essential fatty acids conjugated linoleic acid and α-linolenic acid were regressed on ADHD status (each essential fatty acid was considered in its own model), again controlling for fatty acid supplementation status.

Results

Descriptive statistics

Participants were on average 30.49 years old (SD = 5.0; range = 18–41.25 years) at enrollment. Ninety-one percent of the sample was non-Hispanic White (7% Native Hawaiian/Pacific Islander, 2% Multiple Races). The median completed education was a Bachelor's degree, with a range from some college but no degree to receiving a doctorate. See Table 1 for demographic information presented separately for the heightened ADHD symptoms and control groups.

Table 2 presents the raw means for all focal study variables, presented for the complete sample as well as separately for the heightened ADHD symptoms and control groups. Results from preliminary analyses that used *t*-tests and chi squared tests to compare the ADHD and control groups on these variables indicated that the ADHD group differed from controls for the following plasma concentrations: the heightened ADHD symptoms group had higher n-6:n-3s ($p = 0.04$), higher AA:EPAs ($p = 0.007$), lower EPA concentrations ($p = 0.03$) and higher TNF-α concentrations ($p < 0.001$). ADHD-associated increases in plasma AA ($p = 0.06$) and plasma total n-6s ($p = 0.07$) were marginally significant. None of the 24-h recall dietary data differed between heightened ADHD symptoms and control groups ($ps > 0.33$). The heightened ADHD symptoms and control groups also did not differ in the percentage of individuals who reported taking a fish oil or fatty acid supplement in the previous 30 days (34 vs. 53%, $p = 0.18$).

Hypothesis 1: Plasma fatty acid concentrations

The results of the regressions used to test Hypothesis 1 are presented in Table 3. The heightened ADHD symptoms group had a significantly higher n-6:n-3 than controls, as indexed by both the n-6:n-3 ($\beta = 0.30$, $p = 0.008$) and the AA:EPA ($\beta = 0.30$, $p = 0.001$). These estimates are adjusted for whether the participant was taking a fish oil or fatty acid supplement, which was not significantly related to the n-6:n-3 ($p = 0.72$) but was negatively associated with the AA:EPA ($\beta = -0.55$, $p < 0.001$) in these models.

In supplemental analyses, we also examined whether the ADHD-associated differences in the n-3 and n-6 fatty acids listed in Table 2 were still present when we controlled for fatty acid supplementation. Results indicate that plasma EPA concentrations were lower ($\beta = -0.24$, $p = 0.017$) and that

TABLE 2 Means for all study variables ($N = 68$).

	Mean (SD) or %	Mean (SD) or %	Mean (SD) or %
	Total ($N = 68$)	**Control ($n = 32$)**	**ADHD ($n = 36$)**
Pre-pregnancy body mass index (kg/m^2)	26.77 (6.88)	23.58 (2.72)	29.12 (8.04)
3rd Trimester Plasma Fatty Acid Levels			
n-3			
Sum of omega-3 fatty acids (nmol/ml)	532.51 (171.79)	538.45 (179.69)	526.87 (168.42)
α-linolenic acid (nmol/ml)	138.91 (49.91)	137.80 (54.54)	139.79 (47.11)
Eicosapentaenoic acid (EPA) (nmol/ml)	55.09 (48.35)	72.23 (65.69)	43.47 (27.42)*
Docosahexaenoic acid (nmol/ml)	336.36 (113.62)	342.98 (116.41)	331.53 (113.60)
n-6			
Sum of omega-6 fatty acids (nmol/ml)	1,199.47 (338.32)	1,098.53 (236.94)	1,270.13 (382.20)$^+$
Conjugated linoleic acid (nmol/ml)	35.88 (19.34)	35.86 (11.85)	35.89 (23.28)
γ-linolenic acid (nmol/ml)	31.60 (14.56)	28.42 (13.59)	33.76 (15.01)
Dihomo-γ-linolenic (nmol/ml)	269.60 (82.57)	254.58 (66.78)	279.78 (91.39)
Arachidonic acid (AA) (nmol/ml)	861.58 (264.53)	779.66 (189.44)	918.93 (296.03)$^+$
n-6:n-3			
Omega-6:omega-3	1.95 (0.93)	1.64 (0.56)	2.17 (1.06)*
AA:EPA	22.09 (11.1)	17.15 (9.41)	25.54 (11.09)**
Dietary Intake			
% taking omega-3 fatty acid supplement	41%	34%	53%
Total energy (Kcal)	2,532.03 (574.83)	2,453.16 (457.96)	2,566.74 (624.81)
Total fat (g)	103.13 (32.06)	99.47 (23.92)	104.73 (99.48)
Percent of energy from Fat (%)	36% (6%)	37% (6%)	36% (7%)
Polyunsaturated fatty acids (PUFA) (g)	22.37 (9.05)	21.31 (7.26)	22.84 (9.83)
Omega-3 fatty acids (g)	2.55 (1.15)	2.56 (0.92)	2.55 (1.26)
Saturated fatty acids (SFA) (g)	37.06 (15.76)	35.82 (7.61)	27.60 (18.36)
Monounsaturated fatty acids (MUFA) (g)	35.69 (11.20)	34.49 (9.39)	35.89 (12.19)
PUFA: SFA	0.68 (0.28)	0.63 (0.15)	0.70 (0.33)
Inflammation (raw values)			
IL-6 (pg/ml)	1.65 (0.76)	1.45 (0.67)	1.78 (0.80)
TNF-α (pg/ml)	11.38 (3.48)	9.34 (3.09)	12.39 (3.07)**

AA, Arachidonic Acid; EPA, Eicosapentaenoic Acid; IL-6, Interleukin-6; TNF-α, Tumor Necrosis Factor-alpha; PUFA, Polyunsaturated Fatty Acids; SFA, Saturated Fatty Acids; MUFA, Monounsaturated Fatty Acids.
Cytokine concentrations presented in this table are raw values, to aid in interpretation; these were log-transformed prior to analysis. In preliminary analyses, the ADHD and Control groups were compared to one another using t-tests or chi squared tests. Significant and marginally significant groups differences based on these analyses are denoted in the table: $^+p < 0.10$, $^*p < 0.05$, $^{**}p < 0.01$.

plasma AA concentrations were higher ($\beta = 0.29$, $p = 0.01$) among individuals with ADHD, relative to controls. There were no significant differences in concentrations of total n-3s or total n-6s, or in any of the other individual fatty acid (*p*s ranged from 0.16 to 0.98) when supplementation was considered.

Hypothesis 2: Plasma cytokine concentrations

As can be seen in Table 3 and in Figure 1, TNF-α concentrations ($\beta = 0.35$, $p < 0.001$) were higher in individuals with heightened ADHD symptoms, even after controlling for both fatty acid supplementation status ($\beta = -0.03$, $p = 0.79$) and pre-pregnancy BMI ($\beta = 0.35$, $p = 0.001$). Contrary to expectation, IL-6 concentrations were not significantly different between the heightened ADHD symptoms and control groups ($\beta = 0.03$, $p = 0.83$) in this model, though pre-pregnancy BMI was associated with higher IL-6 concentrations ($\beta = 0.44$, $p < 0.001$), as expected.

As presented in Table 4 and depicted in Figure 2, in a model that does not include covariates, the AA:EPA ratio was associated with greater IL-6 ($\beta = 0.32$, $p = 0.004$) and with greater TNF-α concentrations ($\beta = 0.43$, $p = 0.001$). However, these

TABLE 3 Results from regression models relating ADHD status and n-6:n3 metrics and pro-inflammatory cytokines in pregnant individuals ($N = 68$).

	Omega-6:Omega-3		AA:EPA		TNF-α		IL-6	
	β (SE)	*p*	β (SE)	*p*	β (SE)	*p*	β (SE)	*p*
ADHD status[a]	0.30(0.11)	0.008	0.30(0.09)	0.001	0.35 (0.13)	0.01	0.03(0.15)	0.83
Fatty acid supplement status[b]	0.06(0.18)	0.72	−0.55(0.08)	<0.001	−0.03	0.79	0.02(0.15)	0.90
Pre-pregnancy BMI	–	–	–	–	0.35 (0.11)	0.001	0.44(0.13)	<0.001

[a]0 = Control, 1 = Heightened ADHD Symptoms.
[b]0 = not taking a fish oil or fatty acid supplement, 1 = taking a fish oil or fatty acid supplement.
BMI, body mass index; AA, Arachidonic Acid; EPA, Eicosapentaenoic Acid; TNF-α, Tumor Necrosis Factor-alpha; IL-6, Interleukin-6.
Results of these regression models suggest that individuals in the heightened ADHD symptoms group had, on average, higher ratios of omega-6-to-omega-3 fatty acids (as measured by the total ratio of total omega-6s to total omega-3s, as well as by the ratio of AA to EPA), and that they had higher mean concentrations of TNF-α.

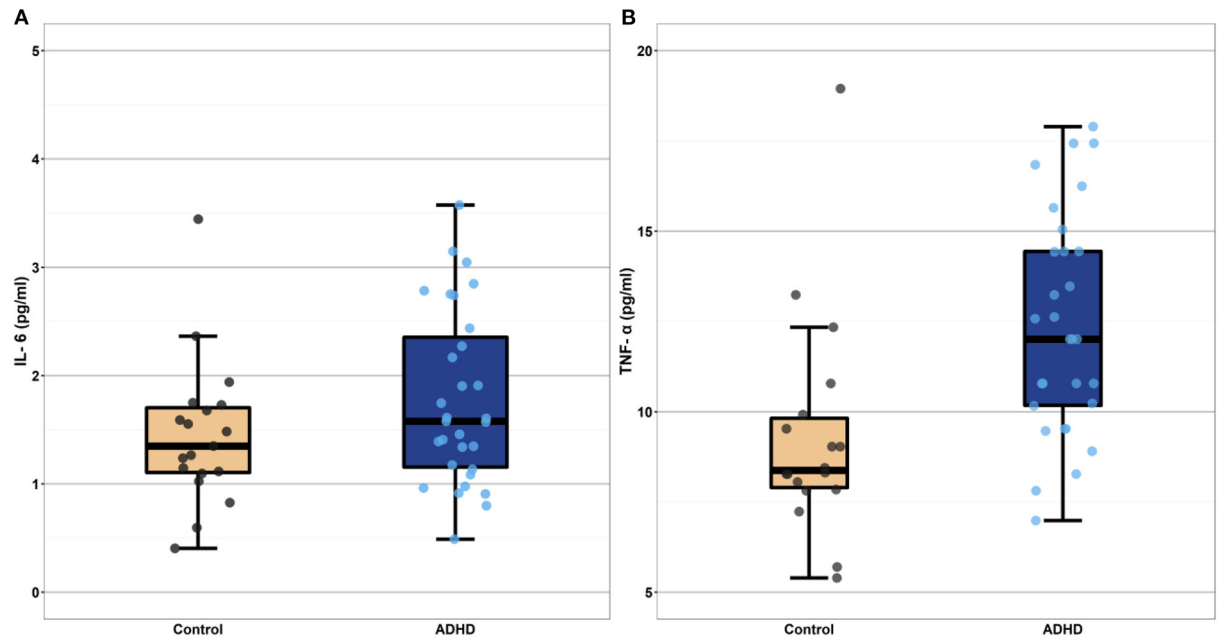

FIGURE 1
Boxplots depicting mean level cytokine concentrations in the heightened ADHD symptoms and control groups (Panel A depicts IL-6 values and Panel B depicts TNF-a values). Cytokine concentrations depicted in these boxplots are raw values, selected for ease of interpretation. These raw values were log-transformed prior to all analyses. The heightened ADHD symptoms and control groups differed significantly in their mean TNF-α concentrations ($p < 0.001$) even after controlling for participant pre-pregnancy BMI. Mean IL-6 concentration did not differ between the ADHD and control groups ($p = 0.83$). IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha.

associations did not survive after controlling for BMI and fatty acid supplement use (though, as expected, BMI was significantly associated with cytokine concentrations in these models, *p*s < 0.002). The n-6:n-3 was not significantly associated with IL-6 or TNF-α concentrations in either model.

Hypothesis 3: Dietary intake measures

The heightened ADHD symptoms and control groups did not differ from one another on any of the 24-h recall dietary intake measures (total calories, total fat, percent of calories from fat (%), polyunsaturated fatty acids, n-3 fatty acids, saturated fatty acids, and the ratio of polyunsaturated fatty acids to saturated fatty acids), *p*s ranged from 0.20 to 0.94. They also did not differ in essential fatty acid concentrations in plasma (conjugated linoleic acid $p = 0.90$, α-linolenic acid $p = 0.81$).

Discussion

Utilizing data from a unique sample of pregnant individuals, the current study examined whether there are ADHD-associated differences in polyunsaturated fatty acid or pro-inflammatory

TABLE 4 Results from regression models relating omega-6:omega3 metrics and pro-inflammatory cytokines ($N = 68$).

	TNF-α		IL-6	
	β (SE)	*p*	β (SE)	*p*
Models Considering Σ Omega-6:Σ Omega-3				
Model 1: Without Covariates				
Omega-6:omega-3	0.03 (0.02)	0.21	0.11 (0.12)	0.39
Model 2: With Covariates				
Omega-6:omega-3	0.10 (0.12)	0.39	0.01 (0.10)	0.94
Fatty acid supplement status[a]	−0.09 (0.14)	0.51	0.02 (0.14)	0.91
Pre-pregnancy BMI	0.44 (0.11)	<0.001	0.46 (0.13)	<0.001
Models Considering AA:EPA				
Model 3: Without Covariates				
AA:EPA	0.43 (0.12)	0.001	0.32 (0.11)	0.004
Model 4: With Covariates				
AA:EPA	0.22 (0.16)	0.16	0.08 (0.14)	0.58
Fatty acid supplement status[a]	0.01 (0.15)	0.94	−0.01 (0.17)	0.99
Pre-pregnancy BMI	0.37 (0.12)	0.002	0.40 (0.12)	0.001

AA, Arachidonic Acid; EPA, Eicosapentaenoic Acid; TNF-α, Tumor Necrosis Factor-alpha; IL-6, Interleukin-6.
[a]0 = not taking a fish oil or fatty acid supplement, 1 = taking a fish oil or fatty acid supplement. BMI, body mass index. Results of these regression models suggest that while the ratio of AA-to-EPA was associated with greater TNF-α and IL-6 in models that did not considered covariates, these associations were no longer significant after controlling for pre-pregnancy BMI and whether the individual was taking a fatty acid supplement.

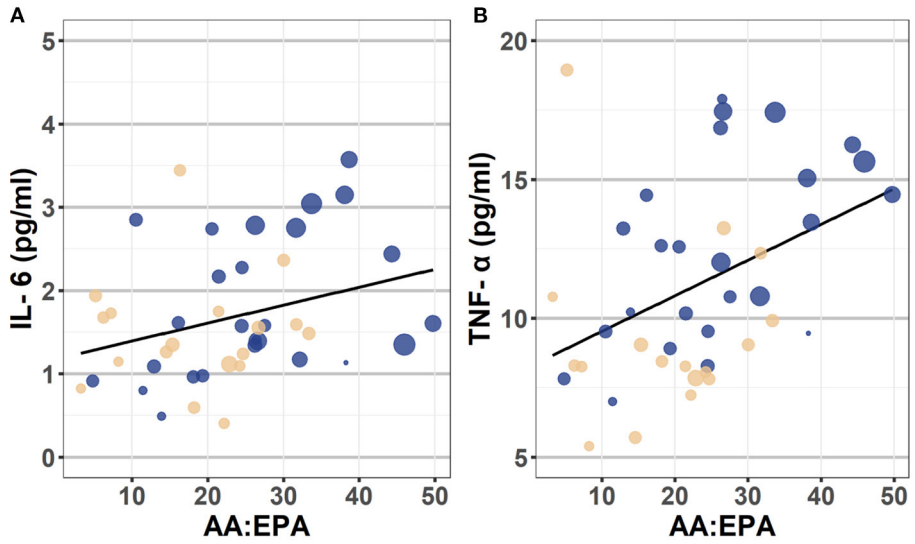

FIGURE 2
Scatterplot depicting the association between AA:EPA ratio and IL-6 (Panel A) and TNF-α (Panel B). These scatterplots depict the bivariate association between the AA:EPA and plasma concentrations of IL-6 (on the left) and TNF-α (on the right). The individual data points are color coded to reflect participant ADHD status (Yellow = Control, Blue = heightened ADHD symptoms). The diameter of each circle reflects participant pre-pregnancy BMI values, with larger circles indicating individuals with higher BMI than smaller diameter circles. Though BMI was treated as a continuous variable in analyses, they were categorized into following categories for the purposes of this plot: Healthy = 18.5–24.9, Overweight = 25.0–29.9, and Obese = 30.0 and above categories. AA, arachidonic acid; EPA, eicosapentaenoic acid; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha; BMI, body mass index.

cytokine concentrations during pregnancy, a developmental period when maternal fatty acid status and inflammation have implications for the health of both the pregnant person and the developing child (27, 56–59). Results suggest that pregnant individuals with heightened ADHD symptoms, on average, had higher plasma n-6:n-3s and higher plasma concentrations of

TNF-α, when compared to controls. The two groups did not differ in their dietary intake of fatty acids or other relevant nutrients, nor did they differ in concentrations of essential fatty acids (those that can only come from diet). While not conclusive, these results are consistent with the hypothesis that the observed differences in the plasma n-6:n-3 are the result of ADHD-associated differences in fatty acid metabolism, rather than simply reflecting differences in dietary intake.

The current study found that pregnant individuals with ADHD differed from controls in the n-6:n-3 using two metrics that have been used in previous research, the sum of total n-6s divided by the sum of total n-3s and the ratio of arachidonic acid to eicosapentaenoic acid concentrations (AA:EPA). Unsurprisingly, results were similar across these metrics. The observed ADHD group differences in the n-6:n-3 persisted even after controlling for whether the participant was taking a fish oil or fatty acid supplement or not. Despite n-3 supplementation being well-studied as a potential complementary treatment for ADHD (1, 60), few previous studies examining ADHD associated differences in n-3s or the n-6:n-3 have controlled for supplementation in their analyses.

The ADHD and control groups did not differ reliably in their self-reported 24-h dietary recalls of PUFA intake (total PUFAs and total n-3 fatty acids) or on other relevant nutrients, including total calories, total fat, MUFAs, SFAs and the PUFA:SFA ratio. There also were no reliable differences in plasma concentrations of conjugated linoleic acid and α-linolenic acid, fatty acids that can only come from dietary intake. Though not conclusive, our observation that there are differences in the plasma n-6:n-3 in the absence of differences in dietary intake is consistent with the purported ADHD-associated differences in the metabolism of fatty acids or in the conversion of essential fatty acids to longer-chain fatty acids (29). For example, single nucleotide polymorphisms in the fatty acid desaturase enzyme which have been shown to modulate circulating n-3 levels (61) have been associated with ADHD (30).

This study also examined whether pro-inflammatory cytokine concentrations differed between pregnant individuals with and without ADHD. We found that pregnant individuals with heightened ADHD symptoms, on average, had higher plasma concentrations of TNF-α, relative to controls. Previous research has shown that heightened gestational inflammation is associated with other types of psychiatric symptoms during pregnancy (e.g., anxiety, depression) (42, 51), but this is the first study to report that pregnant individuals with heightened ADHD symptoms have greater concentrations of pro-inflammatory cytokines. We did not see differences in plasma IL-6 between pregnant individuals with and without heightened ADHD symptoms, though this study may not have been adequately powered to detect such an effect. This is in contrast to previous research which reports differences in IL-6 between children with and without an ADHD diagnosis (38–40), but is consistent with findings from Corominas-Roso et al. which reports that adults with and without ADHD did not differ from one another in serum IL-6 concentrations (though, of note, these authors also did not find a differences in serum TNF-α) (62).

Consistent with the assertion that a greater n-6:n-3 is indicative of a more pro-inflammatory state, we found a positive association between the AA:EPA ratio and plasma concentrations of both IL-6 and TNF-α in models that did not include covariates. However, these effects did not survive controlling for pre-pregnancy BMI. Interestingly, in their study of pregnant individuals with prenatal depression, Chang et al. (12) similarly did not find a correlation between the n-6:n-3 and TNF-α, despite finding that the n-6:n-3 and TNF-α were increased in depression. The fact that individuals with ADHD are at heightened risk for obesity [see (63) for a systematic review]—which could be the long-term consequence of an increased n-6:n-3 rather than simply a confound–makes it challenging to fully interpret these findings.

The precise mechanistic connection between ADHD, PUFAs and inflammation is not fully elucidated, but see (64) for a comprehensive review on the association between PUFAs and inflammation. Fatty acids are critical nutrients in maintaining neuronal signaling (65). Additionally, the n-3 PUFA membrane content has been shown to influence neurotransmitter receptor functionality in dopaminergic neurons (66) which has implications for behaviors related to ADHD. In glial cells, levels of n-3 PUFAs can influence microglial phagocytosis (67) as well as astrocyte differentiation (68). Notably, microglia are the main cell type able to detect and respond to inflammatory signals in the brain. Further, microglia express several inflammatory receptors that have also been suggested to be sensitive to lipids (69). Animal models show that n-3 PUFA supplementation can inhibit microglial activation and the expression of pro-inflammatory cytokines, including TNF-a and IL-6 (70). It is plausible that altering microglial functional states *via* the n-6:n-3 in the brain may provide a connection between ADHD, PUFAs, and inflammation.

This study had a number of strengths. This is the first study to examine fatty acid or cytokine differences in pregnant individuals with and without heightened ADHD symptoms. Though differences in n-6:n-3 and pro-inflammatory cytokine concentrations have been observed in children with ADHD (6, 38, 39), no previous study has examined these associations during pregnancy, despite the profound implications that fatty acids and cytokines during pregnancy have for both the health of the pregnant person and for fetal development (26–28, 71). Methodological strengths of this study include assessment of fatty acids in plasma, the use of 24-h recall data collected by trained dietitians, and our inclusion of covariates that may impact fatty acid status and inflammation (most studies in this area have not considered covariates in their analyses).

This study also had limitations, including our small sample size. Though samples of this size are common in this literature,

we may have lacked sufficient power to detect some effects, for example a difference in plasma IL-6 based on ADHD status. Thus, the null findings in this study should be viewed as inconclusive. In this first study investigating these associations in pregnant individuals, we focused on plasma concentrations of fatty acids, which reflect current fatty acid levels. Future studies on this topic should also examine fatty acid levels in red blood cell membranes, which reflect more chronic (i.e., the previous 3 months) levels of fatty acids. Examination of other plasma lipid levels (i.e., triglycerides) during pregnancy and may also yield important insights. While the unique nature of our sample (of pregnant individuals living in the US) is a strength of this study, it may limit generalizability of our findings. Future research should examine these associations utilizing data from participants residing in other countries as well as in non-pregnant populations. We examined two pro-inflammatory cytokines in our analyses (selected because they were shown to be related to ADHD in previous research) that were assessed at one timepoint in pregnancy. Future research should examine a wider range of relevant immune factors, assessed at multiple times in pregnancy. While our study included measurement of ADHD symptomatology assessed using a well-validated and widely used scale with established norms, we did not assess ADHD diagnosis, which also may limit the generalizability of our findings. Last, while metrics such as the n-6:n-3 offer a more comprehensive picture of fatty acid status than absolute values of n-3s or n-6s, the n-6:n-3 is also not a perfect or complete measure of fatty acid status. For example, trans fatty acids interfere with the desaturation and elongation of both omega-6 and omega-3 fatty acids (10). Future research should include other such metrics (including measures of SFAs, MUFAs, and measures of PUFAs not examined here, including docosapentaenoic acid) in their analyses.

Summary and conclusion

This study found that, on average, pregnant individuals with heightened ADHD symptoms had higher plasma n-6:n-3s and higher TNF-α concentrations in plasma relative to controls. The heightened ADHD symptoms and control groups did not differ statistically on self-reported dietary intake (assessed using 24-h recalls), nor on essential fatty acid concentrations in blood. Though not conclusive, these findings are consistent with a picture of altered fatty acid metabolism in ADHD.

Data availability statement

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

Ethics statement

All study procedures were reviewed and approved by the Oregon Health & Science University Institutional Review Board. Written informed consent to participate in this study was provided by the participants.

Author contributions

ES and JN conceived the project. HG, ES, JN, and JL designed the research. HG, ES, and JL analyzed the data. AM data created the plots. HG and ES wrote the manuscript, with contributions from JN, JL, GD, AM, and KH. All authors discussed the data. All authors contributed to the article and approved the submitted version.

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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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EDITED BY
Margit Burmeister,
University of Michigan, United States

REVIEWED BY
Justin L. Bollinger,
College of Medicine, University of Cincinnati, United States
Sumit Sarkar,
National Center for Toxicological Research (FDA), United States
Tertia Purves-Tyson,
Neuroscience Research Australia, Australia

*CORRESPONDENCE
Marianne L. Seney
seneyml@upmc.edu

†These authors have contributed equally to this work

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Brain region- and sex-specific transcriptional profiles of microglia

Kelly Barko[1†], Micah Shelton[1†], Xiangning Xue[2], Yvette Afriyie-Agyemang[1,3], Stephanie Puig[4,5], Zachary Freyberg[1,3,6], George C. Tseng[2], Ryan W. Logan[4,5,7] and Marianne L. Seney[1,3*]

[1]Translational Neuroscience Program, Department of Psychiatry, University of Pittsburgh School of Medicine, Pittsburgh, PA, United States, [2]Department of Biostatistics, University of Pittsburgh School of Public Health, Pittsburgh, PA, United States, [3]Center for Neuroscience, University of Pittsburgh, Pittsburgh, PA, United States, [4]Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, Boston, MA, United States, [5]Center for Systems Neuroscience, Boston University, Boston, MA, United States, [6]Department of Cell Biology, University of Pittsburgh, Pittsburgh, PA, United States, [7]Genome Science Institute, Boston University School of Medicine, Boston, MA, United States

Microglia are resident macrophages of the brain, performing roles related to brain homeostasis, including modulation of synapses, trophic support, phagocytosis of apoptotic cells and debris, as well as brain protection and repair. Studies assessing morphological and transcriptional features of microglia found regional differences as well as sex differences in some investigated brain regions. However, markers used to isolate microglia in many previous studies are not expressed exclusively by microglia or cannot be used to identify and isolate microglia in all contexts. Here, fluorescent activated cell sorting was used to isolate cells expressing the microglia-specific marker TMEM119 from prefrontal cortex (PFC), striatum, and midbrain in mice. RNA-sequencing was used to assess the transcriptional profile of microglia, focusing on brain region and sex differences. We found striking brain region differences in microglia-specific transcript expression. Most notable was the distinct transcriptional profile of midbrain microglia, with enrichment for pathways related to immune function; these midbrain microglia exhibited a profile similar to disease-associated or immune-surveillant microglia. Transcripts more highly expressed in PFC isolated microglia were enriched for synapse-related pathways while microglia isolated from the striatum were enriched for pathways related to microtubule polymerization. We also found evidence for a gradient of expression of microglia-specific transcripts across the rostral-to-caudal axes of the brain, with microglia extracted from the striatum exhibiting a transcriptional profile intermediate between that of the PFC and midbrain. We also found sex differences in expression of microglia-specific transcripts in all 3 brain regions, with many selenium-related transcripts more highly expressed in females across brain

regions. These results suggest that the transcriptional profile of microglia varies between brain regions under homeostatic conditions, suggesting that microglia perform diverse roles in different brain regions and even based on sex.

KEYWORDS

microglia, RNA-sequencing, Tmem119, disease-associated microglia, sex difference

Introduction

As the brain's resident macrophage, microglia are instrumental to the regulation of parenchyma health, surveilling the local environment using a complex network of ramified processes through which they identify potential threats (i.e., cellular debris, microorganisms, misfolded proteins). In response to infection or injury, microglia rapidly change morphology to take on the classic activated ameboid form characterized by retracted, thickened processes and increased soma size. Activated microglia swarm the site of injury, release pro-inflammatory cytokines [i.e., interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α (1, 2)], and recruit peripheral macrophages to the site of injury. Microglia clear or engulf dead and dying cells (3) which prevents further damage caused by the release of cellular contents. Once the threat has been addressed, microglia release anti-inflammatory cytokines and growth factors to repair damage and restore homeostasis (1). Beyond their disease- and injury-associated functions, microglia play roles in shaping neuronal circuits during specific developmental periods, and evidence suggests that this process continues throughout the lifespan. In the healthy brain, microglia interact with and eliminate synapses and clear apoptotic neurons (3, 4), but crucially, they also induce synapse formation and regulate neurogenesis (5, 6). Microglia release synaptic factors (e.g., BDNF, glycine, L-serine) and prune synapses in an activity-dependent manner with implications for learning and behavior (4, 6–9).

Evidence from both preclinical and clinical studies reveal that microglia are heterogenous across brain regions in their density (10), morphology (11–14), gene expression (13–18), and proliferation (19–21). While not completely understood, the wide regional heterogeneity of microglia suggests they play distinct roles in specific regions of the brain. An early study by Lawson et al. demonstrated a greater than 5-fold variation in the density of microglial processes between regions (10). Frontal regions, including the cortex, striatum, and hippocampus, exhibit high levels of microglial ramification (11, 12), while microglia in hindbrain regions (i.e., cerebellum, brainstem) and regions which do not have a protective blood brain barrier (i.e., median eminence, circumventricular organs, subventricular zone) have low ramification tending more toward the activated ameboid morphology (10, 13, 14). The expression of molecular markers is region-specific. For instance, expression of the fractalkine receptor C-X3-C Motif Chemokine Receptor 1 (CX3CR1), a major component of the signaling pathway between microglia and neurons in the healthy brain, is highest in frontal regions and midbrain but comparatively low in hindbrain and circumventricular regions (18). The opposite pattern is true for phagocytic or immune activating genes, with, for instance, higher expression of markers associated with microglia reactivity in the blood brain barrier-lacking circumventricular organ of the mouse brain (13–17).

Further, evidence suggests that microglia may be phenotypically distinct between males and females (22–27). Brain sexual dimorphism is regulated by gene expression and hormonal surges during discrete developmental windows. By birth, microglia exhibit sex differences in number, morphology, and expression of activation markers/receptors. For instance, there are sex differences in levels of microglia with activated morphology during early postnatal development in several brain regions [e.g., in preoptic area, paraventricular nucleus, dentate gyrus, amygdala (26, 27)]. In these regions, sex differences in microglia number are dependent on steroid hormones produced during development; treating female mice with estradiol in the first two postnatal days produces the masculine pattern of microglia number and morphology (26). Evidence suggests sex differences in microglia phenotype as well. *Ex vivo*, microglia derived from male and female brains show divergent inflammatory signaling to lipopolysaccharide and estradiol (28). Evidence also suggests sex differences in the transcriptional profile of microglia [e.g., (23–25)], with female microglia showing a neuroprotective phenotype which is retained after transfer into male brains (23). There are also sex differences in morphology and transcript expression of microglia in the prefrontal cortex (25, 29), one of our regions of interest in the current study. Together, this prior evidence for sex differences in microglia motivates our current study to investigate the transcriptional profile of isolated microglia, focusing on whether any sex differences are consistent across brain regions.

Previous attempts to identify microglia in the CNS relied on morphology, relative marker expression as assessed by flow cytometry, or generating bone marrow chimeras [e.g., (10–16, 30–33) reviewed in (34)]. However, these approaches cannot be used to identify and isolate microglia in all contexts. Some commonly used markers are not cell type exclusive.

For example, the commonly used marker for the fractalkine receptor CX3CR1 is also expressed by circulating monocytes and peripheral macrophages (35–37). Similarly, both microglial morphology and the expression of common microglial markers may change in response to disease or injury (e.g., expression of the purinergic receptor P2RY12 is lower in response to immune activation, while expression of CD68, a lysosomal-associated membrane protein, increases) (38–40). This adds an additional confounding factor to analyses which are aimed at generating a transcriptional profile across all microglia regardless of pathological state. Here, we make use of the microglia specific marker, transmembrane protein 119 (TMEM119), a robustly expressed cell-surface protein, to distinguish microglia from infiltrating macrophages (41). We used mice that conditionally expressed a fluorescent reporter only in TMEM119 expressing cells to sort microglia for RNA-sequencing (42). These mice enabled us to create transcriptomic profiles of purified microglia from male and female mice across the PFC, striatum, and midbrain to investigate potentially unique populations of microglia by sex and brain region. The three regions are reciprocally connected, functionally related, and activity across these regions underlies a multitude of complex behaviors. The direct connections between the dorsal and ventral striatum in the forebrain and the ventral tegmental area (VTA) and substantia nigra (SNc) in the midbrain represent a conserved dopaminergic circuit which is central to regulating movement, motivation, reinforcement, and learning (43–46). While the vast body of work has examined the ways that microglia in these regions respond to infection, disease, and degeneration, comparatively few groups have investigated the regional heterogeneity of this important cell type under homeostatic conditions, which we directly assess at the transcriptional level in the current study.

Materials and methods

Mice

Mice were group-housed (3–5 mice/cage) and maintained under standard conditions (12:12 h light/dark cycle; lights on 7 a.m.; 22 ± 1°; food and water *ad libitum*), in accordance with University of Pittsburgh Institutional Animal Care and Use Committee. Heterozygous Tmem119-2A-CreERT2 (Jackson Labs; RRID:IMSR_JAX:031820) female mice were crossed with a homozygous Ai14(RCL-tdTomato)-D (Jackson Labs; RRID:IMSR_JAX:007914; contains *loxP* flanked STOP cassette to prevent transcription of tdTomato reporter) male mouse to produce a Tmem119-2A-CreERT2/Ai14(RCL-tdTomato)-D mouse strain that allowed for conditional activation of the tdTomato reporter in TMEM119 labeled cells in the brain using tamoxifen. Adult mice heterozygous for Tmem119-2A-CreERT2 and Ai14(RCL-tdTomato)-D, 17–18 weeks in age were used for experimentation (n = 6 mice per sex). Mice were group-housed in 12-h light/dark with food and water *ad libitum*. Tamoxifen (Sigma−Aldrich, order no. 10540−29−1) solution preparation and administration were followed accordingly to instructions from The Jackson Laboratory, Bar Harbor, Maine[1] (47, 48). Mice were administered 75 mg tamoxifen/kg body weight via intraperitoneal injection once every 24 h, for five consecutive days. Mice were sacrificed 10–21 days post final injection to allow for effective tamoxifen induction.

Tissue extraction

Mice were sacrificed by live-cervical dislocation without anesthesia. Brains were extracted and rinsed with chilled 1X artificial cerebrospinal fluid (ACSF). Brain regions (prefrontal cortex; Bregma + 2.96 to + 1.42 mm, striatum; Bregma + 1.42 to −0.46 mm, and mid-brain; Bregma −2.88 to −3.88 mm) were separated by using a stainless-steel mouse brain matrix (1 mm) and single edge blades, kept on wet ice (**Figure 1A**). Sectioned tissue was transferred to designated 15 ml conical tubes filled with approximately 3 ml of chilled 1X ACSF.

Tissue dissociation and preparation

Individual cell suspensions of harvested tissue for fluorescent activated cell sorting (FACS) were prepared by utilizing the following Miltenyl Biotec products: Adult Brain Dissociation Kit, mouse and rat (order no. 130-107-677), gentleMACS Octo Dissociator with Heaters (no. 130-096-427), gentleMACS C Tubes (no. 130-093-334), and MACS SmartStrainer (70 um) (no. 130-098-462). Cells were resuspended in 200 μL of 1X phosphate buffered saline (Gibco, order no. 70011-004) and transferred to designated 1.5 mL tubes for FACS. One sample from the PFC was lost during tissue dissociation due to a cracked tube, resulting in N = 5 male PFC samples.

Fluorescent activated cell sorting

Individual cell suspensions were sorted by the Unified Flow Core FACS facility at the University of Pittsburgh. Cells containing the microglia marker of interest, Tmem119-2A-CreERT2, expressing Ai14(RCL-tdTomato)-D were sorted with a BD FACSAria II; cells were excited by a 532 nm laser and detected with 610/20 bandpass filter. The isolation and gating strategy is shown in **Figure 1B**; only positive cells were collected for RNA-seq. We confirmed using qPCR that positive cells exhibited high expression of the microglia-specific markers *Tmem119* and *Aif1*, and negligible expression

1 https://www.jax.org/research-and-faculty/resources/cre-repository/tamoxifen

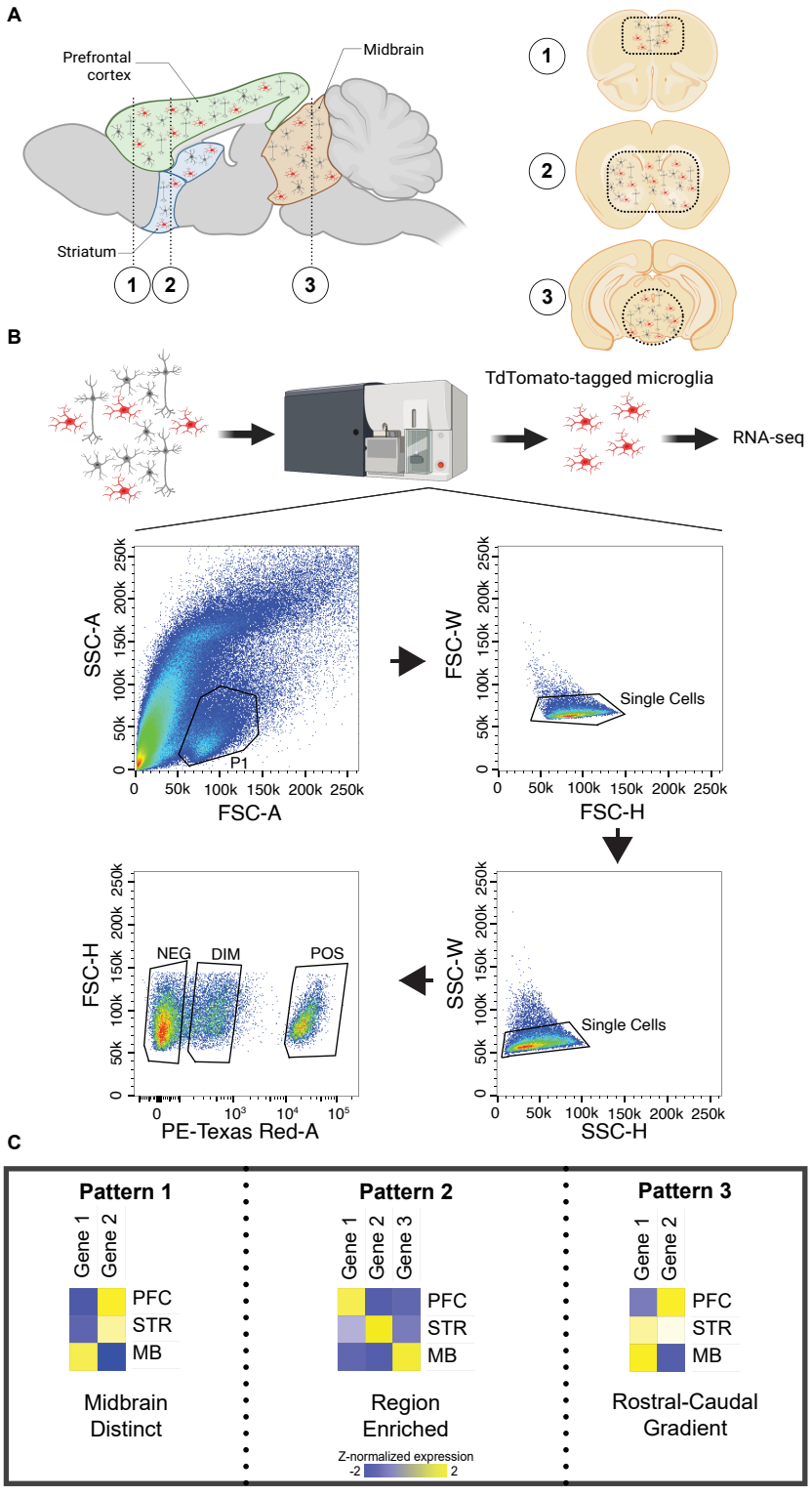

FIGURE 1
Brain regions investigated and patterns of differential expression across regions. **(A)** Left. Sagittal section of the mouse brain with prefrontal cortex (PFC), striatum (STR), and midbrain (MB) labeled. **(A)** Right. Coronal sections of the mouse brain corresponding to the numbered regions in the left sagittal section. Dashed lines indicate the approximate location of tissue isolation for each region. **(B)** Experimental design for microglia isolation with fluorescent-activated cell sorting, including the gating strategy. Only cells identified as Td-Tomato positive were sequenced. **(C)** Patterns of transcript expression across investigated brain regions. Pattern 1 was associated with distinct expression in the midbrain. Pattern 2 was associated with enrichment in either PFC or striatum. Pattern 3 was associated with a rostral-to-caudal gradient of expression. Created with BioRender.com.

of the neuronal marker *Rbfox2* (**Supplementary Figure 1**). Cells were sorted into designated 1.5 mL tubes with 250μL solution of Buffer RLT Plus (Qiagen, order no. 1030963) and 2-mercaptoethanol (MilliporeSigma, order no. 444203). Isolated microglia samples were stored at −80°C until further use. The mean number of isolated cells was 33,399 ± 4,272 (mean ± SEM; **Supplementary Extended data 1**).

RNA sequencing

RNA extractions, cDNA generation, and library preparation were performed by the University of Pittsburgh Health Science Sequencing Core at the UPMC Children's Hospital of Pittsburgh, Pittsburgh, PA, United States. RNA was extracted using the Qiagen RNeasy Plus Micro extraction kit (Qiagen:74034) following manufacturer's instructions, including the use of DNA elimination columns. RNA was assessed for quality on an Agilent Fragment Analyzer 5300 using the High sensitivity RNA kit (Agilent: DNF-472-1000). The mean RNA integrity number (RIN) was 8.4, indicating excellent quality for RNA sequencing. 4.5 μl of RNA was used from each sample for cDNA generation using the Takara Smart-Seq HT kit (Takara: 634438) following manufacturer's instructions, with 15 cycles of cDNA amplification. Smart-Seq cDNA was assessed for quality on an Agilent Fragment Analyzer 5300 using the High sensitivity NGS kit (Agilent: DNF-474-1000). All samples passed QC with full length cDNA (mean concentration of 12.9 ng/μl; primary peaks ∼2000 bp; absence of short length cDNA with bimodal peaks including second peak at ∼300 bp). Library preparation was performed using 1 ng of cDNA input with the Illumina Nextera XT kit (Illumina: FC-131-1096) and UDI indexes (Illumina: 20027215) added using 12 PCR cycles. Libraries were assessed using an Agilent High sensitivity NGS kit (Agilent: DNF-474-1000), and then normalized and pooled by calculating the nM concentration based on the fragment size (base pairs) and the concentration (ng/μl). Prior to sequencing, library pools were quantified by quantitative polymerase chain reaction (qPCR) on the LightCycler 480 using the KAPA qPCR quantification kit. Libraries were sequenced on a NovaSeq 6000 at UPMC Genome Center on an S2 100 cycle flow cell, 2 × 50 bp, for an average of ∼30 million reads per sample. Microglia-specific cell type markers (e.g., *Tmem119*, *P2ry12*, *Aif1*, *Itgam*) are highly expressed in our sorted cells compared to other cell type markers, indicating that our cell sorts are indeed effective at isolating microglia (**Supplementary Figure 2**).

Data analysis

FastQC (version 0.11.9) was used to determine the per base sequence quality, with a mean score of 36 across samples. Paired-end reads were preprocessed, adapters removed using trimmomatic (version 0.38), and trimmed reads were mapped to Mus musculus Ensembl GRCm38 using HISAT2 (version 2.2.0), with a mean overall alignment rate of 92% across samples.

After mapping, the total 46,078 Ensembl transcripts were filtered to remove low expression transcripts. Specifically, we divide samples into subgroups by sex and brain region, then we keep only transcripts with at least one count per million (CPM) in at least one subgroup. After the filtering, 21,236 remained for DE analysis. RNA-seq data were analyzed using DESeq2 using brain region and sex as the main effects. Principal component analysis was performed using ggplot and the function prcomp. For brain region comparisons, we first determined if there was a main effect of region on microglial transcript expression (adjusted $p < 0.05$). If there was a main effect of brain region, we then performed two-group *post hoc* comparisons; transcripts with *post hoc* $p < 0.05$ and fold change > 1.2 were considered differentially expressed (DE). We identified 3 patterns of transcript expression, which we then probed in more detail: (1) distinct expression in the midbrain; (2) enrichment in either striatum or PFC; (3) gradients of expression (**Figure 1C**). We then determined which transcripts exhibited a main effect of sex ($p < 0.05$; fold change > 1.2). From the full interaction model, we then extracted data for sex differences within each brain region ($p < 0.05$; fold change > 1.2). Pathway over representation was assessed using Metascape, with expressed transcripts as background. Rank Rank hypergeometric overlap (RRHO) was used as a threshold-free approach to determine if there were similar patterns of sex differences across brain regions (49).

Results

Differential expression identifies brain region-specific transcriptional patterns

We first determined whether there were brain region differences in transcript expression of isolated microglia by performing principal component analysis (PCA). A clear separation between transcripts enriched in the midbrain compared to those enriched in the PFC and striatum was observed; this effect is consistent for both males and females (**Figure 2A**). ANOVA detected 2,372 transcripts exhibiting a main effect of brain region (**Supplementary Extended data 2**) and *post hoc* two brain region comparisons revealed transcripts that were differentially expressed between: (1) PFC and midbrain ($p < 0.05$: 1368 transcripts; $p < 0.01$: 1226 transcripts; **Figure 2B** and **Supplementary Extended data 3**); (2) striatum and midbrain ($p < 0.05$: 1574 transcripts; $p < 0.01$: 1381 transcripts; **Figure 2C** and **Supplementary Extended data 4**); and (3) striatum and PFC ($p < 0.05$: 718 transcripts; $p < 0.01$: 486 transcripts; **Figure 2D** and **Supplementary Extended data 5**) (fold change for both *p*-value cutoffs > 1.2). These

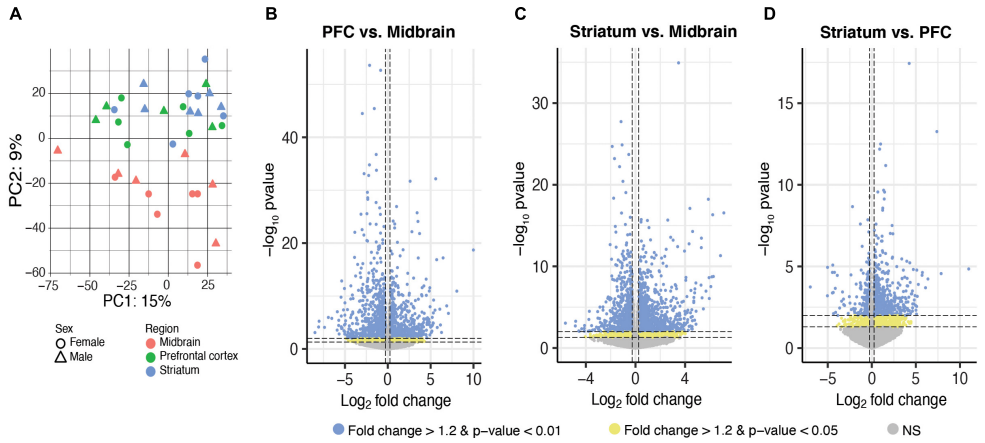

FIGURE 2
Differential expression of microglia-specific transcripts across brain regions. **(A)** Principal component (PC) analysis indicated distinct transcriptional profile in midbrain-isolated microglia compared to prefrontal cortex (PFC) and striatum. Log_2FoldChange plotted relative to $-log_{10}$pvalue by volcano plots for differentially expressed transcripts between PFC and midbrain **(B)**, striatum and midbrain **(C)**, and striatum and PFC **(D)**. Horizontal dashed lines represent p-value significance cutoffs of $p < 0.01$ and $p < 0.05$, while vertical dashed lines represent log_2FC cutoffs of ≤ -0.26 or ≥ 0.26 (FC ≥ 1.2). Blue dots represent DE transcripts with $p < 0.01$ and FC ≥ 1.2. Yellow dots represent DE transcripts with $p < 0.05$ and FC ≥ 1.2.

results suggest that microglia in the midbrain exhibit a distinct transcriptional profile compared to the PFC and striatum.

Three patterns of differential transcript expression were observed across brain regions in isolated microglia (Figure 1C). In line with the PCA, the first pattern was higher expression in the midbrain compared to PFC and striatum, with no difference between PFC and striatum [midbrain > (PFC = striatum)], or lower expression in the midbrain compared to PFC and striatum, with no difference between PFC and striatum [midbrain < (PFC = striatum)]. The second pattern included transcripts enriched in PFC or striatum. The final pattern included transcripts with a rostral-to-caudal gradient of expression, or the reverse of this gradient (i.e., caudal-to-rostral).

Distinct transcriptional profile of microglia in the midbrain

Given evidence for a distinct transcriptional profile in the midbrain compared to the PFC and striatum, we searched for transcripts exhibiting distinct expression in midbrain (Figure 3A). First, we considered transcripts with higher expression in the midbrain compared to PFC and striatum, with no difference between PFC and striatum [midbrain > (PFC = striatum)]; 533 transcripts fit this pattern, including the taurine transporter gene *Slc6a6* and beta-2 microglobulin (*B2m*; Figure 3B and Supplementary Extended data 6). Pathway analysis of the transcripts more highly expressed in midbrain, but equal expression in PFC and striatum [midbrain > (PFC = striatum)], identified pathways related to immune function, such as the MHC protein complex and positive regulation of immune response (Figure 3C and Supplementary Extended data 6).

We also considered the opposite expression profile in which transcripts were expressed at lower levels in the midbrain compared to PFC and striatum, with no difference between PFC and striatum [midbrain < (PFC = striatum)]; 500 transcripts fit this pattern, including the lysosomal enzyme cathepsin A (*Ctsa*) and the transcriptional regulator, *P2ry12*, which encodes purinergic receptor P2Y12 (Figures 3A,D and Supplementary Extended data 7). Overall, transcripts more lowly expressed in the midbrain with similar expression in PFC and striatum [midbrain < (PFC = striatum)] were involved in pre- and post-synapse function, and synapse organization (Figure 3E and Supplementary Extended data 7).

Transcriptional profiles of microglia in the PFC and striatum

While the midbrain was most strikingly different from the PFC and striatum in terms of the transcriptional profile of microglia, we also identified transcripts enriched in PFC or striatum. We found 169 transcripts enriched in the PFC (Figure 4A and Supplementary Extended data 8), including *Acox1*, which codes for peroxisomal acyl-coenzyme A oxidase 1, and protein kinase cAMP-dependent type I regulatory subunit alpha (*Prkar1a*; Figure 4B). These PFC-enriched transcripts were associated with many synapse-related pathways, including long-term memory and regulation of synaptic vesicle endocytosis (Figure 4C and Supplementary Extended data 8). There were 279 transcripts enriched in the striatum compared to the PFC

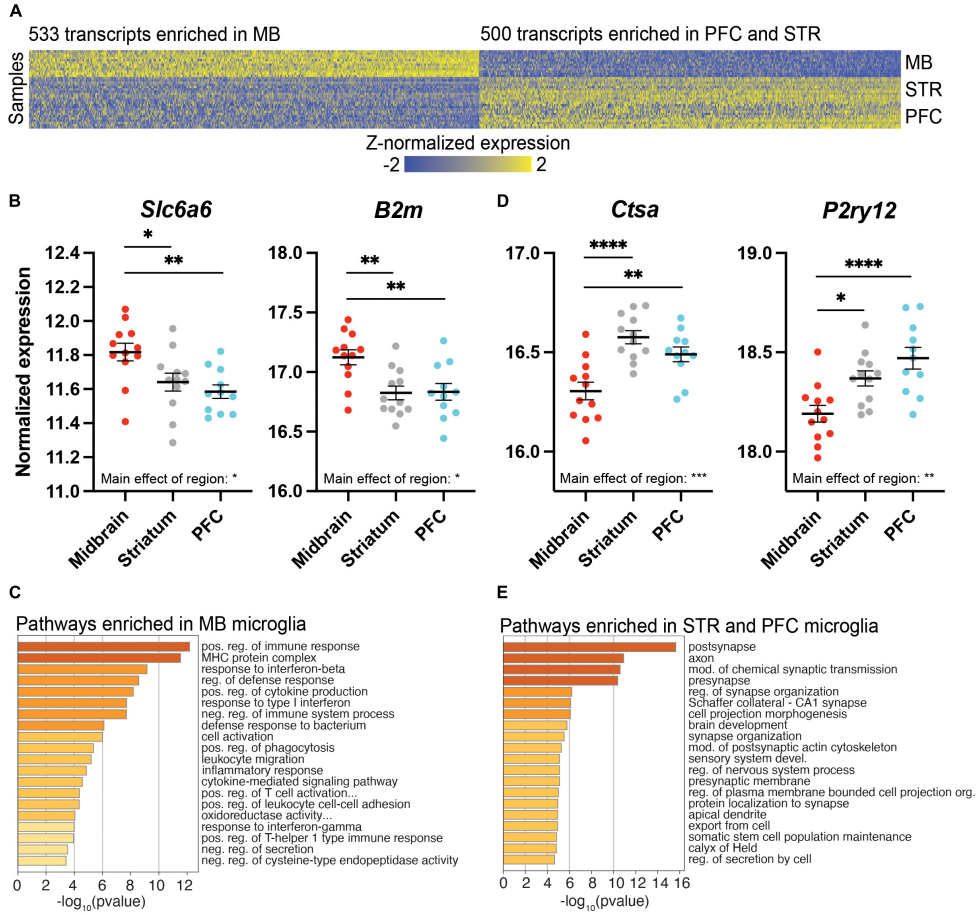

FIGURE 3
Differentially expressed (DE) transcripts that distinguish the midbrain (MB) from the prefrontal cortex (PFC) and striatum (STR). **(A)** Heatmap of DE transcripts between MB and both PFC and STR. The 533 transcripts more highly expressed in MB compared to both PFC and STR are indicated on the left of the heatmap, while the 500 transcripts expressed at lower levels in MB compared to both PFC and STR are indicated on the right of the heatmap. DE transcripts are plotted on the x-axis and individual subject samples on the y-axis. **(B)** *Slc6a6* and *B2m* fit the pattern of being more highly expressed in MB compared to PFC and STR. **(C)** Top pathways represented by transcripts expressed more highly in the midbrain compared to PFC and STR. **(D)** *Ctsa* and *P2ry12* fit the pattern of being expressed at lower levels in midbrain compared to PFC and STR. **(E)** Top pathways represented by transcripts expressed at lower levels in midbrain compared to PFC and STR. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

and midbrain (Figure 4D and Supplementary Extended data 9), including interleukin-6 receptor alpha (*Il6ra*) and the gene coding for P-selectin glycoprotein ligand 1 (*Selplg*) (Figure 4E). Transcripts enriched in striatal microglia were associated with pathways related to microtubules and the cytoskeleton (Figure 4F and Supplementary Extended data 9).

Transcriptional profiling reveals expression gradients among microglia across brain regions

We also identified transcripts that exhibited a gradient of expression across the rostral-to-caudal axis of the brain. These transcripts had highest expression in the midbrain, intermediate expression in the striatum, and lowest expression in the PFC; 162 transcripts fit this pattern (Figure 5A and Supplementary Extended data 10), including C-type lectin domain family 7 member 7 (*Clec7a*) and AXL receptor tyrosine kinase (*Axl*; Figure 5B). Pathway analysis revealed that these transcripts are involved in mitotic nuclear division and external side of plasma membrane (Figure 5C and Supplementary Extended data 10). We also considered the opposite gradient pattern, with transcripts that were most highly expressed in the PFC, with intermediate expression in the striatum, and lowest expression in the midbrain; 70 transcripts fit this pattern of expression (Figure 5D and Supplementary Extended data 11). The homeostatic microglia marker *Fcrls* which encodes the Fc receptor-like S, scavenger receptor, fits this pattern, as does the chemotaxis-related gene *Cd164* (Figure 5E). Pathway analysis indicated involvement in

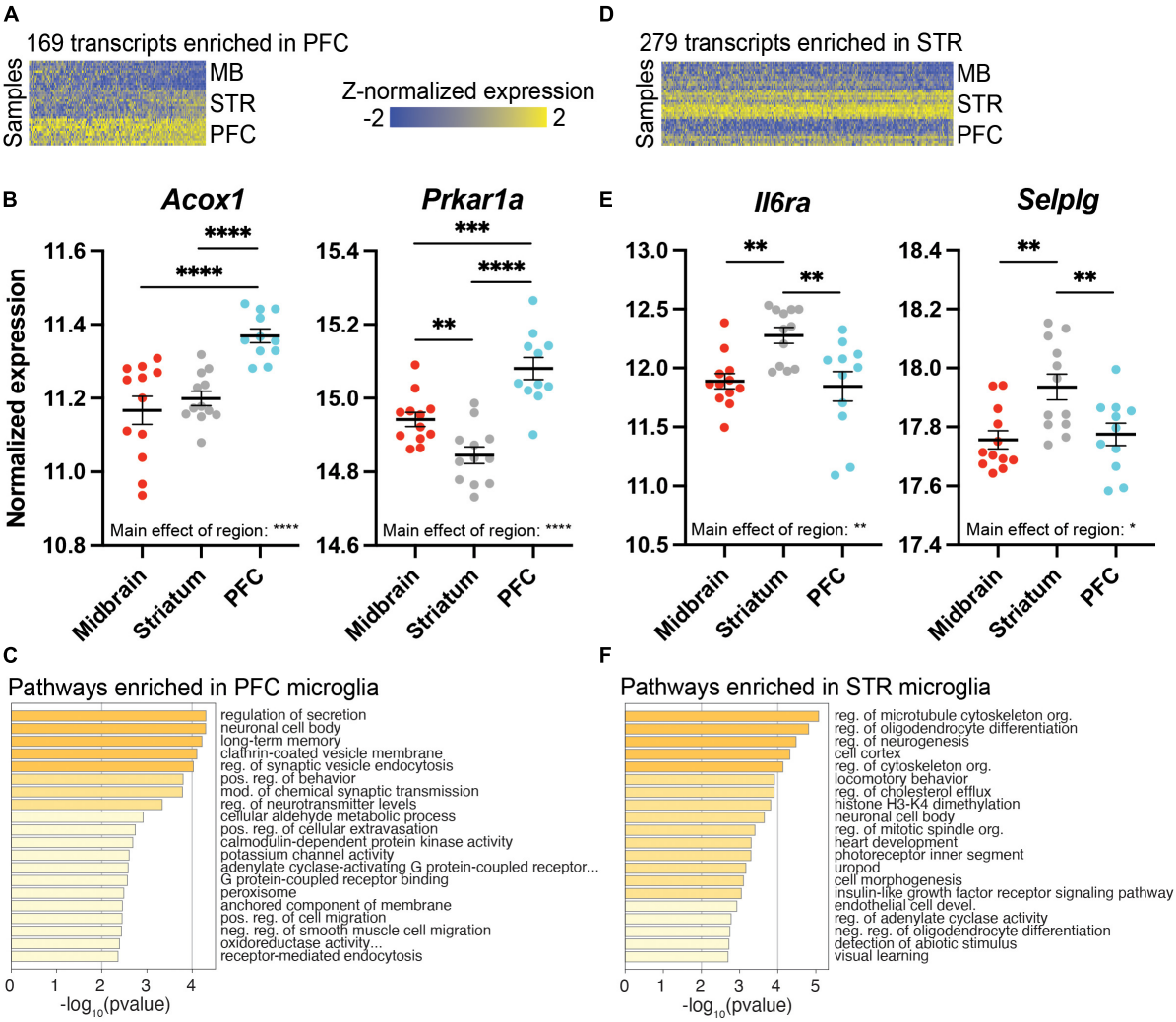

FIGURE 4
Differentially expressed (DE) transcripts enriched in prefrontal cortex (PFC) or striatum (STR). **(A)** Heatmap of 169 DE transcripts between PFC and both STR and midbrain (MB). DE transcripts are plotted on the x-axis and individual subject samples on the y-axis. **(B)** *Acox1* and *Prkar1a* fit the pattern of being more highly expressed in PFC compared to STR and MB. **(C)** Top pathways represented by transcripts expressed more highly in the PFC compared to STR and MB. **(D)** Heatmap of 279 transcripts enriched in STR compared to both PFC and MB. **(E)** *Il6ra* and *Selplg* fit the pattern of being more highly expressed in the STR compared to PFC and MB. **(F)** Top pathways represented by transcripts enriched in STR compared to PFC and MB. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

axons and long term memory (Figure 5F and Supplementary Extended data 11).

Sex differences in transcript expression in microglia

Although our PCA (Figure 2A) indicated that brain region explained most of the variance in transcript expression in microglia, previous studies suggest that microglia exhibit sex differences in morphology and gene expression (22, 23, 26–28). Given evidence that some sex differences in microglia are brain region specific, we first looked for sex differences in transcript expression within each brain region separately. In the midbrain, there were 924 DE transcripts by sex (male > female: 535 transcripts; female > male: 389 transcripts; Figure 6A and Supplementary Extended data 12). In the striatum, 454 transcripts were differentially expressed by sex (male > female: 288 transcripts; female > male: 166 transcripts; Figure 6B and Supplementary Extended data 13). In the PFC, there were 982 DE transcripts by sex (male > female: 534 transcripts; female > male: 448 transcripts; Figure 6C and Supplementary Extended data 14). As expected, many sex chromosome transcripts were among the top DE transcripts in all three brain regions (e.g., *Eif2s3y*, *Ddx3y*, *Uty*, *Kdm5d* more highly expressed

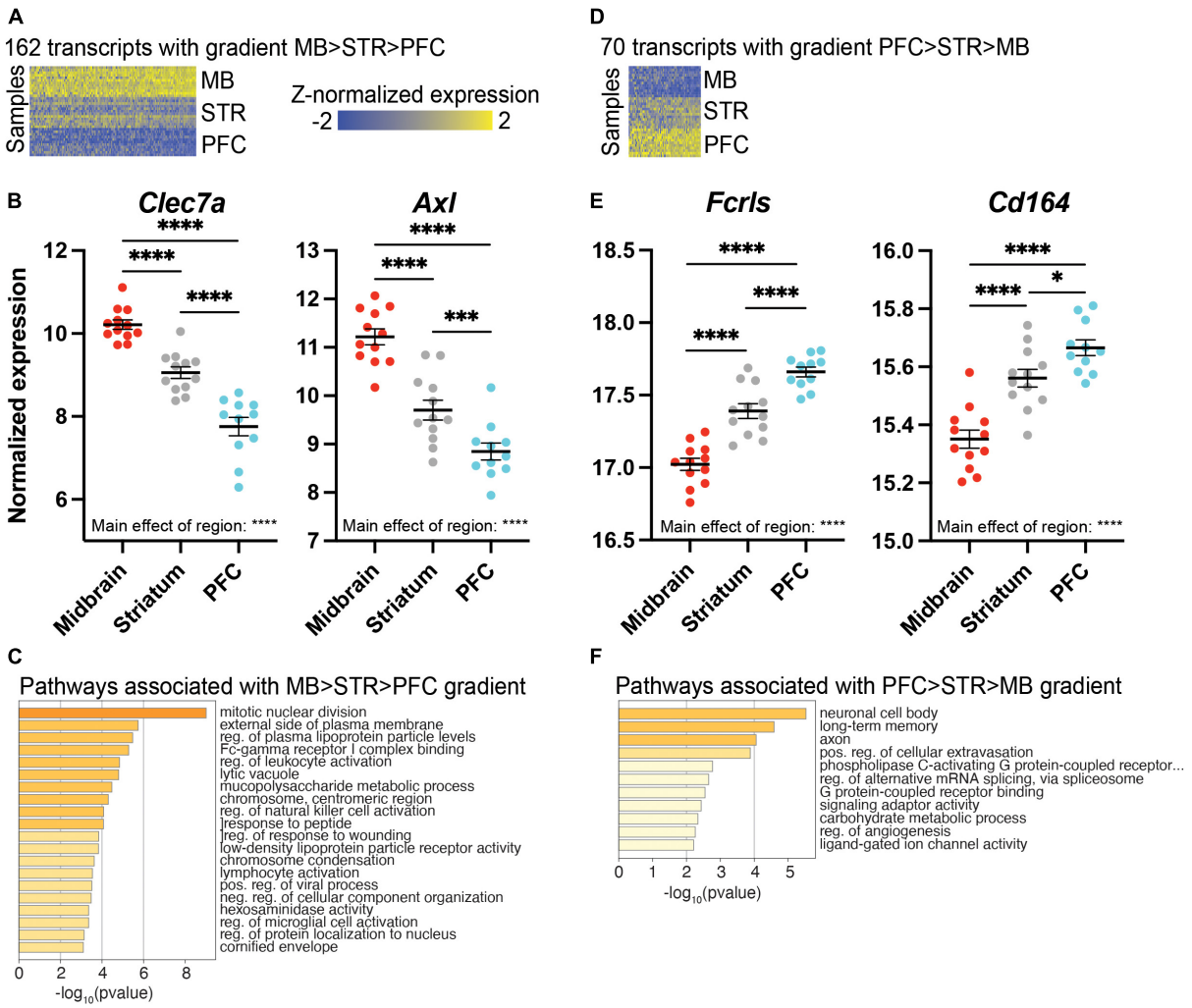

FIGURE 5

Differentially expressed (DE) transcripts exhibiting a gradient of expression across brain regions. **(A)** Heatmap of 162 DE transcripts with highest expression in the midbrain (MB), intermediate expression in the striatum (STR), and lowest expression in the prefrontal cortex (PFC). DE transcripts are plotted on the x-axis and individual subject samples on the y-axis. **(B)** *Clec7a* and *Axl* exhibit a gradient of expression of MB > STR > PFC. **(C)** Top pathways represented by transcripts exhibiting a gradient of expression of MB > STR > PFC. **(D)** Heatmap of 70 transcripts exhibiting highest expression in the PFC, intermediate expression in STR, and lowest expression in MB. **(E)** *Fcrls* and *Cd164* exhibit a gradient of expression of PFC > STR > MB. **(F)** Top pathways represented by transcripts exhibiting a gradient of expression of PFC > STR > MB. *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

in males; *Xist*, *Tsix* more highly expressed in females). Given the overlap in the top DE transcripts across brain regions, we more globally examined the overlap of transcripts that are DE by sex across regions using the threshold-free approach RRHO. Indeed, we found that the pattern of DE transcripts was quite similar between midbrain and striatum, between PFC and striatum, and between PFC and midbrain (Figure 6D). Thus, we next went back to our full model and determined that 891 transcripts exhibited a main effect of sex, with most of these transcripts being more highly expressed in males (male > female: 586 transcripts; female > male: 305 transcripts; Supplementary Extended Data 15). Pathway analysis indicated that transcripts enriched in male microglia across brain regions were associated with immune-related pathways (e.g., positive regulation of lymphocyte proliferation) and G protein-coupled receptor activity (Figure 6E and Supplementary Extended data 15). Transcripts more highly expressed in female microglia across brain regions were associated with response to selenium ion (Figure 6F and Supplementary Extended data 15), including *Selenow*, *Selenoh*, *Selenom*, and *Gpx1* (Figure 6G). Similar pathways were found when assessing each brain region separately (Supplementary Figure 3; Supplementary Extended datas 12–14).

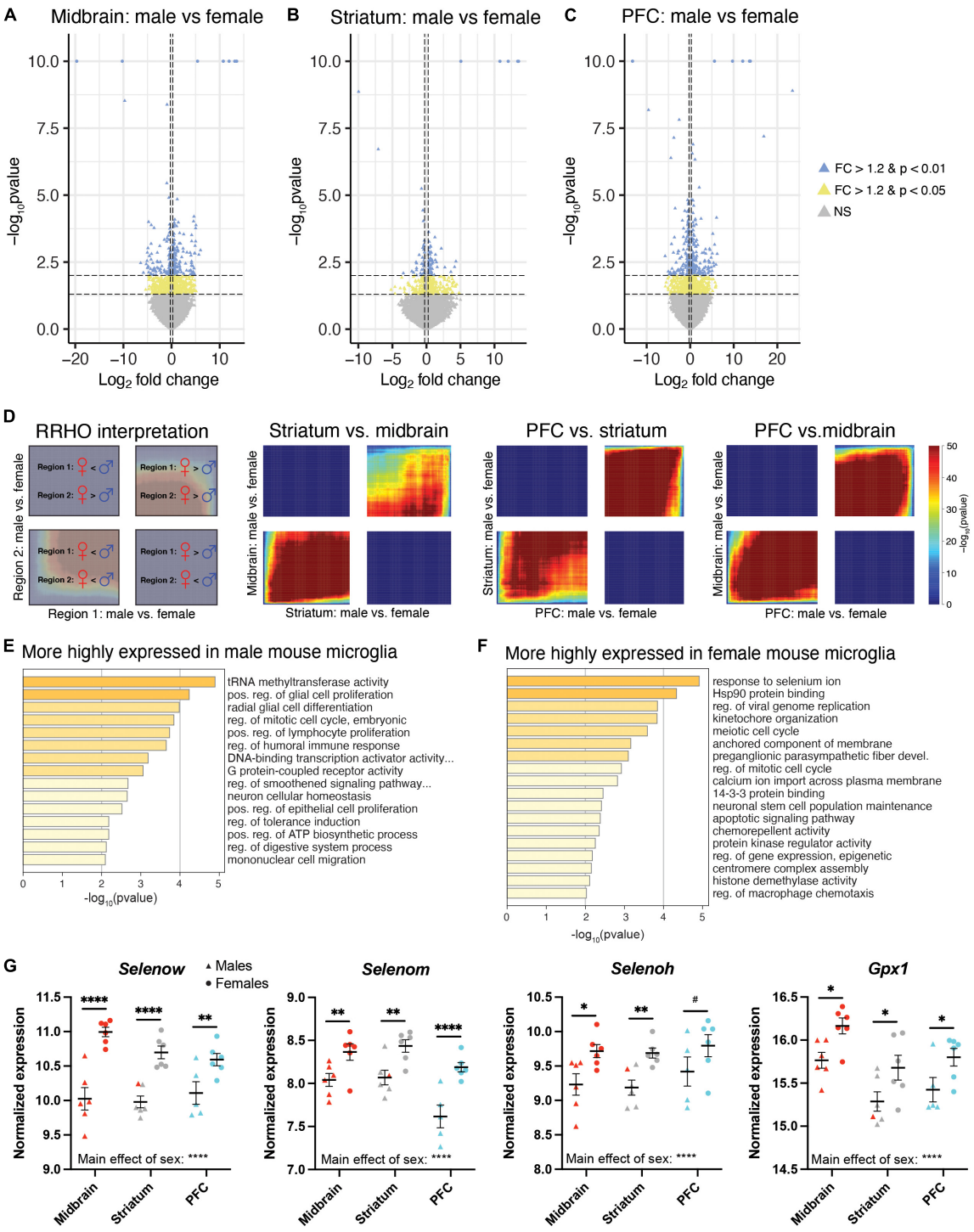

FIGURE 6

Differential expression of microglia-specific transcripts between males and females. Log_2FoldChange plotted relative to $-log_{10}$pvalue by volcano plots for differentially expressed (DE) transcripts between males and females in the midbrain (MB; **A**), striatum **(B)**, and prefrontal cortex (PFC; **C**). Horizontal dashed lines represent *p*-value significance cutoffs of $p < 0.01$ and $p < 0.05$, while vertical dashed lines represent log_2FC cutoffs of ≤ -0.26 or ≥ 0.26 (FC ≥ 1.2). Blue triangles represent DE transcripts with $p < 0.01$ and FC ≥ 1.2. Yellow triangles represent DE transcripts with $p < 0.05$ and FC ≥ 1.2. **(D)** Rank rank hypergeometric overlap (RRHO) plots indicating high degree of overlap of DE transcripts between males and females across brain regions. The interpretation of RRHO plots is indicated on the left, followed by RRHO plots representing 2 brain region comparisons. Enrichment in the bottom left and top right quadrants indicates consistent sex differences across regions. **(E)** Top pathways associated with transcripts more highly expressed in male microglia. **(F)** Top pathways associated with transcripts more highly expressed in female microglia. **(G)** The selenium-related transcripts, *Selenow*, *Selenom*, *Selenoh*, and *Gpx1* were all more highly expressed in female microglia across all three brain regions. *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$; #, $p < 0.1$.

Discussion

Summary of findings

Here we examined the transcriptional profile of microglia in three brain regions of treatment-naïve adult mice: the PFC, striatum, and midbrain. Our findings demonstrate that transcript expression between these regions differs substantially and follows one of 3 patterns. In the first and most common pattern, the transcriptional profile of midbrain microglia was distinct from the PFC and striatum. Our analysis identified many transcripts which were enriched in the midbrain with similar expression between PFC and striatum. Midbrain-enriched microglia were associated with pathways related to immune function, such as positive regulation of immune response, MHC protein complex, and response to interferon beta. We also identified transcripts with lower expression in the midbrain compared to the other two regions. These forebrain-enriched microglia were part of pathways related to synaptic function, including synapse organization and post synaptic genes. While the greatest difference in transcriptional profile was between the midbrain and forebrain, we also identified transcripts exhibiting other patterns of expression. In the second pattern, transcripts were enriched in either PFC or striatum compared to the other two regions. PFC-enriched transcripts were found in pathways associated with synapses, including regulation of synaptic vesicle endocytosis and modulation of chemical synaptic transmission. Transcripts enriched in striatal microglia were enriched for pathways associated with microtubules and cytoskeleton organization. In the third expression pattern, a subset of transcripts exhibited gradients in expression. Transcripts exhibiting a midbrain > striatum > PFC gradient were involved in mitotic nuclear division and external side of plasma membrane, as well as pathways associated with neuroinflammation. On the other hand, transcripts exhibiting a PFC > striatum > midbrain gradient were involved in axons and long-term memory. Finally, we found consistent sex differences in microglia-specific transcript expression across all three brain regions, with notable enrichment for selenium-related transcripts in female microglia.

Brain region differences in the transcriptional profile of microglia

Though these three regions are related, we found that midbrain microglia were distinct from microglia in the PFC and striatum. Notably, several immune-related pathways were enriched in midbrain microglia compared to microglia isolated from the PFC or striatum. For instance, the taurine transporter gene, *Slc6a6*, is enriched in midbrain compared to PFC and striatum, and has previously been shown to be upregulated during M1 macrophage polarization (50). Additionally, *B2m*, a component of major histocompatibility complex (MHC) class 1 which we find to be more highly expressed in midbrain microglia, exhibits elevated expression in disease associated microglia [DAM (51, 52)]. Transcripts associated with homeostatic microglia, including the transcriptional regulator, *P2ry12* (53), were more lowly expressed in midbrain microglia. Our findings fall in line with previous studies of microglial regional heterogeneity in which others have demonstrated that *P2ry12* is higher in cortex and striatum, and comparatively lower in the midbrain (17, 34), while the expression of phagocytic and immune-activating genes was higher in midbrain compared to forebrain regions (16, 17, 34). The purinergic receptor P2Y12 is an important cell surface protein which microglia use to interact with other cell types. P2Y12 is responsible for sensing ATP in the environment released by both overactive neurons and by dead or dying neurons and initiates the movement of microglial processes toward the site of injury. P2Y12 signaling is thus necessary for not only mounting the microglial neuroinflammatory response (54, 55), but also for normal synaptic function and neurophysiology (56). Together, our findings support the conclusion that midbrain microglia exhibit a more immune-vigilant signature, while microglia in the forebrain are more homeostatic.

There were also transcripts enriched within the PFC or striatum as compared to the other two regions. PFC-enriched transcripts were enriched for synapse-related pathways. *Prkar1a* was enriched in PFC microglia, and this transcript has been shown to be elevated in surveillant microglia (57). Striatal-enriched microglia were associated with microtubule- and cytoskeleton-related pathways. *Il6ra* and *Selplg* were enriched in microglia isolated from the striatum; notably, *Selplg* plays a role in microglia's ability to sense the environment (58). Together, these findings suggest midbrain microglia have a more disease-associated or immune-vigilant transcriptional profile, while cortical and striatal microglia have a transcriptional profile oriented toward remodeling synaptic and neuronal architecture as well as sensing the local environment. Because plasticity in these regions underlies learning and memory, it is possible that microglia in forebrain areas spend more time surveilling the local environment and performing functions related to synapse dynamics.

It is difficult to say with certainty what it means functionally for midbrain microglia to have a disease-associated transcriptional profile in treatment-naïve, healthy mice. However, we can look at other microglia with a similar transcriptional expression profile for clues. Previous work has demonstrated that microglia from regions with a more fenestrated BBB, such as the median eminence, the subventricular zone (SVZ), and circumventricular organs (CVOs), also have a disease-associated profile under

normal homeostatic conditions. Microglia in regions with an incomplete BBB are characterized by downregulation of identity markers and upregulation of immune markers such as CD16/32 and CD86 (13–15, 31); however, there was no effect of brain region on these markers in our dataset. Morphologically, these microglia have shortened, thicker processes, a phenotype which is typically associated with an activated state (10, 13, 14). Other research has demonstrated that midbrain microglia have more sparse branching with smaller tissue coverage compared to striatal microglia (16). It is interesting to speculate that microglia in these regions with an incomplete BBB could be constitutively activated because they are exposed to more potential threats from the periphery without the protection afforded by a complete BBB. Indeed, systemic administration of lipopolysaccharide, which induces an inflammatory response, produces robust microglial proliferation, and an accompanying increase in microglial density, exclusively in CVOs and adjacent regions (59). For microglia in the midbrain, one could speculate that the local environment demands them to be in a similarly immune-vigilant state. However, it is important to note that pro-inflammatory cytokines released by microglia such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α participate in the inflammatory process when faced with an immune challenge; however, under homeostatic conditions, these same cytokines regulate synaptic transmission and potentially work to regulate long-term synaptic plasticity (60). Along these lines, microglia in regions with an incomplete BBB express lower levels of the purinergic receptor P2Y12. Microglia in these regions extend their processes more slowly toward administered ATP than cortical microglia which express higher levels of P2Y12 (13). Consistently, midbrain microglia in our study also expressed lower levels of P2Y12. Functionally, this suggests that regulation of neurotransmission and synaptic functions by microglia could be region specific and regulated in part by markers typically associated with neuroinflammation.

Microglia-specific transcript expression follows a gradient across regions

We found that there was a hierarchical pattern of expression across the brain regions that we assayed. While expression levels in the PFC and striatum were similar and expression in the midbrain was distinct, there were transcripts in which expression was highest in the PFC and lowest in the midbrain with an intermediate level of expression in the striatum. Transcripts which followed this pattern are associated with axons, long term memory, and G protein-coupled receptor binding. Two transcripts that fit this pattern are the homeostatic microglia marker *Fcrls* and the chemotaxis-related gene *Cd164* (61). There was also expression which followed the opposite pattern, with the highest expression in the midbrain and lowest in the PFC with the striatum being intermediate. Transcripts which fit this pattern were enriched for pathways such as mitotic nuclear division and external side of plasma membrane. Several neuroinflammatory pathways were identified in our gradient analysis, including regulation of leukocyte activation, regulation of natural killer cell activation, lymphocyte activation, and regulation of microglial cell activation, consistent with midbrain-enriched microglia exhibiting a more immune-surveillant phenotype. Consistent with these immune-related pathways, *Clec7a* and *Axl* exhibit this gradient of expression (midbrain > striatum > PFC), and these transcripts are more highly expressed in DAM (51, 52).

Others have demonstrated a similar rostro-caudal gradient in expression. Identity markers (i.e., CX3CR1, P2RY12) and immune-inhibitory genes such as *Sirpa* and *Cd206* are highly expressed in forebrain regions including the cortex, hippocampus, and striatum, and exhibit lower levels in midbrain and hindbrain regions such as VTA, cerebellum, and brainstem (15, 17, 34, 62). Phagocytic or immune activating genes show the opposite pattern of enrichment: high in hindbrain and midbrain structures but lowly expressed in forebrain regions (16, 17, 63). The gradient in transcript expression is concomitant with a gradient in microglial density and morphology. The density of microglia is high in cortex and hippocampus, intermediate in midbrain nuclei, and low in the hindbrain. The same pattern is repeated with regards to the ramification of microglial processes (10–12, 16). These rostral-caudal changes in microglia could reflect the increasing complexity of the dendritic arbor and a related increase in spine density as you move more rostral through the parenchyma (64).

Work by De Biase et al. suggests that microglial diversity may be more nuanced than just a rostral-to-caudal relationship. The authors focused on regional differences within the basal ganglia circuit nuclei. Like our work, they demonstrated that midbrain VTA microglia were distinct from cortical and striatal microglia. However, variation in cell density, process complexity, and lysosome content was largest between immediately adjacent midbrain nuclei. Substantia nigra pars reticulata (SNr) microglia exhibited a transcriptional profile distinct from substantia nigra pars compacta (SNc) and VTA microglia which were comparatively much more similar to NAc microglia (16). This indicates that while a rostro-caudal gradient in microglia transcriptional profiles exists across brain regions, local environments exist along the neuroaxis, and microglia respond to cues within those discrete areas. De Biase et al. further demonstrate that the ratio of microglia to neurons and other glial cell types, particularly astrocytes, changes in accordance with differences in microglial function and morphology; regional microglial phenotype was restored after pharmacologic ablation. These findings suggest that rather

than epigenetic programing set during development, cell-extrinsic regulatory signals produce and maintain regional microglial identity in the adult organism. The midbrain contains other important structures, including perhaps most notably the dorsal raphe nuclei (DRN), which is responsible for serotonin synthesis within the brain. These serotonergic cells receive a range of brainstem inputs and project to an array of forebrain nuclei and play a crucial role in modulating complex behaviors (i.e., mood, reward, motivation, and learning). This nucleus consists of a diversity of cell types which contain multiple neurotransmitter types that are transcriptionally heterogenous (65–68). The periaqueductal gray (PAG) is another midbrain region relevant to autonomic function, motivation, defensive behavior, and pain modulation. Interestingly, evidence suggests that microglia within the PAG exhibit sex differences in levels of activation (female > male), and that female PAG microglia are more responsive to an immune challenge (69). Thus, the DRN and PAG represent discrete, local environments that may have distinct pools of microglia which might vary by sex or exhibit different transcriptional profiles compared to other brain regions. While we did not differentiate between subregions of the midbrain, such analyzes might be interesting in the future to discern whether microglia isolated from these discrete midbrain regions exhibit transcriptional differences.

Other research has corroborated this conclusion in both human and mouse. It has been consistently demonstrated that microglia express a composite transcriptional profile across regions which distinguishes them from other cell types such as peripheral macrophages, but which is expressed at different ratios within discrete structures. The maintenance of microglial phenotype depends on yet to be identified signals *in vivo*, and microglia lose their phenotype *ex vivo* and *in vitro* (15, 70–73). All these lines of evidence suggest that microglial regional heterogeneity depends on local signaling from other cell types which vary from region to region in terms of density, morphology, and function.

Sex differences in transcript expression in microglia

While brain region explained most of the variance associated with the transcriptional profile of microglia, we also found sex differences in expression. This finding is consistent with a growing literature indicating sex differences in microglia (22, 23, 26–28).

The top pathway associated with transcripts more highly expressed in female microglia across all three brain regions was response to selenium ion (e.g., *Selenow*, *Selenoh*, *Selenom*, *Gpx1*). Gene products of these transcripts represent members of a class of Selenium (Se)-dependent proteins which participate in glucose metabolism and protect cells from oxidative stress by reducing reactive oxygen and nitrogen species (74, 75). Glutathione peroxidase 1 (GPX1) may play a further role in regulating the inflammatory response. For instance, overexpression of GPX1 results in fewer activated microglia after ischemic injury (76). There are many members of this diverse family with tissue-specific patterns of expression. Interestingly, expression of GPX1 and SelenoW is brain enriched. Spatial expression profiling indicates enrichment of SelenoW in 90% of brain regions assayed in the adult mouse (77). SelenoW has strong antioxidant properties and evidence suggests it plays a functional role in neuronal synapses and is highly expressed in the synaptic compartment (78, 79). In line with our findings, multiple lines of research demonstrate strong sex differences in selenoprotein expression and activity across domains including intracellular selenium metabolism, selenium recycling, absorption, and secretion. Selenium also directly influences the production of the sex hormone testosterone and, in return, sex hormones regulate selenium distribution and metabolism (74). For GPX1, female mice demonstrate greater efficiency in use of dietary selenite and higher expression of Gpx1 mRNA in peripheral tissue (80, 81). In humans, *Gpx1* SNPs also show sex differences leading to lower enzyme activity in males (82, 83). However, less is known about sex differences in activity and functional consequences of SelenoW expression. Further, existing research links GPX1 and SelenoW to risk for Alzheimer's Disease (AD) (84). Selenium levels are significantly decreased in AD patients and carriers of the risk allele apolipoprotein E (ApoE4), making selenoproteins an intriguing site of inquiry (84). Both GPX1 and SelenoW are expressed in regions associated with the pathophysiology of AD and polymorphisms in human *Gpx1* have been significantly correlated with AD in two South American populations (85, 86). Chen et al. found SelenoW can form a disulfide bond to inhibit tau aggregation, which suggests this protein may play a crucial neuroprotective role (87). Microglia are directly linked to the pathophysiology of AD; activated microglia respond to the buildup of Aβ plaques, and the overactivation of microglia may lead to the pathological loss of synapses in the disorder. Outside of *APOE*, the majority of AD-associated risk loci are expressed exclusively or preferentially by microglia (88). It is interesting to speculate that sex differences in selenoproteins, possibly even within microglia, might contribute to sex differences in AD.

While we see mostly consistent sex differences in the microglia transcriptional profile across the PFC, striatum, and midbrain, there are some differences. Notably, we find that many synapse-related transcripts and associated pathways (e.g., "glutamatergic synapse" and "GABA receptor binding") are more highly expressed in females in only the midbrain. Interestingly, a previous study by Guneykaya et al., examined sex differences in microglia transcript expression and identified "GABA and Glutamate receptor activity" associated with

transcripts more highly expressed in females (25). Guneykaya et al., also found that male microglia are enriched for pathways associated with transcriptional activity in the cortex, similar to our findings in the PFC. We also found that across brain regions, male microglia exhibit a more inflammatory profile. This result is consistent with a previous study which examined the transcriptional profile of microglia isolated from the whole mouse brain (23). Overall, our results associated with sex differences in microglia transcript expression are consistent with previous studies.

Limitations

One limitation to the current study is that we did not assess phase of estrous cycle in female mice at the time of sacrifice, which would have given us insight into whether levels of circulating ovarian hormones might influence transcript expression in females. Given our findings for sex differences in expression of microglia-specific transcripts, in future studies, it will be important to determine whether circulating gonadal hormones drive these sex differences. However, a previous study reported that phase of estrous cycle did not influence microglia-specific gene expression in the hippocampus (24). Future studies will also use similar methodology to probe for sex differences in brain regions more traditionally defined as being sexually dimorphic (e.g., hypothalamus). Another limitation is that the transgenic strain we used required tamoxifen injections to drive Cre expression. Tamoxifen can act as a potent estrogen receptor-alpha agonist and antagonist, and administration of tamoxifen may disrupt cyclicity in females [e.g., (89–93)]. Thus, it is possible that tamoxifen influenced some of our findings related to sex differences. However, we waited 10–21 days after tamoxifen injection to sacrifice mice, reducing the possibility for acute effects of tamoxifen on microglia-specific transcript expression. Further, evidence suggests that tamoxifen used for Cre-induction does not cause long-term effects or sex differential responses in the brain transcriptome (94). We report findings related to transcript expression within isolated microglia; thus, it is unclear if associated proteins will exhibit similar patterns. Future studies will assess protein levels of identified transcripts within microglia. Another limitation is that we might have missed a population of TMEM119-negative microglia, which could not be determined using the method employed here. Finally, it is likely that several factors contribute to inform regional phenotype, which cannot be divorced from each other. It is hard to disentangle the effects of local cues in the environment from putative identity differences inherent to microglia within different compartments. Factors from the local environment (e.g., from neurons, glia, as well as infiltrating blood-derived macrophages) all interact with microglia, which may contribute to regional heterogeneity. Indeed, there are regional differences in the density, relative cell ratio, and function of these other cell types which creates unique signaling milieus within discrete local environs to which microglia respond.

Conclusion

Here, we find that the transcriptional profile of isolated microglia differs based on brain region and sex, even under homeostatic conditions. Future studies will use similar methodology to assess whether these transcriptional profiles shift when mice are exposed to stress, with a specific focus on whether males and females exhibit similar alterations. Microglia have in the past been thought of as a homogenous cell type which was alternatively quiescent during homeostasis and activated when the parenchyma was faced with threat or neurodegeneration. However, microglia play several roles in the healthy organism as well, and evidence suggests that there are several subtypes of microglia which can be distinguished by the transcripts they express. These putative subtypes can be found in different ratios within and across regions and may perform specific functions in response to stimuli (73). Indeed, our findings support the hypothesis that microglia perform diverse functions based on both brain region and sex.

Data availability statement

Raw and processed RNA-sequencing data have been deposited into the NCBI Gene Expression Omnibus database (GEO; GSE203553).

Ethics statement

The animal study was reviewed and approved by University of Pittsburgh Institutional Animal Care and Use Committee.

Author contributions

MLS, RL, and ZF designed and coordinated the study. KB and SP obtained and processed samples using FACS sorting. XX, MLS, KB, GT, and YA-A conducted statistical analyzes and data interpretation. MS, YA-A, KB, and MLS drafted the manuscript. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2022.945548/full#supplementary-material

SUPPLEMENTARY EXTENDED DATA 1
Mean number of isolated microglia across brain regions.

SUPPLEMENTARY EXTENDED DATA 2
Microglia-specific transcripts exhibiting a main effect of brain region.

SUPPLEMENTARY EXTENDED DATA 3
Microglia-specific transcripts exhibiting significant differences between prefrontal cortex and midbrain in *post hoc* analyzes.

SUPPLEMENTARY EXTENDED DATA 4
Microglia-specific transcripts exhibiting significant differences between striatum and midbrain in *post hoc* analyzes.

SUPPLEMENTARY EXTENDED DATA 5
Microglia-specific transcripts exhibiting significant differences between striatum and prefrontal cortex in *post hoc* analyzes.

SUPPLEMENTARY EXTENDED DATA 6
Microglia-specific transcripts with enrichment in midbrain (MB) compared to both prefrontal cortex (PFC) and striatum (STR), with no difference between prefrontal cortex and striatum [MB > (PFC = STR)].

SUPPLEMENTARY EXTENDED DATA 7
Microglia-specific transcripts with lower expression in midbrain (MB) compared to both prefrontal cortex (PFC) and striatum (STR), with no difference between prefrontal cortex and striatum [MB < (PFC = STR)].

SUPPLEMENTARY EXTENDED DATA 8
Microglia-specific transcripts with enrichment in prefrontal cortex compared to both striatum and midbrain.

SUPPLEMENTARY EXTENDED DATA 9
Microglia-specific transcripts with enrichment in striatum compared to both prefrontal cortex and midbrain.

SUPPLEMENTARY EXTENDED DATA 10
Microglia-specific transcripts with highest expression in midbrain (MB), intermediate expression in striatum (STR), and lowest expression in prefrontal cortex (PFC) [MB > STR > PFC].

SUPPLEMENTARY EXTENDED DATA 11
Microglia-specific transcripts with lowest expression in midbrain (MB), intermediate expression in striatum (STR), and highest expression in prefrontal cortex (PFC) [MB < STR < PFC].

SUPPLEMENTARY EXTENDED DATA 12
Microglia-specific transcripts exhibiting a sex difference in expression in the midbrain.

SUPPLEMENTARY EXTENDED DATA 13
Microglia-specific transcripts exhibiting a sex difference in expression in the striatum.

SUPPLEMENTARY EXTENDED DATA 14
Microglia-specific transcripts exhibiting a sex difference in expression in the prefrontal cortex.

SUPPLEMENTARY EXTENDED DATA 15
Microglia-specific transcripts exhibiting a main effect of sex across brain regions.

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EDITED BY
Ryan W. Logan,
Boston University, United States

REVIEWED BY
Anilkumar Pillai,
University of Texas Health Science Center at Houston, United States
Stewart Alan Anderson,
University of Pennsylvania, United States

*CORRESPONDENCE
Anthony Christopher Vernon
anthony.vernon@kcl.ac.uk
Deepak Prakash Srivastava
deepak.srivastava@kcl.ac.uk

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Interferon-γ exposure of human iPSC-derived neurons alters major histocompatibility complex I and synapsin protein expression

Adam Pavlinek[1,2], Rugile Matuleviciute[1,2], Laura Sichlinger[1,2], Lucia Dutan Polit[1,2], Nikolaos Armeniakos[1,2], Anthony Christopher Vernon[1,2]* and Deepak Prakash Srivastava[1,2]*

[1]Department of Basic and Clinical Neuroscience, Institute of Psychiatry, Psychology and Neuroscience, King's College London, London, United Kingdom, [2]MRC Centre for Neurodevelopmental Disorders, King's College London, London, United Kingdom

Human epidemiological data links maternal immune activation (MIA) during gestation with increased risk for psychiatric disorders with a putative neurodevelopmental origin, including schizophrenia and autism. Animal models of MIA provide evidence for this association and suggest that inflammatory cytokines represent one critical link between maternal infection and any potential impact on offspring brain and behavior development. However, to what extent specific cytokines are necessary and sufficient for these effects remains unclear. It is also unclear how specific cytokines may impact the development of specific cell types. Using a human cellular model, we recently demonstrated that acute exposure to interferon-γ (IFNγ) recapitulates molecular and cellular phenotypes associated with neurodevelopmental disorders. Here, we extend this work to test whether IFNγ can impact the development of immature glutamatergic neurons using an induced neuronal cellular system. We find that acute exposure to IFNγ activates a signal transducer and activator of transcription 1 (STAT1)-pathway in immature neurons, and results in significantly increased major histocompatibility complex I (MHCI) expression at the mRNA and protein level. Furthermore, acute IFNγ exposure decreased synapsin I/II protein in neurons but did not affect the expression of synaptic genes. Interestingly, complement component 4A (*C4A*) gene expression was significantly increased following acute IFNγ exposure. This study builds on our previous work by showing that IFNγ-mediated disruption of relevant synaptic proteins can occur at early stages of neuronal development, potentially contributing to neurodevelopmental disorder phenotypes.

KEYWORDS

interferon-γ, MHCI, synapsin, iPSC, maternal immune activation, inflammation, C4A, schizophrenia

Introduction

Human epidemiological studies and animal models suggest a link between maternal immune activation (MIA) and an increased risk for psychiatric disorders with a putative neurodevelopmental origin, including schizophrenia and autism (1). Although there are many plausible factors that are critical for establishing neurodevelopmental resilience or susceptibility to MIA (2), there is evidence to suggest that the intensity of the maternal immune response is one important factor linking maternal infection to the potential for differential brain development and behavioral phenotypes (3–5). Indeed, animal MIA models display deficits in cognitive and social behaviors (6), which are accompanied by altered synaptic plasticity, decreased synaptic protein levels, and reduced dendritic spine density, predominantly in the prefrontal cortex and hippocampus (7–11). These findings are consistent with *in vivo* neuroimaging evidence for reduced synaptic density, as measured by reduced binding of positron emission tomography (PET) radioligands targeting synaptic vesicle glycoprotein 2A (SV2A) in schizophrenia (12, 13), reduced dendritic spines (14), and a meta-analysis confirming decreased expression of synaptic proteins in *post-mortem* brain tissue from individuals with schizophrenia (15).

One key feature of the maternal immune response that shapes these phenotypes is the elevation of numerous cytokines in the maternal serum, placenta and fetal brain (16, 17). Consistent with this view, elevated levels of cytokines in the maternal serum are predictive of the risk for the affected offspring to develop schizophrenia (18). The emerging theme from such studies is that changes in maternal cytokines during pregnancy can have long-lasting consequences (19–21). However, to what extent specific cytokines are necessary and sufficient for these effects remains unclear. Moreover, the underlying molecular mechanisms the are exerted on the developing brain and on specific cell types, remain to be fully elucidated. Evidence from animal models of MIA provides support for the involvement of altered levels of interleukins, particularly interleukin-(IL)-6, IL-1beta and IL-10 but also for the cytokines TNF-alpha, and interferon-γ (IFNγ) (6, 22–24). Of these, IFNγ has been found to have increased levels in the plasma of first-episode schizophrenia patients (25). In addition to its key role in the response to viral infection, IFNγ has also been shown to induce retraction of dendrites and inhibit synapse formation in the central nervous system (26, 27). Despite these findings, it is unclear whether and how elevated levels of IFNγ impact the development of neurons, and if this could contribute to increased risk for schizophrenia.

We previously demonstrated that acute exposure of neural progenitor cells (NPCs) and neurons derived from human induced pluripotent stem cells (iPSCs) to IFNγ results in gene expression changes in genes associated with schizophrenia and autism, and altered neuronal morphology in exposed neurons (28). In particular, IFNγ treatment increased major histocompatibility complex I (MHCI) expression (28). Class I MHC family molecules are best known for their function in presenting antigens to T-cells (29). MHCI is however also expressed in neurons and neural progenitors and has been found to be important in neuronal plasticity and for the co-regulation of synapse pruning in mice (29, 30). Furthermore, MHCI negatively regulates synapse density in developing cortical neurons, with *in vitro* manipulations of MHCI expression inversely affecting the density of both GABAergic and glutamatergic synapses in rat and mouse cultures (31). In a mouse model of MIA, synapse number in cultured cortical neurons were decreased, and MHCI was found to be required for this MIA-induced effect on synapse density (32). Genome-wide association studies (GWAS) also demonstrate that genetic variation within the MHC loci links with schizophrenia risk (33, 34). For example, variation of complement component 4A (C4) at the MHCIII locus and human leukocyte antigen-B (HLA-B) at the MHCI locus is strongly associated with increased risk for schizophrenia (35).

In our previous work, gene expression changes following IFNγ treatment included increased expression of MHCI genes and downregulation of genes related to the gene ontology (GO) term "synapses" in exposed iPSC-neurons (28). Given that IFNγ has been shown to affect expression of synaptic genes in iPSC-neurons in the absence of glial cells, we aimed to further characterize the effect of IFNγ treatment in developing human glutamatergic neurons, and specifically on MHCI and synaptic protein expression. Using Neurogenin 2 (NGN2) optimized inducible overexpression ioGlutamatergic iNeurons (*NGN2*-iNs) (36), we find that acute exposure to IFNγ activates a STAT1-signaling pathway in immature *NGN2*-iNs. Furthermore, we observed that IFNγ exposure increased MHCI protein and *HLA-B* and *C4A* expression but decreased the expression of the synaptic proteins synapsin I and synapsin II in cell bodies without altering the expression of a select panel of synaptic genes. These data further demonstrate that elevated levels of IFNγ are capable of disrupting the expression of synaptic proteins and impacting the development of immature glutamatergic neurons in the absence of glial cells.

Methods

Human iPSC culture, neuralization, and treatment

The ioGlutamatergic male neurotypical stem cell line (36) was obtained from BitBio (Cambridge, UK) under MTA agreement. ioGlutamatergic cells were maintained in Stemflex media (Gibco; A3349401) on six-well plates coated with 1:100

Geltrex (Life technologies; A1413302). Media was changed every 48 h and passaged when 70–80% confluent with HBSS and Versene (Gibco; 15040066) at 37°C before being transferred into new Stemflex medium. Neuralization was conducted based on the protocol used by Pawlowski et al. (36). Cells for experiments were terminally plated onto 6-well-plates (for RNA and protein extraction) or glass coverslips in 24-well-plates (for immunocytochemistry) coated with Poly-D-Lysine (5 μg/ml, PDL, A-003-E; Millipore) and laminin (1 mg/ml Sigma L2020). Human iPSCs were dissociated with accutase (A11105-01; Thermo Fisher Scientific) before being diluted with medium and subsequently resuspended in N2 medium with 1 μg/ml doxycycline hyclate and 10 μM ROCK inhibitor (Sigma; Y0503). Cells were plated at a density of 900,000 cells/well for RNA extraction and 25,000 cells/well for ICC. The cells were incubated at 37°C; 5% CO2; 20% O2 with daily N2 media changes supplemented with 1 μg/ml doxycycline hyclate. Either 25 ng/ml IFNγ (Abcam, AB9659; diluted in DMEM) for treatment conditions or vehicle (DMEM) was added at day 3 to the N2 medium. The cells were incubated for 24-h before sample collection (28). For western-blotting, total protein was extracted 15 min after treatment with IFNγ or vehicle on day 3.

In parallel, the 127_CTM_01 human iPSC male neurotypical line (37) was differentiated into NPCs using a dual SMAD inhibition protocol (37, 38). Briefly, the NPCs were expanded from day 18 frozen stocks in maintenance medium (1:1 N2:B27, 10ng/ml bFGF) for seven days. Before treatments, the cells were plated on 12-well NUNC^{TM} tissue culture plates (Thermo Scientific; 150628) at a density of 500,000 cells/well, with dedicated wells for treatment and vehicle treatments. The day after plating, the cells were exposed to 25 ng/ml IFNγ or vehicle and incubated for 24-h before sample collection.

Western blotting

Cell lysates from treated *NGN2*-iNs were prepared from day 3 cells following treatment. Cells were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris-HCl (pH 7.2), 5 mM EDTA, 0.1% SDS (weight/volume), 1% Triton X-100 (volume/volume), 1% deoxycholate (weight/volume), and inhibitors), before being sonicated with 10 short bursts. Sample buffer was added to all samples, which were then denatured for 5 min at 95°C and stored at −80°C until used further. All samples (5 μg) were subsequently separated by SDS-PAGE and analyzed by Western Blotting with antibodies against phospho-STAT1, phosphor-ERK1/2, ERK1/2, and GAPDH (Supplementary Table 1). Western blots were visualized using Clarity Western ECL substrate (Bio-Rad) before protein detection using the ChemiDoc XRS+ imaging system using ImageLab^{TM} software. Quantification of bands was performed by measuring the integrated intensity of each band and normalizing to the housekeeper GAPDH using ImageStudioLite.

Immunocytochemistry

Cells were fixed with 4% formaldehyde in PBS-sucrose for 10 min at room temperature, washed 2× with Dulbecco's PBS (DPBS, Gibco), and then fixed with ice cold Methanol at 4°C for 10 min, then washed 2× with DPBS. Cells were permeabilized and blocked using 2% normalized goat serum (NGS) in DPBS with 0.1% triton x-100 for 2 h. Antibody solutions (Supplementary Table 2) were prepared in 2% NGS in DPBS. The coverslips were incubated with primary antibody solution at 4°C overnight, then washed 3× with DPBS for 10 min each and incubated with secondary antibodies for 1 h at room temperature. The coverslips were washed 3× with DPBS for 10 min each and incubated for 5 min in DAPI solution, followed by two DPBS washes, then mounted onto glass slides using ProLong Gold antifade reagent (Invitrogen P36930).

Microscopy and image analysis

Coverslips were imaged using a Leica SP5 confocal microscope. The gain and other imaging parameters were set using the vehicle control and were not changed during subsequent imaging of the control and IFNγ exposed coverslips with 246.5x246.5 μm regions imaged. The Z-stack thickness was kept at 0.5 μm and Z-stacks were then maximally projected to form a single image in FIJI. Prior to measuring fluorescent intensity, the background of each image and channel was measured in FIJI by selection of 10 25×25-pixel areas of background and measuring the mean and standard deviation (SD) of staining intensity of each area. The mean of these measurements + 2SD was then subtracted from the image. Cell Profiler (39) was used to identify the nuclei, cells, cell bodies, processes, and the cytoplasm and to measure the mean intensity of the MHC and synapsin I/II channels. Mean intensity values of 0 were excluded from the analysis. The pipeline is provided as a Supplementary file.

Quantitative PCR

Cells for RNA extraction were lysed in TRI Reagent (T3809, Merck) for 5 min at room temperature and RNA was extracted from TRI Reagent according to the manufacturer's protocol. Isolated RNA was cleaned by precipitation with 3% sodium acetate in ethanol at −80°C overnight, washed as in the isolation protocol, and resuspended in H_2O. A nanodrop spectrophotometer was used to measure RNA concentration and quality.

For cDNA synthesis, a mixture of 1 μl of oligo(dT)20 (50 μM) (Invitrogen; 18418020), 2 μg total RNA, 1 μl 10 mM dNTP Mix (10 mM each) (Invitrogen; 18427013), and water to make up a total of 13 μl per sample was heated to 65°C

for 5 min and incubated on ice for 1 min. Next, superscript mastermix (Invitrogen; 18080093) was added to each sample (4μl 5X First-Strand Buffer, 1 μl 0.1 M DTT, 1 μl RNaseOUT Recombinant RNase Inhibitor (Invitrogen; 10777019), 1 μl of SuperScript III RT (200 units/μl)) and the mixture was incubated at 50°C for 50 min and then 70°C for 15 min. qPCR was done in a 348 well-plate, with two technical replicates per sample, and also a blank well-containing no cDNA for each primer pair (Supplementary Table 3). Three housekeeping genes (HPRT, SDHA, RPL27) were used. A mastermix consisting of 2 μl 5x qPCR Mix Plus, 1.5 μl Primer mix, and 4.5 μl RNAse free per well was added to the plate. 2 μl cDNA were added to each well. qPCR was run using a QuantStudio7 thermocycler with one cycle for 12 min at 95°C and 40 cycles of 95°C for 15s, 60–65°C for 20s and 72°C for 20 s.

The data were analyzed using the $2^{-\Delta\Delta Ct}$ method (40). For each gene, the technical replicates were averaged. The three housekeeping genes were averaged and the ΔCt (difference between the housekeeper average and gene of interest average) was calculated for each gene of interest. The ΔΔCt was calculated as ΔCt-[Calibrator] where the calibrator is the average of the ΔCt of the controls. The final result is $2^{-\Delta\Delta Ct}$. This value was log-transformed prior to statistical analysis.

Statistical analysis

For both the ICC and qPCR experiments, three biological replicates ($N = 3$) were analyzed, where each replicate is the same cell line but with a different passage number and differentiated on a different day. The number of replicates was decided prior to the conducting of the experiments. Statistical analysis was done in Prism 9.0.2. The exposed and control mean intensity values (ICC) or log ($2^{-\Delta\Delta Ct}$) values (qPCR) were compared using multiple 2-tailed unpaired *t*-tests, and corrected for multiple comparisons using the Holm-Šídák method.

Results

Acute IFNγ exposure downregulates presynaptic genes associated with synaptic vesicles

In the RNA sequencing data from our previous study, we found downregulation of genes related to the GO term "synapses" in human iPSC-NPCs exposed to IFNγ for 24 h (28). To explore this further, a curated database of synaptic genes, SynGO (41), was used to identify significantly enriched biological processes (BP) and cellular component (CC) ontologies related to synaptic function. Analyses were carried out with the complete list of significantly down-regulated genes in day 30 neurons acutely exposed to IFNγ (25 ng/ml, 24 h) compared with vehicle-exposed neurons. The results reveal 18 genes mapping to SynGO synaptic proteins with significant enrichment for 3 CC and 5 BP terms (Figure 1). Most of these proteins ($n = 12$) were annotated in the presynapse cluster with four genes enriched for the synaptic vesicle membrane term. These results suggest that acute IFNγ exposure leads to the downregulation of 18 genes that exert presynaptic functions and regulate synaptic vesicle mechanisms in iPSC-neurons.

Ngn2 overexpression generates early glutamatergic neurons at day 4

We used ioGlutamatergic line cells with *NGN2* optimized inducible overexpression to allow for rapid and reliable generation of *NGN2*-induced neurons (*NGN2*-iNs) upon treatment with doxycycline (Figure 2A) (36, 42). We first validated whether the ioGlutamatergic line expresses relevant markers of glutamatergic neurons after the activation of the *NGN2* gene. By day 7 of differentiation, the cells express the pan-neuronal marker microtubule-associated protein 2 (MAP2) and excitatory presynaptic marker vesicular glutamate transporter 1 (VGLUT1) (Supplementary Figure 1). After 28 days of differentiation >99% of DAPI+ cells were immune-positive for MAP2 and also expressed TBR1, VGLUT1, CAMKIIA, and SV2A, consistent with the generation of forebrain glutamatergic neurons (Supplementary Figure 2). This is consistent with evidence that the majority of mature ioGlutamatergic neurons represent cortical excitatory neurons (42, 43). Analysis was conducted on cells at day 4 of differentiation, hereafter referred to as Day 4 *NGN2*-iNs. At this developmental timepoint, the *NGN2*-iNs resemble NPCs or early neurons with synapse growth cones (43), suitable for analysis of synaptic vesicles and synapse development.

We characterized Day 4 *NGN2*-iNs using immunocytochemistry (ICC) and quantitative PCR (qPCR). We stained for the post-mitotic neuron marker neuronal nuclei antigen (NeuN) and mature neuron marker microtubule-associated protein 2 (MAP2) and the neuroprogenitor markers nestin (NES) and PAX6. In addition, staining was conducted for the early neuron/late progenitor marker Class III β-Tubulin (TUBB3). Qualitatively, all imaged Day 4 *NGN2*-iNs expressed both the neuroprogenitor markers nestin and PAX6 and the neuronal markers NeuN and MAP2 (Supplementary Figure 3), indicating that the Day 4 *NGN2*-iNs represent early post-mitotic neurons. Morphologically, the Day 4 cells had extensive processes, and some resembled young neurons with a pyramidal cell body. Other cells had a bipolar neuroprogenitor-like morphology (Supplementary Figure 3).

qPCR for the neuronal markers *NeuN*, *TBR1*, and *MAP2* and the neuroprogenitor markers nestin and *PAX6*

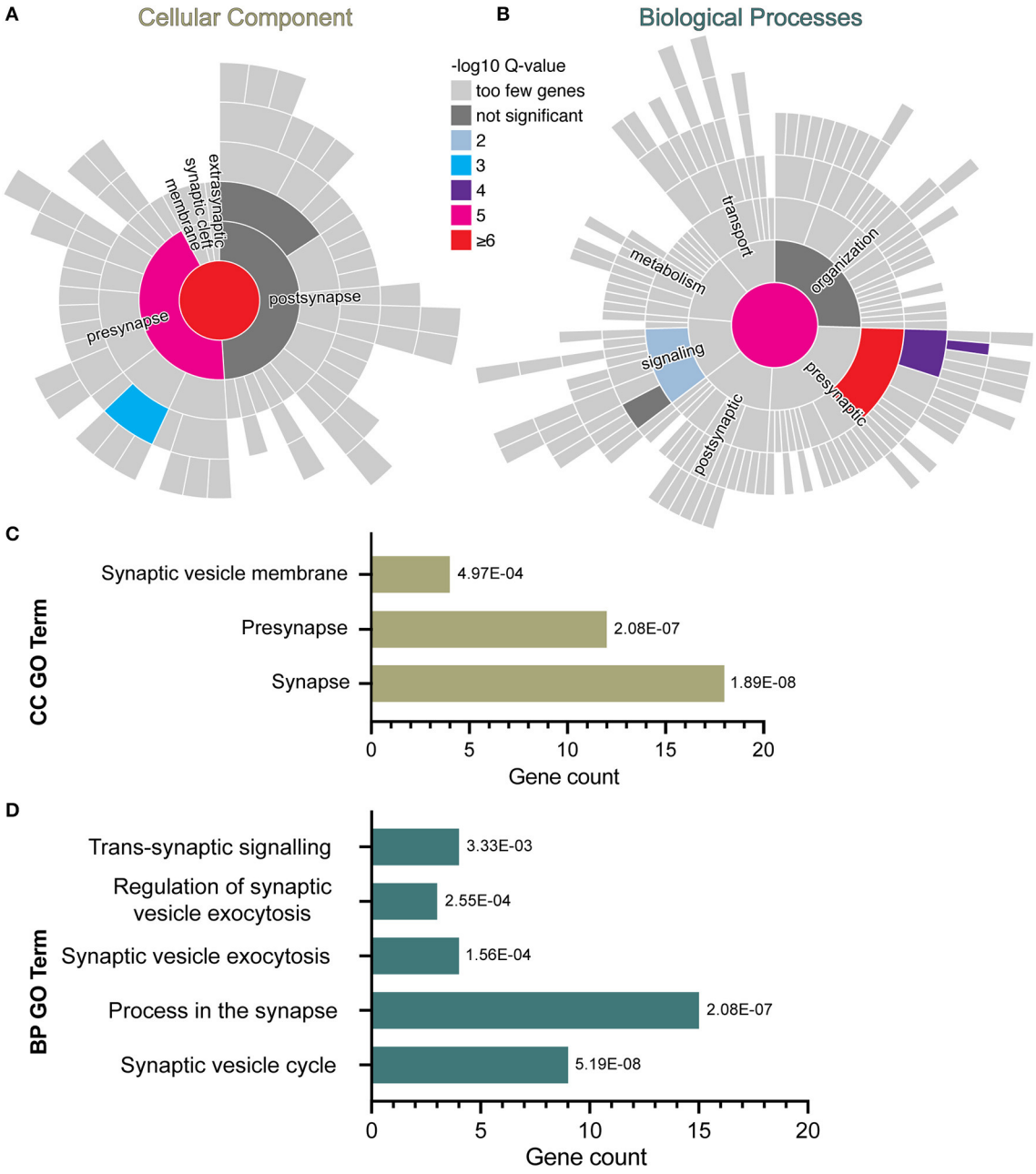

FIGURE 1
SynGO synaptic gene ontology analyses of down-regulated DEG in D30 neurons acutely exposed to IFNγ. The sunburst plots represent synaptic annotated ontologies for CC **(B)** and BP **(A)** terms. The key color scale indicates -log10 FDR adjusted *p*-values. **(A)** Significantly enriched CC ontologies include synapse (red) presynaptic (magenta) clusters. **(B)** Annotated BP terms include synaptic (magenta), presynaptic (red) and signaling (blue) terms. **(C,D)** Plots of synaptic GO output showing the 3 CC **(C)** and 5 BC **(D)** significantly (FDR-adjusted) enriched terms for D30 IFNγ exposed neurons. The bar length indicates the number of genes, the order of each bar and numbers adjacent to each are the FDR adj. *P*-value. Analysis of data previously published inWarre-Cornish et al. (28).

(Supplementary Figure 3) shows that neural genes had a higher expression level compared to the progenitor genes, in particular *TBR1* and *NeuN* were highly expressed. Overall, these results indicate that Day 4 *NGN2*-iNs resemble early neurons.

Acute IFNγ signals through a canonical signaling pathway in NGN2-iNs

In neurons, IFNγ is thought to signal via a signal transducer and activator of transcription 1 (STAT1)-dependent pathway,

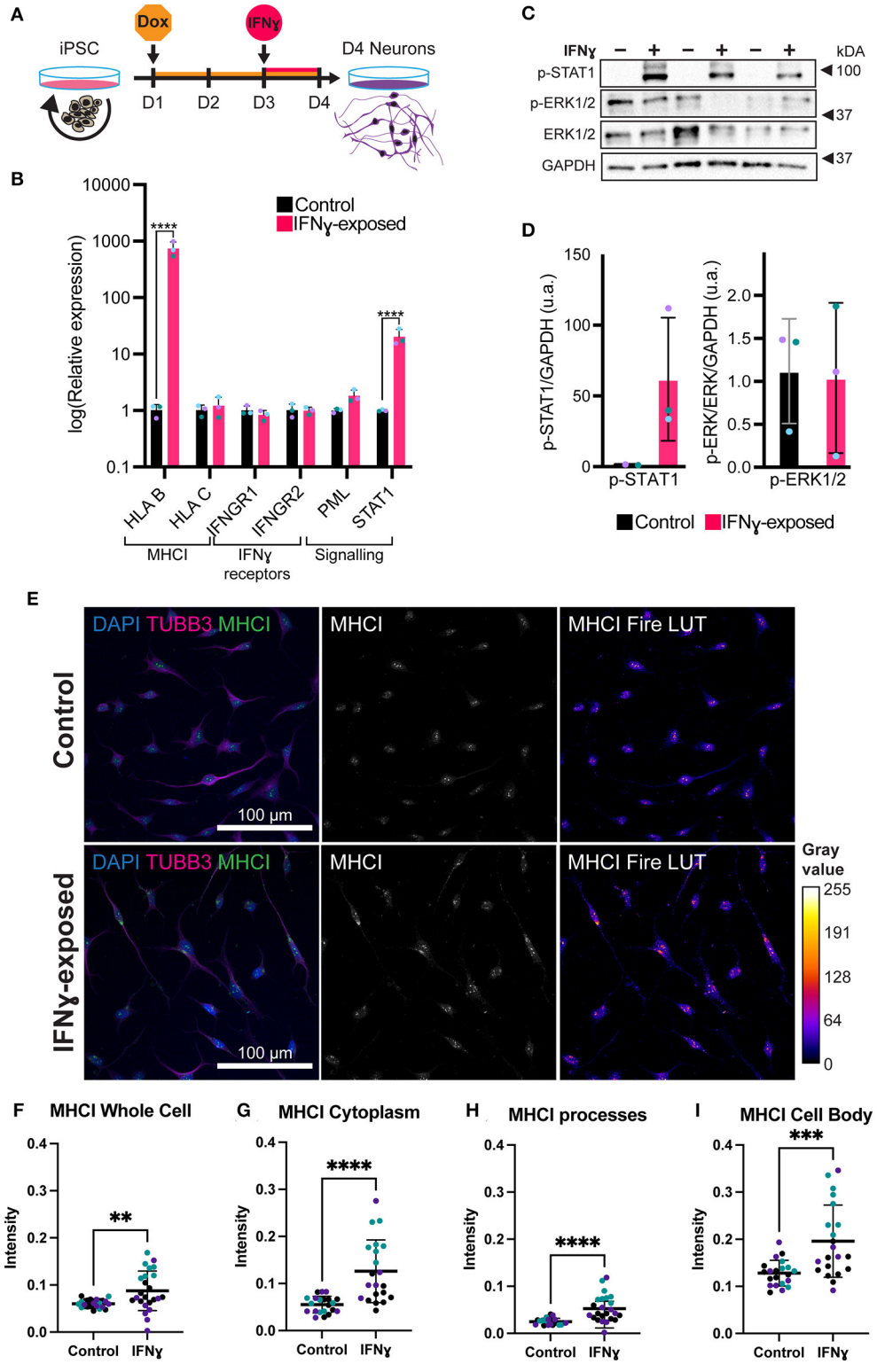

FIGURE 2
Exposure of neurons to IFNγ results in increased MHCI. **(A)** Schematic of Opti-OX neural induction and IFNγ exposure at day 3 for 24 h. **(B)** Bar graphs of relative expression of selected IFNγ signaling-related genes, showing increased *HLA-B* and *C4A* expression in IFNγ-exposed neurons. The bars indicate the $\log(2^{-\Delta\Delta CT})$, which indicates the expression relative to housekeepers and normalized to the housekeepers of the control samples (See methods for details.) Expression in Day 4 *NGN2*-iNs exposed at day 3. $N = 3$. **** indicates $P < 0.0001$, *** indicates $P = 0.000291$ (unpaired *t*-test). **(C)** Western blot for p-STAT1, p-ERK1/2, ERK1/2, and GAPDH protein in Day 3 *NGN2*-iNs exposed to IFNγ (+) or vehicle (-) at day 3 for 15 min. Three biological replicates with different passage numbers are shown **(D)** Quantification of p-STAT1 and p-ERK1/2 blots shown
(Continued)

FIGURE 2 (Continued)
in **(C)**. The different data point colors represent biological replicates with different passage numbers. **(E)** ICC for MHCI. The top row shows control cells, the bottom row shows cells exposed to IFNγ at day 3 for 24hrs. The MHCI Fire LUT pseudo color shows higher intensity with warmer colors and lower intensity with cooler colors. The gray values corresponding to the colors are shown on the calibration bar on the right. **(F–I)** Scatter plots of MHCI intensity in control and IFNγ-exposed neurons. The horizontal bars represent the mean, the error bars represent the standard deviation. Each point in the intensity plots represents the mean intensity of one field of view i.e., image, of the respective object. The different data point colors represent biological replicates with different passage numbers. The IFNγ and control were compared using an unpaired *T*-test, where $N = 3$ and ****indicates $P < 0.0001$, ***indicates $0.001 < P > 0.001$, **indicates $0.001 < P > 0.01$.

which in turn regulates the transcription of target genes (44). We thus tested whether IFNγ signaled through this canonical pathway in *NGN2*-iNs (Figures 2C,D). First, we assessed phosphorylated STAT1 levels following 15 min of IFNγ exposure. As expected, we observed increased phosphorylation of STAT1 in *NGN2*-iNs after 15 min of IFNγ-exposure (Figures 2C,D). No increased phosphorylation of extracellular signal-regulated protein kinase 1/2 (ERK1/2) was observed after 15 min of IFNγ exposure. Consistent with our previous work (28), we further observed an increase in *STAT1* and *HLA-B* expression after 24 h of treatment with IFNγ (Figure 2B). IFNγ treatment has no effect on the expression levels of the IFNγ receptors *IFNGR1* and *IFNGR2*; a trend toward increased expression of PML was also observed (Figure 2B). We further measured the expression of downstream target genes that show a robust response to IFNγ, *HLA-B* and *HLA-C*, using qPCR. Of these, *HLA-B* ($t_{(4)} = 27.97$, $P = 0.00001$) was significantly increased in the exposed neurons (Figure 2B). Together, these data indicate that IFNγ is capable of signaling via the canonical STAT1-dependent signaling pathway in *NGN2*-iNs.

Acute IFNγ-treatment increased MHCI but decreased synapsin I/II expression in *NGN2*-iNs

We next examined the distribution of MHCI in *NGN2*-iNs following treatment with IFNγ for 24 h. Under baseline conditions, MHCI localized to the cell body, processes, and growth cones of all Day 4 *NGN2*-iNs (Figure 2E). Consistent with our previous work (28), IFNγ-exposure caused a higher expression of MHCI in Day 4 *NGN2*-iNs compared to the control (Figure 2E). There appeared to be increased expression of MHCI in the cell body and increased MHCI localization to the processes in the IFNγ-exposed neurons. Analysis of MHCI in different sub-cellular compartments revealed that mean MHCI expression was increased by 31.2% in the cells as a whole ($t_{(43)} = 2.920$, $P < 0.0001$); increased in the cytoplasm by 56.3% ($t_{(40)} = 4.723$, $P < 0.0001$); cell body by 34.6% ($t_{(40)} = 3.819$, $P = 0.0005$); and in neurite processes by 52.5% ($t_{(40)} = 4.331$, $P < 0.0001$) (Figures 2F–I). MHC I intensity in the nucleus was not significantly different ($t_{(43)} = 1.937$, $P = 0.06$). No change in neurite morphology was observed 24 h after IFNγ exposure (Supplementary Figure 4).

Given the effects of IFNγ on synaptic genes and particularly on synaptic vesicle mechanisms, we next directly tested the effect of acute IFNγ exposure on the synaptic vesicle regulators synapsin I and II, in Day 4 *NGN2*-iNs. Synapsin was selected as an early synaptic marker, since this protein is expressed in NPCs and colocalizes with constitutively recycling vesicles along the whole surface of developing axons that then localize to forming synapses (45, 46). In day 4 *NGN2*-iNs, synapsin I/II staining was localized to the cell body, processes, and growth cones (Figure 3A). Staining was particularly evident in the cell body, with synapsin I/II asymmetrically localized within the shaft of one process in many neurons (Figure 3A), presumably in vesicles being transported to the processes (Figure 3A, arrowhead). Synapsin I/II was primarily localized to the cytoplasm of the cell body. There were also sparse puncta of synapsin I/II within cell processes. Expression of synapsin at day 4 is thus primarily in the cell body of all cells.

In contrast to the effects on MHCI, synapsin I/II appeared to be decreased in IFNγ-exposed neurons. Specifically, the asymmetrically localized clusters of synapsin I/II vesicles in the shaft and cell body appeared reduced in some exposed neurons, while others had intensity that is similar to control neurons (Figure 3B). Quantification showed that synapsin I/II expression was decreased in the whole cell by 21.6% ($t_{(43)} = 2.303$, $P = 0.0261$), cell body by 23.7% ($t_{(43)} = 2.300$, $P = 0.0263$), and cytoplasm by 31.1% ($t_{(43)} = 3.339$, $P = 0.0017$) (Figures 3C–F). The mean intensity difference in IFNγ-exposed processes was not statistically significant ($t_{(43)} = 1.840$, $P = 0.0726$, unpaired t-test) (Figure 3E). These results show that IFNγ increases MHCI in Day 4 *NGN2*-iNs but has an inverse effect on synapsin I/II, which decreases in the cytoplasm and cell body. Cytoplasmic synapsin I/II and MHCI expression in single cells are positively correlated in the vehicle condition ($r = 0.57$, $n = 306$), which did not change ($P = 0.1471$, $z = 1.45$) in the IFNγ-exposed condition ($r = 0.49$, $n = 389$).

Synaptic gene expression is unaltered in IFNγ-exposed neurons

We next were interested in understanding whether an acute exposure to IFNγ was sufficient to alter the expression

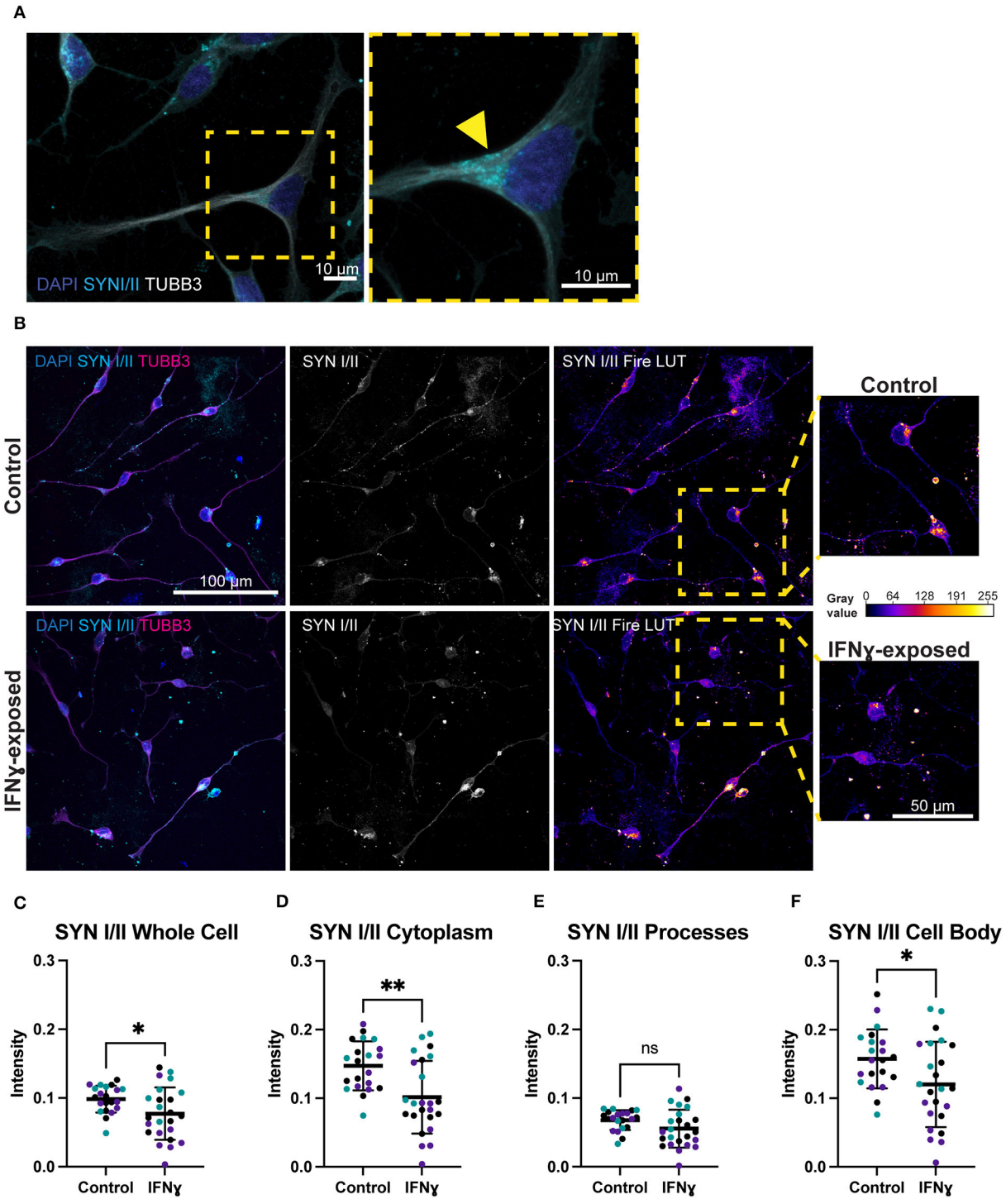

FIGURE 3
Exposure of neurons to IFNγ results in decreased SYNI/II intensity in the cell bodies of some cells. **(A)** Characteristic localization of synapsin I/II in the cell body. Right image shows a detailed view of the highlighted region. The arrowhead indicates apparent synapsin vesicles within the cytoplasm. **(B)** IHC for synapsin I/II. The top row shows control cells, the bottom row shows cells exposed to IFNγ at day 3 for 24hrs. The SYN1 Fire LUT pseudo color shows higher intensity with warmer colors and lower intensity with cooler colors. Detailed view shown on right. The gray values corresponding to the colors are shown on the calibration bar on the right. **(C–F)** Scatter plots of synapsin I/II intensity in control and IFNγ-exposed neurons. The horizontal bars represent the mean, the error bars represent the standard deviation. Each point in the intensity plots represents the mean intensity of one field of view i.e., image, of the respective object. The different data point colors represent biological replicates with different passage numbers. The IFNγ and control were compared using an unpaired *t*-test, where $N = 3$ and **indicates d0.001<P>0.01, *indicates 0.01<P>0.05, and ns indicates $P \geq 0.05$ (not significant).

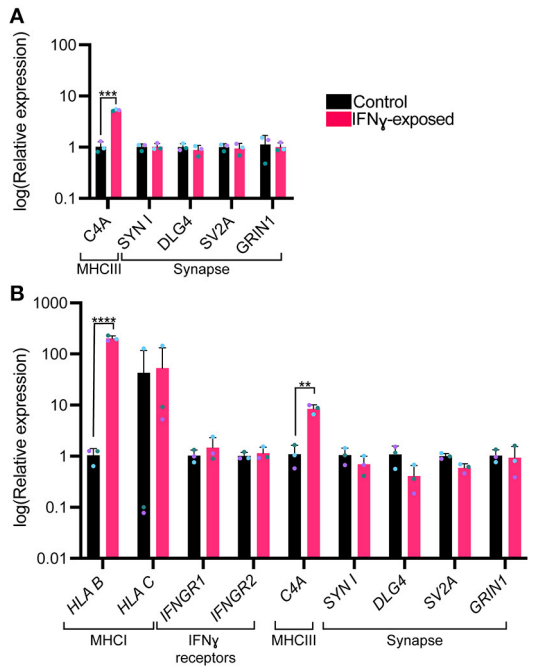

FIGURE 4
Bar graphs of relative expression of selected synaptic genes and *C4A*, showing increased *C4A* expression in IFNγ-exposed neurons. The bars indicate the log($2^{-\Delta\Delta CT}$), which indicates the expression relative to housekeepers and normalized to the housekeepers of the control samples (See methods for details.) **(A)** Expression in Day 4 *NGN2*-iNs exposed at day 3. $N = 3$. ****indicates $P < 0.0001$, ***indicates $P = 0.000291$ (unpaired *t*-test). **(B)** Expression in day 27 conventionally differentiated 127_CTM iPSC line NPCs exposed at day 26. ****indicates $P < 0.0001$, **indicates $P = 0.002947$(unpaired *t*-test). The bar represents the mean, the error bars represent the standard deviation. Points of the same color represent the same biological replicate.

of genes encoding for synaptic genes. Since synapsin I/II decreases in the cell body following IFNγ treatment, we first tested whether expression of synapsin I and other synaptic genes would be decreased following IFNγ treatment. The mean expression level for *SYN1*, *DLG4*, *SV2A*, and *GRIN1* were not significantly different from vehicle conditions (Figure 4A). However, when we examined *C4A*, we observed an significant increase in expression of this gene ($t_{(4)} = 11.84$, $P = 0.000291$). We validated these findings using dual SMAD inhibition differentiated 127_CTM iPSC line NPCs exposed to IFNγ at day 26 to ensure that the observed effects were not cell line specific. As seen in treated NGN2-iNs, IFNγ caused an increase in *HLA-B* ($t_{(4)} = 22.53$, $P = 0.000023$) and *C4A* expression ($t_{(4)} = 6.466$, $P = 0.002947$) in treated NPCs (Figure 4B). No change in *IFNG* receptor expression or of synaptic genes was observed (Figure 4B).

Discussion

In this study, we used *NGN2*-iNs to study the impact of acute IFNγ exposure on immature developing glutamatergic neurons. We find that IFNγ activates an interferon-mediated canonical signaling pathway in the absence of glial cells and demonstrate that synaptic protein expression is disrupted by this cytokine, building on previous studies showing IFNγ affects expression of synaptic genes in iPSC-NPCs and neurons (28).

The observation that acute IFNγ exposure reduced synapsin I/II expression and increased MHCI expression in immature glutamatergic neurons is consistent with previously published findings. For example, Glynn et al. (31) found an increased density of clusters of synaptic vesicles containing synapsin I upon siRNA knockdown of an MHCI subunit and observed significantly decreased synapsin I at inhibitory synapses when MHCI was overexpressed in rodent neurons. The decrease in synapsin observed in our study is therefore likely linked to the concurrently increased *MHCI* expression, although we observed a positive correlation between synapsin I/II and MHCI intensity at the single-cell level. Decreased synapsin may translate to disruptions in synapses subsequently, as synapsin is important for synapse maturation, including the correct localization of synaptic vesicles in growth cones and the regulation of vesicle recycling rate, although this remains to be tested in *NGN2*-iNs (46). Of note however, a gene enrichment study comparing both rat (gestational day 15, MIA) whole-brain and *post-mortem* human brain tissue samples from individuals with autism reported a common downregulation of genes associated with synaptic vesicle exocytosis (47). This is in line with our SynGO analysis of our IFNγ RNAseq datatset (28) and the decrease in synapsin associated with synaptic vesicles observed here. Whether these changes in synaptic protein translate to altered neuronal activity, however, remains to be established. In this context, a previous study suggested that IFNγ treatment of cultured early hippocampal mouse (E15) neurons at 1–4 days *in vitro* had no effect on excitatory transmission, but did not investigate neither other synapse parameters nor whether treatment of NPCs has an effect (24).

Treatment with IFNγ for 24 h did not alter the expression of *selected* synaptic genes. This may be due to several possibilities. For example, changes in proteostasis or mRNA turnover may drive changes in protein levels without affecting mRNA levels. The observed changes in protein levels may reflects a transient change in mRNA expression that is no longer detectable after 24 h. The increase in MHCI gene and protein expression following IFNγ exposure matches our findings in a previous neuroprogenitor cell study that used the same 24-h acute IFNγ exposure of iPSC-NPCs and -neurons (28). This study also described upregulation of *HLA-C* and *HLA-B* expression; however, we only observed a significant increase in *HLA-B*.

Consistent with our previous study, we observed no change in the expression of IFNγ receptors. MHCI is known to be involved in synaptic plasticity and learning (29, 30, 48, 49) and is important for negatively regulating synapses (50). Dysregulation of *MHCI* expression could thus potentially be sufficient for a downstream disruption of synapses even if no change in synaptic genes is present at the point of IFNγ exposure. MHCI has been shown to mediate reduced synaptic connectivity in a mouse MIA model by signaling through myocyte enhancer factor 2 (MEF2) (32). Future work would need to establish whether these changes in MHCI expression persist, if MEF2 is involved and whether other downstream changes arise as the neurons mature.

We also observed increased expression of the complement component *C4A* after acute IFNγ exposure. *C4A* mRNA levels are increased in *post-mortem* cortical brain tissue from individuals with schizophrenia and *C4A* variants are associated with elevated risk for schizophrenia (35). The genes downregulated upon increased C4A expression are also enriched for schizophrenia risk (51). C4A is expressed by neurons and colocalizes with synaptic markers and is thought to play a role in the pruning of synapses during brain development and maturation (35). Consistent with this view, overexpression of C4A in mice resulted in behavioral changes of relevance for schizophrenia, reduced cortical synapse density, and increased engulfment of synapses by microglia (52). Inhibition of microglial activity reverses MIA abnormalities, including synapse loss (53). Co-culture studies with microglia are required to understand if IFNγ exposure leads to increased synaptic engulfment by microglia via increased C4A expression. Deletion of C4A could be used to interrogate the role of this protein in the effects of IFNγ in neurons. Co-culture studies with microglia would thus be particularly informative for future IFNγ exposure studies.

We observed activation of the canonical STAT1 signaling pathway following IFNγ exposure but did not observe non-canonical signaling as there was no altered ERK1/2 phosphorylation. This suggests that our observed effects may be mediated by activation of STAT1 signaling, the primary signaling pathway for IFNγ responses (54–56). IFN-γ signaling through JAK/STAT signaling has been observed *in vivo* in multiple species, with effects that promote GABA-ergic inhibition and regulate neuronal connectivity (57). Further experiments are however required to fully interrogate the dynamics of IFNγ signaling pathways in neurons.

In conclusion, elevated levels of IFNγ were sufficient to activate a canonical interferon-signaling mechanism in immature developing neurons, an increase in MHCI proteins and complement components, and reduced synaptic vesicles in immature glutamatergic neurons. Our findings further support a possible link between IFNγ exposure in immature glutamatergic neurons and cellular phenotypes associated with neurodevelopmental disorders, although further work is needed to understand the mechanistic basis of this link.

Data availability statement

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

Author contributions

DS and AV: conception and design, literature searching, manuscript writing and editing, project supervision, and financial support. AP, RM, LS, LD, and NA: carried out experiments. AP: manuscript writing and editing. All authors approved the final manuscript.

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Conflict of interest

Authors AV and DS receive research funding from bit.bio.

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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2022.836217/full#supplementary-material

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EDITED BY
Zachary Freyberg,
University of Pittsburgh, United States

REVIEWED BY
Elisa Conti,
University of Milano Bicocca, Italy
Artemis Kimon Tsitsika,
Adolescent Health Unit (AHU), Greece

*CORRESPONDENCE
Judith Alferink
Judith.Alferink@ukmuenster.de

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

‡These authors share last authorship

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Expression of CXCR4 on CD4$^+$ T cells predicts body composition parameters in female adolescents with anorexia nervosa

Jana Freff[1,2†], Lisa Bröker[1,2†], Rafael Leite Dantas[1,2], Kathrin Schwarte[1], Judith Bühlmeier[3,4,5], Isabelle Kraft[3], Anke Hinney[3], Ulrike Buhlmann[4], Volker Arolt[6], Udo Dannlowski[6], Georg Romer[7], Bernhard T. Baune[1,8,9], Johannes Hebebrand[3], Manuel Föcker[3,7‡] and Judith Alferink[1,2*‡]

[1]Department of Psychiatry, University of Münster, Münster, Germany, [2]Cells in Motion Interfaculty Cluster, University of Münster, Münster, Germany, [3]Department of Child and Adolescent Psychiatry and Psychotherapy, University Hospital Essen, University of Duisburg-Essen, Essen, Germany, [4]Department of Clinical Psychology and Psychotherapy, University of Münster, Münster, Germany, [5]Faculty of Natural Sciences, Institute of Nutrition, Consumption and Health, University Paderborn, Paderborn, Germany, [6]Institute for Translational Psychiatry, University of Münster, Münster, Germany, [7]Department of Child and Adolescent Psychiatry and Psychotherapy, University Hospital Münster, Münster, Germany, [8]Department of Psychiatry, University of Melbourne, Melbourne, VIC, Australia, [9]The Florey Institute of Neuroscience and Mental Health, University of Melbourne, Melbourne, VIC, Australia

Anorexia nervosa (AN) is a severe eating disorder characterized by excessive weight loss and lack of recognition of the seriousness of the current low body weight. Individuals with AN frequently exhibit an enhanced inflammatory state and altered blood levels of cytokines and chemokines. However, the expression of chemokine receptors in AN and the association with body composition parameters and treatment effects are still unknown. In this study, we examined the expression of CCR4, CCR6, CXCR3, and CXCR4 on peripheral blood T cells in female adolescents with AN before (T0, n = 24) and after 6 weeks of multimodal therapy (T1, n = 20). We also investigated their value to predict body mass index (BMI) and fat mass index (FMI) at baseline. Using multi-parameter flow cytometry, we found increased expression of CCR4, CXCR3, and CXCR4, but not CCR6, on CD4$^+$ T cells in AN at T0 when compared to healthy controls (HC, n = 20). At T1, CXCR3 and CXCR4 expression decreased in AN. We found a close link between CCR4, CCR6 and CXCR4 expression and the adolescent mental health status in the study cohort as determined by the Strengths and Difficulties Questionnaire (SDQ). Specifically, CXCR4 expression correlated positively with emotional symptoms and peer relationship problems, as well as with the total sum score of the SDQ. In addition, CXCR4 expression on CD4$^+$ T cells was a significant predictor of BMI and FMI in female adolescents. Our findings that CXCR4 expression

on T cells is altered in adolescents with AN and predicts body composition parameters in adolescents suggest an impact of this chemokine receptor in the pathogenesis of AN.

KEYWORDS

anorexia nervosa, eating disorder, T cell, immune system, chemokine receptor, inflammation

Introduction

Anorexia nervosa (AN) is a severe eating disorder with peak onset during adolescence and a high incidence in females (1–3). AN has the highest mortality rate among all mental disorders (4–7). Clinical symptoms of AN are characterized by low body weight, distorted body image, and weight phobia associated with restricted food intake and/or weight-controlling behaviors (8–11). Several body composition parameters such as body mass index (BMI) and fat mass (FM) are reduced in AN and related to the severity of malnutrition (12). We and others have shown before that the body composition parameters FM and fat free mass index (FFMI) are also associated with endocrine and immunometabolic adaptations (13–17).

In addition to genetic and environmental risk factors, the immune system is thought to play a pathophysiological role in AN (3, 18–25). A dysregulated immune system due to impaired eating behavior or malnutrition has been shown to impair the activation and function of immune cells, which may have negative effects on the defense against pathogens (22, 26). A bidirectional relationship between eating disorders and autoimmunity is well established (27–29), and genetic risk factors with genome-wide significance associated with immune responses have been identified (22, 23, 30). Changes in pro-inflammatory cytokines and numbers of T and B lymphocytes in the peripheral blood of individuals with AN point toward a pathophysiological involvement of the innate and adaptive immune system (22, 31–34). Accordingly, we recently demonstrated changes in B cell maturation stages in the peripheral blood of adolescents with AN and a strong association with fat mass index (FMI) and FFMI (13).

Chemokines are chemotactic proteins that control chemotaxis, activation and differentiation of immune cells by signaling through cell surface G-protein coupled chemokine receptors (35, 36). A bidirectional relationship between different chemokine-chemokine receptor axes and the secretion of leptin, a hormone that regulates appetite through a post-transcriptional mechanism, was found (37, 38). Recent studies provide insights into blood chemokine levels in AN with controversial results (24, 39–42). Dalton and colleagues found unchanged serum concentrations of several chemokines among those CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL11 (Eotaxin), CCL13 (MCP-4), CCL17 (TARC), and CCL26 (Eotaxin-3) in 23 subjects with AN compared to 13 HC (31). In a cross-sectional study, however, individuals with AN (n = 56) had lower blood CCL4 concentrations as HC (n = 51), and CCL4 levels correlated negatively with BMI and eating disorder psychopathology (43). A close association between chemokine levels and body parameters regarding intracellular, extracellular and total body water balance was also reported for CCL13 in individuals with AN and HC (44). However, knowledge is scarce about the expression profiles of chemokine receptors in AN and their association with body composition parameters.

The chemokine receptors CCR4, CCR6, CXCR3, and CXCR4 control homing and effector functions of T cells implicated in inflammatory responses and behavior (45–52). For example, CCR4 is a key regulator of neuroinflammation by promoting Th17 migration and modulates emotional and cognitive behavior in human and mice (51, 53–58). Also CCR6, the unique receptor for CCL20, mediates chemotaxis of leucocytes and has been functionally involved in emotion-like, cognition-like and sociability behaviors in mice (59–61). The CXC chemokine receptor CXCR3 controls CXCL9, CXCL10, and CXCL11 induced leucocyte trafficking to inflammatory sites (62, 63), and reduced CXCR3 expression on $CD4^+$ T cells and $CXCR3^+$ $CD4^+$ T cell numbers have been found in mental disorders (64, 65). The CXCR4–CXCL12 axis crosstalks with several neurotransmitter systems and is part of the circuits controlling feeding behavior and metabolism (66). In the immune system, CXCR4 controls CXCL12 dependent migration of memory T cells to bone marrow niches which is required for their self-renewal (67). To date, the surface expression of these chemokine receptors and their potential as clinical predictors of body composition parameters in adolescence have not been studied.

In this exploratory study, we examined CCR4, CCR6, CXCR3 and CXCR4 expression on peripheral blood $CD4^+$ T cells in female adolescents with AN and HC by multi-parameter flow cytometry, to detect state or trait related alterations. We investigated the association between receptor expression and mental health, body composition and weight related parameters. Finally, we analyzed the predictive impact of chemokine receptor expression on BMI and FMI in the study cohort.

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Materials and methods

Subjects

Female patients with AN (n = 24) and healthy controls (HC, n = 20) were recruited at the Department of Child and Adolescent Psychiatry, Psychotherapy, and Psychosomatics, University Hospital Essen, University of Duisburg-Essen, Essen, Germany. For a more detailed description of the study group, the treatment regime performed, the anthropometric measurements and the collection of the blood samples, we refer to our recent publication (13). In the same study cohort, we reported earlier maturation stages of peripheral blood B cells in AN and HC (13). In brief, this study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Medical Faculty of the University of Duisburg-Essen (12-5289-BO). All patients and controls and their parents gave written informed consent prior to the study. Inclusion criteria were female gender, age 12–18 years, and European ancestry. An additional inclusion criterion for the patient group was an AN diagnosis confirmed by clinical examination and semi structured interview, the Kiddie Schedule for Affective Disorders and Schizophrenia (K-SADS), according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition, text revision (DSM-IV-TR) (68). One patients' BMI was at the 12th age- and sex adjusted BMI percentile, but as all other DSM-IV-TR criteria for AN were met, the patient was included in our study. Exclusion criteria for patients and HC were a severe comorbid mental disorder, alcohol or drug abuse, chronic endocrinological or inflammatory diseases, cancer, insufficient German language skills and IQ <70. Psychiatric disorders and severe somatic diseases were excluded based mainly on participants' medical and psychiatric history. For assessment of emotional and behavioral items in childhood and adolescence the SDQ was applied in the whole study group (69). All participants were non-smokers (n = 44). All participants in the control group had regular menstrual cycles (n = 20), whereas 21 anorexic patients were amenorrheic. Three patients with AN were taking oral contraceptives and reported withdrawal bleeding. One patient with AN was receiving benzodiazepines and domperidone at T0. Three patients with AN were taking vitamin D because of vitamin D deficiency. None of the subjects in the control group were taking medication.

Treatment

All patients with AN were admitted to psychiatric inpatient care. Treatment was based on the German S3 guideline with a multimodal, cognitive and dialectical-behavioral therapy concept (70, 71). A detailed description of the treatment concept can be found in Freff et al. (13).

Screening variables

Anthropomorphic measurements

As described before, we performed anthropomorphic measurements at the acute state of starvation (T0) and after 6 weeks of inpatient treatment (T1). We conducted body weight, height, chest-, abdominal- and hip circumference measurements using earlier described standard operating procedures. In addition, body composition parameters such as FM assessed by the BodPod®, FMI, BMI, BMI standard deviation scores (BMI SDS) were determined. For details see Freff et al. (13).

Eating disorder examination questionnaire

Dysfunctional eating behavior was assessed using the German version of the Eating Disorder Examination Questionnaire (EDE-Q) (72) to screen the severity of the eating disorder. The EDE-Q is a widely used instrument to assess eating disorder specific psychopathology as well as diagnostically relevant core behaviors. It assesses eating disorder-specific characteristics using four subscales including (i) restrained eating, (ii) eating-related worries, (iii) weight worries, and (iv) figure worries (72). A total of 22 items are assigned to these subscales. Six additional items are used to record diagnostically relevant core behaviors, such as binge eating or self-induced vomiting. All items are to be assessed for the period of the last 28 days using seven-point rating scales according to frequency and intensity (0 = attribute non-existent; 6 = attribute existent every day/in an extreme degree) (72). High internal consistencies are shown for both the subscales as well as for the total score of this questionnaire procedure. Moreover, retest reliability, tested on a non-clinical sample, shows stability over a 3-month period (72).

Strengths and difficulties questionnaire

The SDQ (69) is used to measure symptoms of mental health disorders in 3–16 year olds. The questionnaire consists of 25 items comprising five subscales: (i) emotional symptoms (anxiety and depressive symptoms), (ii) behavioral problems, (iii) hyperactivity/inattention, (iv) relationship problems with peers, and (v) pro-social behavior (positive behaviors such as kindness and helpfulness, rated inversely to the other subscales). Response options are scored using a three-point rating scale (0 = strongly disagree, 1 = somewhat agree, 2 = strongly agree). The SDQ discriminates well between children and adolescents with and without psychopathology symptoms (73–75), and can be used as an effective screen for child/adolescent psychiatric disorders in the general population (76).

Blood sample collection, cryopreservation, and thawing of peripheral blood mononuclear cells

Blood sample collection was performed as described before (13). Briefly, 15 ml freshly drawn peripheral blood was

obtained in sterile sodium heparin-treated tubes regularly from participants in the morning after an overnight fast. For isolation of peripheral blood mononuclear cells (PBMC), a standard density gradient centrifugation was performed according to the manufacturer's instructions using Leucosep^{TM} tubes (Frickenhausen, GER: Greiner Bio-One GmbH). Before cryopreservation of aliquots, isolated PBMC were washed three times with PBS supplemented with 2% fetal calf serum (FCS). Afterward, cells were overnight stored at −80°C and later transferred to the gas phase of liquid nitrogen until further use. For this, 1×10^7 cells in X-Vivo 15^{TM} (Basel, CH: Lonza Group Ltd) supplemented with 10% FCS were mixed 1:1 with freezing medium, which consists of 80% FCS and 20% dimethyl sulfoxide (DMSO). On the day of use, PBMC were carefully thawed in a 37°C water bath and immediately washed with PBS supplemented with 2% FCS.

Flow cytometry

The whole blood staining procedure for surface marker expression was performed similar as described before (77). In brief, for identification of T cells and their subsets, monoclonal antibodies anti-human CD3-PerCP, anti-human CD4-APC-Cy7, and anti-human CD8-BV510 were used. Chemokine receptor expression was analyzed using monoclonal antibodies anti-human CCR4-PE-Cy7, anti-human CCR6-FITC, anti-human CXCR3-APC, and anti-human CXCR4-PE. In brief, thawed PBMCs were stained for CCR4, CCR6, CXCR3, and CXCR4 at 37°C for 15 min. Antibodies for T cell staining (CD3, CD4, CD8) were directly added afterwards to the samples followed by another incubation step for 45 min at room temperature (RT), protected from light. All antibodies were purchased from BioLegend® (San Diego, CA, USA). Samples were acquired on a CytoflexS® flow cytometer (Krefeld, GER: Beckman Coulter GmbH) and analyzed by FlowJo^{TM} Software v10 (Ashland, OR: Becton, Dickinson and Company; 2019).

Statistical procedure

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 28. Data were visualized with GraphPad Prism version 9. For analyses of group differences in demographic data, *t*-tests were performed for continuous variables. For outcome measures, normal distribution of data within the groups was tested by means of Shapiro–Wilk Test. Possible baseline differences in outcome variables between patients (T0 or T1) and HC were calculated using unpaired *t*-tests. Differences within anorectic patients (T0 vs. T1) were evaluated using paired *t*-tests. For correlational studies, Spearman correlation coefficient was used. Furthermore, the influence of chemokine receptor expression on the variance of BMI and FMI was calculated by multivariate linear regression (MLR). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ were considered statistically significant.

Results

Anthropometric and clinical characteristics of participants with AN and HC are summarized in Table 1. In accordance to the earlier published study protocol, all participants were female adolescents with AN of the restricting subtype (13). There was no significant difference ($p = 0.248$) in the mean age between

TABLE 1 Characteristics of the study sample.

	HC ($n = 20$)		AN pre-treatment (T0, $n = 24$)		
	Mean	**± SD**	**Mean**	**± SD**	***P*-value**
Body height (cm)	169.13	± 6.68	165.45	± 5.12	**0.045**
Chest circumference (cm)	86.33	± 4.79	74.85	± 4.43	**<0.001**
Abdominal circumference (cm)	66.43	± 3.81	58.33	± 4.10	**<0.001**
Hip circumference (cm)	89.15	± 6.02	77.06	± 3.98	**<0.001**
BMI (kg/m^2)	20.53	± 1.93	16.01	± 1.46	**<0.001**
BMI SDS (KIGGS)	−0.35	± 0.74	−2.64	± 1.47	**<0.001**
FM (%)	24.95$^{\#}$	± 5.57	16.97^{+}	± 4.26	**<0.001**
FMI (kg/m^2)	5.17$^{\#}$	± 1.47	2.76^{+}	± 0.79	**<0.001**
SDQ emotional symptoms	2.35	± 2.08	6.67	± 1.66	**<0.001**
SDQ conduct problem	1.35	± 1.27	1.41	± 1.21	0.845
SDQ hyperactivity	2.65	± 2.13	3.46	± 1.98	0.200
SDQ peer relationship problem	1.55	± 0.94	3.17	± 1.81	**0.002**
SDQ pro-social	8.90	± 1.02	9.08	± 1.10	0.448
SDQ sum score	7.90	± 3.43	14.58	± 3.96	**<0.001**

$^{\#}n = 19$.

$^{+}n = 23$.

HC, healthy control; AN, anorexia nervosa; BMI, body mass index; SDS, standard deviation scores; KIGGS, German health interview and examination survey for children and adolescents; FM, fat mass; FMI, fat mass index; SDQ, strengths and difficulties questionnaire.

Significant effects are in bold print.

the AN (15.6 ± 1.4 years) and HC group (16.1 ± 1.6). We found significantly lower body composition parameters such as BMI, BMI SDS, percentage FM, and FMI in adolescents with AN than in HC (Table 1). We determined mental health items such as emotional symptoms, conduct problems, hyperactivity, peer relationship problems, and pro-social behaviors by the SDQ. Compared with HC, the mean scores of the SDQ subscales emotional symptoms and peer relationship problems, and the SDQ sum score were higher in patients with AN when compared to HC ($p < 0.001$) (Table 1).

Outcome measures including various body composition parameters before (T0) and after 6 weeks of multimodal treatment (T1) in the AN group are displayed in Table 2. Body composition parameters including chest-, abdominal- and hip circumference, BMI, BMI SDS, FM, and FMI were significantly increased at T1 compared to T0. We also compared the specific eating disorder psychopathology in the treated AN group to baseline measures at admission using the Eating Disorder Examination-Questionnaire (EDE-Q). Scores of EDE-Q subscales such as restraint, eating concern, and weight concern significantly decreased at T1 compared to scores measured at T0. In addition, the EDE-Q sum score in the AN group was lower after treatment (Table 2). These data indicate that multi-modal therapy was effective in the patient group in improving body parameters and health-related eating behavior.

Altered expression profiles of CCR4, CCR6, CXCR3, and CXCR4 on CD4$^+$ T cells in anorexia nervosa

We next examined the expression profile of CCR4, CCR6, CXCR3, and CXCR4 in adolescents with AN at T0 and T1, and HC using multi-parameter flow cytometry. In accordance with our previous findings, we found reduced frequencies of total blood lymphocytes in AN at T1 when compared to T0 and HC (Table 3) (13). We further determined lower CD3$^+$ T cell frequencies in AN at T1 when compared to T0, while proportions of CD4$^+$ and CD8$^+$ T cells were not affected (Table 3). To characterize expression of chemokine receptors on CD4$^+$ T cells, we measured mean fluorescence intensity (MFI) on the cellular surface of these cells. We found increased MFI for CCR4, CXCR3, and CXCR4, but not CCR6, on CD4$^+$ T cells in AN at T0 compared to HC (Figures 1A–D and Table 3). At T1, CXCR3, and CXCR4 expression on CD4$^+$ T cells decreased, while CXCR4 expression levels remained higher when compared to HC (Figures 1C,D and Table 3). CCR6 expression on CD4$^+$ T cells, instead, increased at T1 when compared to T0 and HC (Figure 1B and Table 3). These results indicate that expression of these chemokine receptors on peripheral T cells is altered in female adolescents with AN when compared to HC.

Close link between chemokine receptor expression and mental health problems

To examine the relationship between chemokine receptor expression on CD4$^+$ T cells and adolescent mental health problems, we performed correlational studies (Table 4). Regarding CXCR3, no correlation was found. Expression of CCR6 on CD4$^+$ T cells correlated negatively with hyperactivity ($\rho = -0.357$, $p < 0.05$) and the SDQ sum score ($\rho = -0.315$, $p < 0.05$). Furthermore, we found a positive correlation between CCR4 expression and emotional symptoms ($\rho = 0.405$, $p < 0.01$). In addition, CXCR4 expression correlated positively with emotional

TABLE 2 Adolescents with AN before and after 6 weeks of inpatient treatment.

	AN pre-treatment (T0, $n = 20$)		AN post-treatment (T1, $n = 20$)		
	Mean	**± SD**	**Mean**	**± SD**	***P*-value**
Chest circumference (cm)	75.15	± 4.65	78.28	± 5.16	**<0.001**
Abdominal circumference (cm)	58.93	± 3.76	62.63	± 4.22	**<0.001**
Hip circumference (cm)	77.48	± 3.87	80.23	± 3.97	**<0.001**
BMI (kg/m^2)	16.29	± 1.22	17.42	± 1.17	**<0.001**
BMI SDS (KIGGS)	−2.40	± 1.11	−1.70	± 0.82	**<0.001**
FM (%)	17.14	± 4.13	21.50	± 4.99	**<0.001**
FMI (kg/m^2)	2.80	± 0.75	7.86	± 1.78	**<0.001**
EDE-Q restraint	3.93	± 1.06	2.56	± 1.70	**<0.001**
EDE-Q eating concern	3.15	± 1.07	2.47	± 1.46	**0.023**
EDE-Q weight concern	4.14	± 1.26	3.47	± 1.76	**0.047**
EDE-Q shape concern	4.51	± 1.18	4.43	± 1.46	0.763
EDE-Q sum score	3.93	± 1.03	3.23	± 1.41	**0.007**

AN, anorexia nervosa; BMI, body mass index; SDS, standard deviation scores; KIGGS, German health interview and examination survey for children and adolescents; FM, fat mass; FMI, fat mass index; EDE-Q, eating disorder examination questionnaire.
Significant effects are in bold print.

TABLE 3 Comparison of immunological characteristics between HC and AN at T0 and T1.

	HC (n = 20)		AN pre-treatment (T0, n = 24)		AN post-treatment (T1, n = 20)		
	Mean	± SD	Mean	± SD	Mean	± SD	P-value
Lymphocytes (%)	69.12	± 5.92	70.97	± 6.36	65.35	± 8.32	0.231[a] **0.012**[b,c]
$CD3^+$ T cells (%)	62.02	± 7.44	61.43	± 10.07	57.30	± 10.13	0.888[a] **0.010**[b] 0.125[c]
$CD4^+$ T cells (%)	60.14	± 9.16	56.69	± 9.78	55.50	± 7.41	0.440[a] 0.076[b] 0.332[c]
$CD8^+$ T cells (%)	27.85	± 6.76	27.33	± 6.81	27.39	± 7.45	0.619[a] 0.379[b] 0.782[c]
CCR4 (MFI) on $CD4^+$ T cells	398.62	± 535.16	588.58	± 404.49	610.44	± 620.17	**0.003**[a] 0.823[b] **0.461**[c]
CCR6 (MFI) on $CD4^+$ T cells	2906.35	± 653.55	2797.79	± 425.76	3184.40	± 503.07	0.738[a] **0.002**[b] **0.008**[c]
CXCR3 (MFI) on $CD4^+$ T cells	4549.25	± 1354.69	4963.63	± 1097.06	4760.95	± 1707.82	**0.040**[a,b] 0.127[c]
CXCR4 (MFI) on $CD4^+$ T cells	7097.50	± 1825.90	19049.79	± 7791.31	13771.35	± 4347.93	<**0.001**[a,b] **0.015**[c]

[a]HC vs. AN T0.
[b]AN T0 vs. AN T1.
[c]HC vs. AN T1.
HC, healthy control; AN, anorexia nervosa; MFI, mean fluorescence intensity.
Significant effects are in bold print.

symptoms (ρ = 0.634, $p < 0.001$), peer relationship problems (ρ = 0.488, p = 0.001), and the SDQ sum score (ρ = 0.633, $p < 0.001$). These results demonstrate that CCR4, CCR6 and CXCR4 levels on blood $CD4^+$ T cells in individuals with AN and HC closely correlate with specific mental health problems in adolescence.

CXCR4 receptor expression on $CD4^+$ T cells serves as predictor of body mass index and fat mass index

We next examined whether receptor expression was associated with BMI and FMI in adolescents with AN at the acute stage of starvation and in HC. Therefore, MLR analysis was conducted using CCR4, CCR6, CXCR3 and CXCR4 expression on $CD4^+$ T cells as independent variables to predict BMI and FMI of the participants of the study cohort (HC = 20; AN = 24). CXCR4 expression significantly predicted the participants' BMI and explained 49.7% of variance. CXCR4 expression was also identified as significant predictor of FMI explaining 33.6% of variance (Table 5). In contrast, neither CCR4, CCR6, nor CXCR3 were predictors of body parameters. Furthermore, we found direct linear relationships between CXCR4 expression on $CD4^+$ T cells and BMI (R^2 = 0.470) and FMI (R^2 = 0.323) in female adolescents (Figures 2A,B). These analyses demonstrate a close link between CXCR4 expression on blood $CD4^+$ T cells and severity of AN and that CXCR4 expression is a clinical predictor of BMI and FMI in female adolescents.

Discussion

Alterations of the immune system such as altered chemokine levels in the blood have been found in individuals with AN (22, 31). However, knowledge on the expression of chemokine receptors on T cells in adolescents with AN is scarce and neither treatment effects, nor associations with body composition parameters and mental health items have been studied. In this exploratory study, we determined cell surface levels of CCR4, CCR6, CXCR3, and CXCR4 on blood $CD4^+$ T cells and identified CXCR4 as significant predictor of body composition parameters in adolescents with AN and HC.

Several studies investigated the effect of severe starvation on numbers of lymphocytes and their subsets in peripheral blood and found relative lymphocytosis or lymphopenia (33, 78, 79). Depending on the study design, decreased, equivalent or increased $CD4^+$ T cell counts have been found in AN associated with altered CD4/CD8 ratios (33, 34, 79–81). In our study cohort, we found unaltered $CD4^+$ and $CD8^+$ T cell proportions in female adolescents with AN compared to HC. The observed decrease in blood $CD3^+$ T cells in AN after therapy compared

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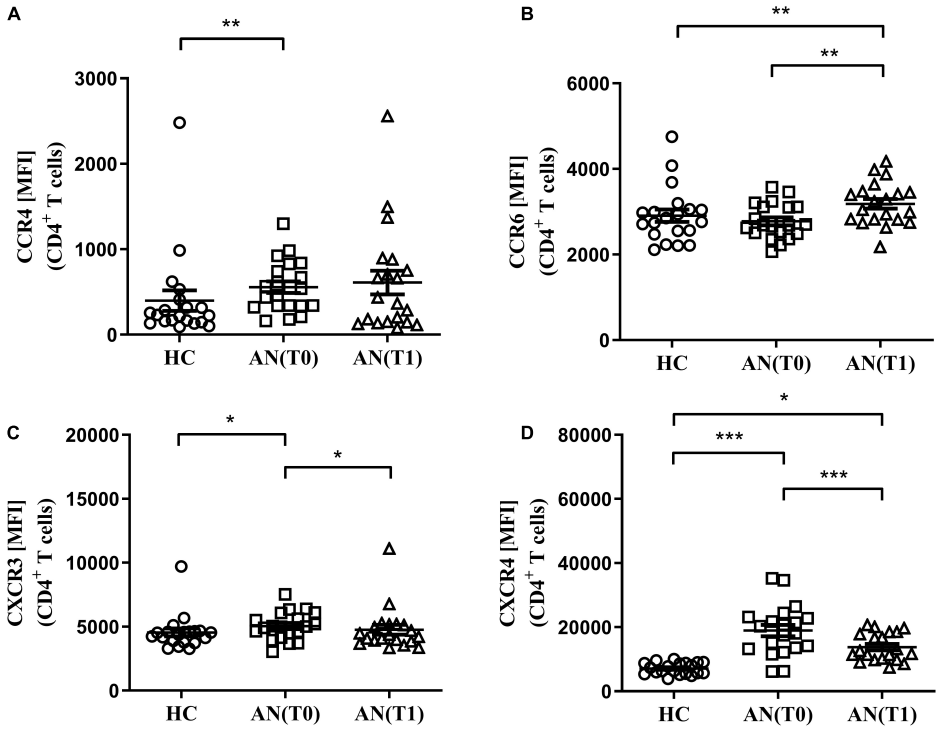

FIGURE 1
Mean fluorescence intensity (MFI) of chemokine receptors on $CD4^+$ T cells. **(A)** CCR4, **(B)** CCR6, **(C)** CXCR3, and **(D)** CXCR4 MFI on $CD4^+$ T cells in healthy controls (HC: $n = 20$), adolescents with anorexia nervosa (AN) at admission (T0, $n = 20$) and after 6 weeks of therapy (T1, $n = 20$), $^*p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$.

TABLE 4 Correlation of immune parameters with psychological characteristics in AN and HC.

	CCR4 (MFI) on $CD4^+$ T cells		CCR6 (MFI) on $CD4^+$ T cells		CXCR3 (MFI) on $CD4^+$ T cells		CXCR4 (MFI) on $CD4^+$ T cells	
	ρ	*P*-value	ρ	*P*-value	ρ	*P*-value	ρ	*P*-value
SDQ emotional symptoms	**0.405**	**0.006**	−0.063	0.685	0.297	0.051	**0.634**	<**0.001**
SDQ conduct problem	−0.164	0.289	−0.152	0.326	−0.167	0.280	0.155	0.315
SDQ hyperactivity	−0.026	0.867	**−0.357**	**0.017**	0.007	0.966	0.216	0.159
SDQ peer problem	0.045	0.769	−0.223	0.145	−0.031	0.842	**0.488**	**0.001**
SDQ pro-social	0.079	0.611	0.169	0.272	0.268	0.078	0.053	0.730
SDQ sum score	0.179	0.245	**−0.315**	**0.037**	0.088	0.571	**0.663**	<**0.001**

$n = 44$ (HC: $n = 20$, AN: $n = 24$).
SDQ, strengths and difficulties questionnaire.
Spearman Correlation Coefficients; Conventions for effect size Cohen's ρ: | ρ| = 0.10 small effect; | ρ| = 0.30 medium effect; | ρ| = 0.50 large effect.
Significant effects are in bold print.

to inpatient admission may be a consequence of altered blood glucocorticoids in AN known to induce lymphocyte apoptosis or a consequence of hypoleptinemia during starvation causing multiple immune system alterations (14, 22, 82).

An important finding of our study was that surface expression of CCR4, CXCR3, and CXCR4 was increased on blood $CD4^+$ T cells in adolescents with AN. To the best of our knowledge, this is the first study investigating the expression of these chemokine receptors in AN. In this context, appetite-regulating properties have been described for chemokine receptors and their ligands. For example, expression of CXCL12 has been found in the lateral hypothalamus of the adult brain, and expression of its cognate receptor, CXCR4, co-localized with melanin-concentrating hormone (MCH)-expressing neurons that regulate feeding behavior (66). In accordance, intracerebral administration of chemokines (CCL2, CCL3, CCL4, CCL5, CXCL4, CXCL8, and CXCL10) has been found to decrease food intake in rodents (83). In addition

TABLE 5 Multivariate linear regression analyses of BMI, FMI, and chemokine receptor expression.

Variable	B	SE	Beta	t	*P*-value
BMI					
(Constant)	24.307	2.493		9.749	<0.001
CCR4 (MFI) on $CD4^+$ T cells	0.001	0.001	0.247	1.311	0.198
CCR6 (MFI) on $CD4^+$ T cells	−0.001	0.001	−0.137	−0.892	0.378
CXCR3 (MFI) on $CD4^+$ T cells	0.000	0.000	−0.132	−0.729	0.471
CXCR4 (MFI) on $CD4^+$ T cells	0.000	0.000	−0.761	−5.991	<**0.001**
$F = 9.628$; df = (4,39); $p \leq$ **0.001**; $R = 0.705$; $R^2 = 0.497$					
FMI					
(Constant)	6.506	1.769		3.677	<0.001
CCR4 (MFI) on $CD4^+$ T cells	0.001	0.001	0.150	0.678	0.502
CCR6 (MFI) on $CD4^+$ T cells	0.000	0.001	−0.118	−0.655	0.516
CXCR3 (MFI) on $CD4^+$ T cells	0.000	0.000	−0.028	−0.132	0.895
CXCR4 (MFI) on $CD4^+$ T cells	0.000	0.000	−0.627	−4.138	<**0.001**
$F = 4.675$; df = (4,37); $p =$ **0.004**; $R = 0.579$; $R^2 = 0.336$					

$n = 44$ (HC: $n = 20$, AN: $n = 24$).
BMI, body mass index; FMI, fat mass index; MFI, mean fluorescence intensity.
Significant effects are in bold print.

to the appetite-regulating capacities of chemokines and their receptors, they are also associated with the regulation of water distribution in the organism. For example, CXCL12 has been found to modulate the firing pattern of arginine-vasopressin neurons through CXCR4. It thus counteracts induced release of arginine-vasopressin that plays an important role in water and electrolyte balance (84). Interestingly, Himmerich and colleagues reported an association between CCL13 levels in peripheral blood and intra- and extracellular water balance in individuals with AN (44).

The molecular mechanism of upregulated receptor expression in AN observed in our study and its functional role in $CD4^+$ T cells is still unresolved. The expression levels of chemokine receptors on human $CD4^+$ T cells are tightly regulated by activating factors such as cytokines, chemokines, and corticosteroids (46). For example, IL-15 has been shown before to increase CCR4 and CXCR3 expression on blood $CD4^+$ T cells *in vitro* (85). Therefore, elevated IL-15 concentrations demonstrated in AN (44, 86) could cause an increase in CCR4 and CXCR3 expression on blood T cells in the AN group of our study cohort. Other studies in AN demonstrated decreased blood levels of CXCL9, one of the ligands of CXCR3, and a correlation with the weight-regulating hormone leptin (41). Thus, upregulated CXCR3 expression on blood T cells in AN as observed in our study may also comprise a compensatory mechanism due to decreased CXCL9 serum levels in AN. It is well established that CXCR4 expression on T cells is tightly controlled by endogenous glucocorticoid receptor signaling (87), and cortisol has been shown to upregulate CXCR4 expression on $CD4^+$ T cells in humans (88). In accordance, hypercortisolism has been determined in urine, plasma and saliva samples from AN subjects and found to be associated with lower hair cortisol concentration as markers for a dysregulated hypothalamic-pituitary-adrenal-axis in this eating disorder (89). Nevertheless, altered expression levels of chemokine receptors may not be a consequence of nutrient deficiencies. Although women with AN consume less dietary zinc and cholesterol than HC, nutrient intake patterns do not contribute significantly to altered concentrations of immune markers in the blood in AN (90).

Upregulation of surface chemokine receptor levels on $CD4^+$ T cells in AN may alter adaptive immune responses. For example, CXCR4 drives migration of memory T cells from the blood to the bone marrow for self-renewal in response to CXCL12 (67). Increased CXCR4 expression on $CD4^+$ T cells in AN may therefore alter sequestration of these cells in the bone marrow and negatively affect the immune response. Regarding CCR4, increased expression in AN on $CD4^+$ T cells, which may represent Th17 cells, could promote autoimmunity and chronic inflammation (51, 55). Increased CCR4 expression in AN on regulatory $CD4^+$ T cells, instead, may enhance their immunosuppressive functions and thus affect immune responses required in host defense (52, 53). However, chemokine receptor expressing T cells have not been further characterized in this study and future research is warranted to define their subtype in AN.

After refeeding during 6 weeks of multimodal therapy, CXCR3 levels normalized on $CD4^+$ T cells in AN suggesting that this immune alteration is linked to the pathophysiology of this eating disorder. CXCR4 expression at T1 was also reduced but maintained at higher levels on $CD4^+$ T cells when compared to HC, possibly due to incomplete weight recovery after therapy or additional stress factors due to refeeding. In the whole study sample, expression of chemokine receptors was significantly associated with social and behavioral problems as determined by SDQ scores. CXCR4 expression on T cells, in particular, correlated positively with emotional problems and peer problems in adolescence. While the cause of this close link is yet unexplained, CXCR4 expression has been found to be highly responsive to changes in the microenvironment and up-regulation of CXCR4 occurred in cell lines subjected to stress conditions such as growth factor deprivation, hypoxia, and space constraints (91).

Because off-label treatment of patients with AN with metreleptin has been associated with a rapid and prominent amelioration of emotional, cognitive and behavioral symptoms of this eating disorder (92, 93), it is tempting to speculate that one of the underlying mechanisms may relate to a metreleptin induced down-regulation of CXCR4. Indeed, it has been shown that caloric restriction alone increased the expression of CXCR4 protein in bone marrow derived mesenchymal stem cells, while co-culturing these cells with leptin following nutrient restriction decreased CXCR4 levels (94).

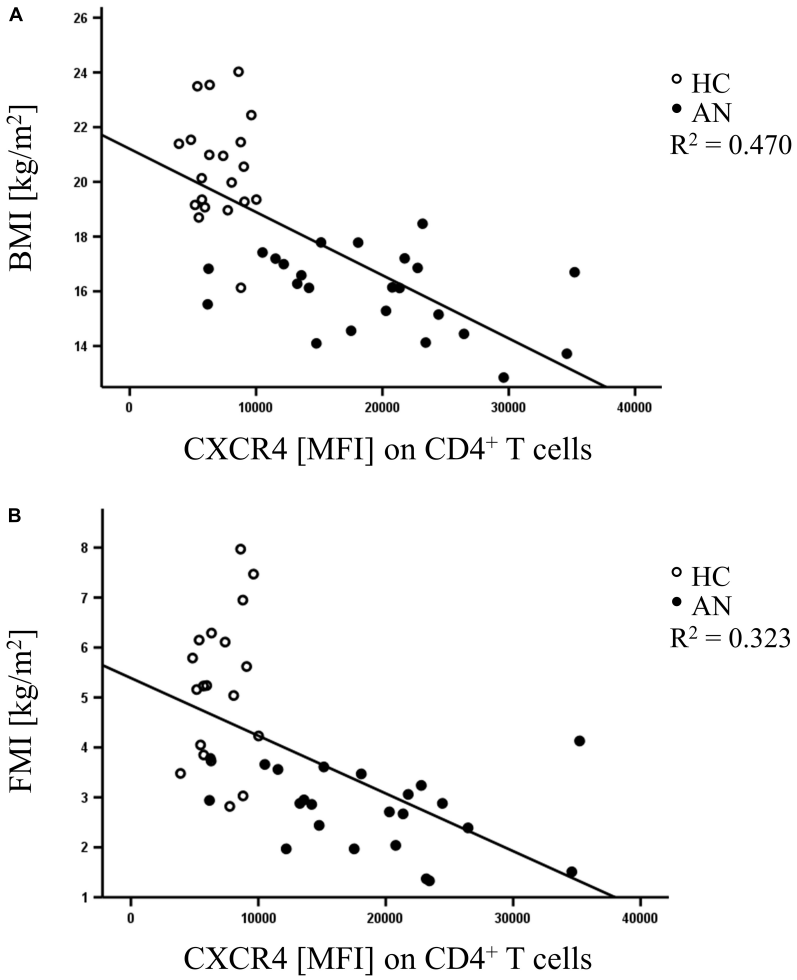

FIGURE 2
Scatter diagram depicting direct linear relationships between mean fluorescence intensity (MFI) of CXCR4 on $CD4^+$ T cells and **(A)** body mass index (BMI), and **(B)** fat mass index (FMI) in healthy controls (HC: open circle, *n* = 20) and anorectic adolescents at admission (AN: black circle, *n* = 24).

A prominent finding of our study was that expression of CXCR4 on $CD4^+$ T cells significantly predicted BMI and FMI in female adolescents. CXCR4 expression levels on $CD4^+$ T cells may therefore be used as a predictive clinical marker in future studies regarding therapy response in AN at admission since the body composition parameters BMI and FMI have been linked before to severity of AN (12, 95).

A limitation of this exploratory study is the small sample size that precludes generalizability of the results. Future studies with larger cohorts are needed to investigate the functional role of blood T cells that express chemokine receptors in AN and their value as clinical predictors for the course and severity of AN. To better understand the specific effects of CXCR4 on immune dysregulation in AN, it is also important to determine the Th17- and Treg-associated CXCR4 expression profiles, and the effector and regulatory abilities of those T cell subsets. In addition, the influence of adipokines, such as leptin, needs to be co-assessed in future studies to disentangle potential moderators and mediators.

Several immune and neuroendocrine factors such as IL-6, leptin, and oxytocin, as well as brain function and gut microbiome have been proposed as potential biomarkers to assess the severity and disease course of AN (96). Therefore, our study complements this active area of research by demonstrating that measurement of CXCR4 expression levels on peripheral blood T cells can be used as a rapid assay and as predictive marker for assessing the severity of AN.

In conclusion, our study demonstrates altered chemokine receptor expression in AN, and that the level of receptor expression on $CD4^+$ T cells is associated with adolescent health problems. Upregulation of CXCR4 on blood T cells serves as clinical predictor for BMI and FMI in adolescence and may therefore be associated with pathophysiology of AN.

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 the Medical Faculty of the University of Duisburg-Essen. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

MF and JA: conceptualization. JF, KS, JB, IK, AH, MF, and JA: methodology. JF, LB, JB, IK, MF, and JA: formal analysis. JF, KS, JB, IK, MF, and JA: investigation. JF, LB, MF, and JA: writing – original draft preparation. RL, AH, UB, VA, UD, GR, BB, JH, MF, and JA: writing – review and editing. JF, LB, and JA: visualization. AH, MF, and JA: supervision. JA: funding acquisition. All authors reviewed the manuscript.

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Conflict of interest

VA was member of advisory boards and/or gave presentations for the following companies: Astra-Zeneca, Eli Lilly, Janssen-Organon, Lundbeck, Otsuka, Servier, and Trommsdorff. BB was member of advisory boards and/or received speaker/consultation fees from AstraZeneca, Lundbeck, Pfizer, Takeda, Servier, Bristol Myers Squibb, Otsuka, and Janssen-Cilag. JH gave presentations for Novo-Nordisk and Amryt Pharmaceuticals. JA gave presentations for Servier.

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.

Publisher's note

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EDITED BY
Brenda Penninx,
Academic Medical Center, Netherlands

REVIEWED BY
Benjamin Pickard,
University of Strathclyde,
United Kingdom
Tássia Brena Costa,
State University of Campinas, Brazil

*CORRESPONDENCE
Huafang Li
lhlh_5@163.com
Wenjuan Yu
wenjuanyu2004@163.com

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

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Plasma metabolomics of schizophrenia with cognitive impairment: A pilot study

Yihe Jiang[1†], Xiujia Sun[1†], Miaowen Hu[1], Lei Zhang[1], Nan Zhao[2], Yifeng Shen[1,3], Shunying Yu[1,4], Jingjing Huang[1], Huafang Li[1,3,4*] and Wenjuan Yu[1*]

[1]Shanghai Mental Health Center, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [2]Shanghai Pudong New Area Mental Health Center, School of Medicine, Tongji University, Shanghai, China, [3]Shanghai Clinical Research Center for Mental Health, Shanghai, China, [4]Shanghai Key Laboratory of Psychotic Disorders, Shanghai, China

Schizophrenia (SCZ) acts as a complex and burdensome disease, in which the functional outcome can be validly predicted by cognitive impairment, as one of the core features. However, there still lack considerable markers of cognitive deficits in SCZ. Based on metabolomics, it is expected to identify different metabolic characteristics of SCZ with cognitive impairment. In the present study, 17 SCZ patients with cognitive impairment (CI), 17 matched SCZ patients with cognitive normal (CN), and 20 healthy control subjects (HC) were recruited, whose plasma metabolites were measured using ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC–MS/MS). The result of metabolic profiling indicated the identification of 46 differentially expressed metabolites between HC, CN, and CI groups, with 7 differentially expressed metabolites between CN and CI groups. Four differential metabolites (imidazolepropionic acid, Homoserine, and Aspartic acid) were repeatedly found in both screenings, by which the formed biomarker panel could discriminate SCZ with cognitive impairment from matched patients (AUC = 0.974) and health control (AUC = 0.841), respectively. Several significant metabolic pathways were highlighted in pathway analysis, involving Alanine, aspartate and glutamate metabolism, D-glutamine and D-glutamate metabolism, and Citrate cycle (TCA cycle). In this study, several differentially expressed metabolites were identified in SCZ with cognitive impairment, providing novel insights into clinical treatment strategies.

KEYWORDS

Schizophrenia, cognitive impairment, metabolomics, UPLC-MS/MS, biomarker

Introduction

Schizophrenia (SCZ) is a chronic and serious mental illness, usually running in families accompanied with some complex symptoms, such as delusions, hallucinations, disorganized speech, and so on (1). According to a systematic review, the prevalence of this disease is 4.6 per 1,000 persons globally (2). The abnormal manifestations and public stigma result in a huge burden on patients, accompanied with their families and society. Among the core symptoms of SCZ, cognitive impairment acts as the prime

driver affecting the therapy and prognosis, predicting the disease development and prognostic global functional outcome in SCZ (3, 4). Cognitive impairment in SCZ is ubiquitous. Empirical reviews have consistently demonstrated that patients with SCZ generally exhibited significantly decreased cognitive performance in comparison with healthy individual, referring to working memory, attention, and processing speed (5). Meanwhile, this feature in SCZ is relatively stable and independent compared to the other core symptoms, which will not be appreciably aggravated or moderated with illness duration (6). With the generality and heterogeneity, early diagnosis and detection of cognitive dysfunction is critical for SCZ treatment. However, there still lack the accurate and reliable pathophysiological criteria for SCZ with cognitive impairment.

Metabolomics serves as a powerful technique for the comprehensive study of low molecular weight molecules or metabolites identified within cells and biological systems, which has been widely used in the discovery of psychiatric biomarkers with high accuracy, resolution, and sensitivity (7, 8). In studies related to SCZ, metabolomics has shown great potential in the etiology, diagnosis, and treatment. For example, in a recent study this technology was adopted was adopted to successfully select four metabolites of SCZ and establish a high accuracy diagnosis model, providing a valuable reference for early diagnosis and intervention (9). Besides, it is well-known that drug therapy is adopted as the main treatment for SCZ, but antipsychotic drugs produce significant metabolic side effects, involving weight gain, abnormal blood-lipid level, and so on. Metabolomics has also efficiently contributed to understanding the underlying mechanism of antipsychotic drugs in SCZ (10). Among the various types of biological samples applied in metabolomics, the blood sample is widely chosen with its convenience and less invasive, and has demonstrated excellent accuracy (9–12). Therefore, with the objective to search biomarkers of SCZ with cognitive impairment, the plasma metabolomics analysis was conducted on SCZ patients with different degrees of cognitive impairment and healthy individuals.

The separation, detection, and determination of metabolites are considered the core of metabolomics technology. Currently, the commonly used analytical platforms for metabolomics refer to liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR), and enzyme assays (13). In recent years, ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC–MS/MS) has displayed obvious advantages in sensitivity, rapidness, efficiency, and cost-saving, which increasingly applied in human plasma metabolomics research (12, 14). For example, a previous study demonstrated the high accuracy of UPLC-MS/MS in detecting serum concentration in SCZ (12). Hence, UPLC-MS/MS was performed in the present study.

Taken together, we hypothesized that SCZ with cognitive impairment would exhibit different metabolic characteristics compared to SCZ with cognitive normality and healthy individual. In this study, plasma samples were collected from the three groups. Based on UPLC-MS/MS technology, we expected to screen out the primary plasma biomarkers in the SCZ with cognitive impairment and explore their potential pathophysiological mechanisms. These differentially expressed metabolites could contribute to providing novel insight for clinical diagnosis, intervention, and treatment of the SCZ with cognitive impairment.

Materials and methods

Participants

The patients of the present study were collected from An Observational Study on Atypical Antipsychotics Long-term Treatment Patients with Schizophrenia (SALT-C), a large sample, non-interventional and long-term prospective clinical study in China (15). The study was approved by the Institutional Review Board of Shanghai Mental Health Center, with the number of 2010-35, 2016-23. The study has been registered on Clinicaltrials.gov under the trial registration number NCT02640911. Written informed consent was obtained from all recruited participants and their guardians.

The diagnosis of all patients was performed based on the Structured Psychiatric Interview using the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). The severity of SCZ was measured by Positive and Negative Symptoms Scale (PANSS) (16). The Montreal Cognitive Assessment Scale (MoCA) was applied to assessing the cognitive performance, in which a score of 26 or above indicated normal cognitive function, while a score below 26 indicated impaired cognitive function (17). In the present study, the MoCA was measured at baseline and follow-up of 52 $\pm$ 2 weeks, based on which SCZ patients were categorized into the impaired cognitive group (MoCA $<$ 26 at both time points, group CI) and the normal cognitive group (MoCA $\geq$ 26 at both time points, group CN). To adjust for the differences in baseline characteristics, the propensity score matching (PSM) was adopted to select matching subjects in the CI and CN groups. The exclusion criteria referred to any organic brain disorder, substance dependency, or other factors that would influence cognitive performance. In addition, the healthy control subjects were additionally recruited from the staff of the Shanghai Mental Health Center, who had no first-degree relative with psychiatric illness.

Blood sample preparation

The blood samples were sampled from participants in a fasting state in the morning, and centrifuged at 2,000 *g* for

10 min. After separation, all plasma samples were stored at −80°C. Prior to metabolomic analysis, the plasma samples were pretreated with liquid-liquid extraction, which were thawed at 4°C to minimize sample degradation. Briefly, each sample was added ice cold methanol with partial internal standards. After vortex, centrifuge, and redissolve, the supernatant was frozen to dry for subsequent analysis.

Metabolomic analysis

In this study, the Q300 Kit provided by Metabo-Profile (Shanghai, China) was used in metabolomics analysis. All targeted metabolites were quantitated by UPLC-MS/MS system (Waters Corp., Milford, MA, USA). For the high-performance liquid chromatography (HPLC), ACQUITY HPLC BEH C18 1.7×10^{-6} M VanGuard precolumn (2.1 × 5 mm) and ACQUITY HPLC BEH C18 1.7×10^{-6} M analytical column (2.1 × 100 mm) were adopted, with column temperature of 40°C and sample manager temperature of 10°C. The mobile phase was composed of 0.1% formic acid solution (A) and acetonitrile/IPA (70:30) (B). Gradient elution was performed as follows: 0–1 min (5% B), 1–11 min (5–78% B), 11–13.5 min (78–95% B), 13.5–14 min (95–100% B), 14–16 min (100% B), 16–16.1 min (100–5% B), 16.1–18 min (5% B). The flow rate was 0.40 ml/min with the injection volume of 5.0 μl. For mass spectrometer, capillary 1.5 (ESL+), 2.0 (ESL-) Kv, source temperature 150°C, de-solvation temperature 550 °C, and de-solvation gas flow 1,000 L/h.

Statistical analysis

The raw data files generated by UPLC-MS/MS were processed using the TMBQ software (v1.0; Human Metabolomics Institute, Shenzhen, Guangdong, China), so as to perform peak integration, calibration, and calculate the concentration of each analyte in samples. Multivariate statistical analyses and univariate analyses were carried out with iMAP (v1.0) to identify differences between groups, composed of principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA). The variable significance in projection (VIP) generated in OPLS-DA processing served as the criterion for metabolites screening. Metabolites with VIP >1 and $p < 0.05$ (univariate analyses were based on whether the data were normally distributed) were considered the statistically significant differentially expressed metabolites. Binary logistic regression analysis was conducted to analyze the predictors of the differentially expressed metabolites. The receiver operating characteristic (ROC) curve was plotted based on the available data, and the area under the ROC curve (AUC) was used to evaluate the diagnostic capability of differentially expressed metabolites. Metabolic pathway analysis was performed using the *Homo sapiens* (Hsa) sets on the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp). Pathway enrichment analysis was performed on MetaboAnalyst 4.0 (http://www.metaboanalyst.ca/MetaboAnalyst/). Other statistical analyses were performed using SPSS 23.00 statistical software. The data were expressed as the mean ± standard deviation (x ± SD), and analyzed by the one-way ANOVA test or the independent sample *t*-test. Differences were considered statistically significant at $p < 0.05$.

Results

Characteristics of participants

A total of 842 SCZ patients were recruited for the SALT-C (15). After careful screening, 34 SCZ patients were enrolled in the present study eventually, covering SCZ patients with cognitive impairment ($n = 17$, it was shown that MoCA < 26 at both time points, group CI), and SCZ patients with cognitive normal ($n = 17$, MoCA ≥ 26 at both time points, group CN). The group CI and group CN were matched in age, gender, education, body mass index (BMI), and PANSS scores ($p > 0.05$), indicating the suitability for the comparison. The detailed screening process is presented in Figure 1. In addition, 20 healthy individuals were recruited as healthy control. The demographics and clinical characteristics of participants are listed in Table 1.

Discovery of discriminatory metabolites

After sampling and processing the plasma metabolite, 168 kinds of metabolites were successfully detected in each sample. The relative abundance of each metabolite class is depicted in Supplementary Figure 1. For the multivariate analyses, the PCA and PLS-DA were performed to explore the separation of metabolites between the three groups, obtaining a relatively clear classification, as shown in Figure 2. To visually illustrating the patterns of differences between groups, three groups of samples were pairwise compared by OPLS-DA. Scores plots and permutation plots of HC *vs.* CI and HC *vs.* CN demonstrated a robust quality for statistical models establishment (HC *vs.* CI, $R^2 = 0.863$, $Q^2 = 0.644$; HC *vs.* CN, $R^2 = 0.818$, $Q^2 = 0.438$), but lower quality parameters in model CN *vs.* CI ($R^2 = 0.771$, $Q^2 = -0.128$) (Supplementary Figure 2).

Subsequently, the screening of potential biomarkers was carried out based on multi-dimensional statistics with VIP > 1 and univariate statistics with $p < 0.05$. With the comparison among the three groups, 46 differentially expressed metabolites were screened out (Supplementary Table 1), with the heatmap shown in Figure 3. To further explore the potential cognitive biomarkers, the identification of differences between group CN and CI was performed. Given

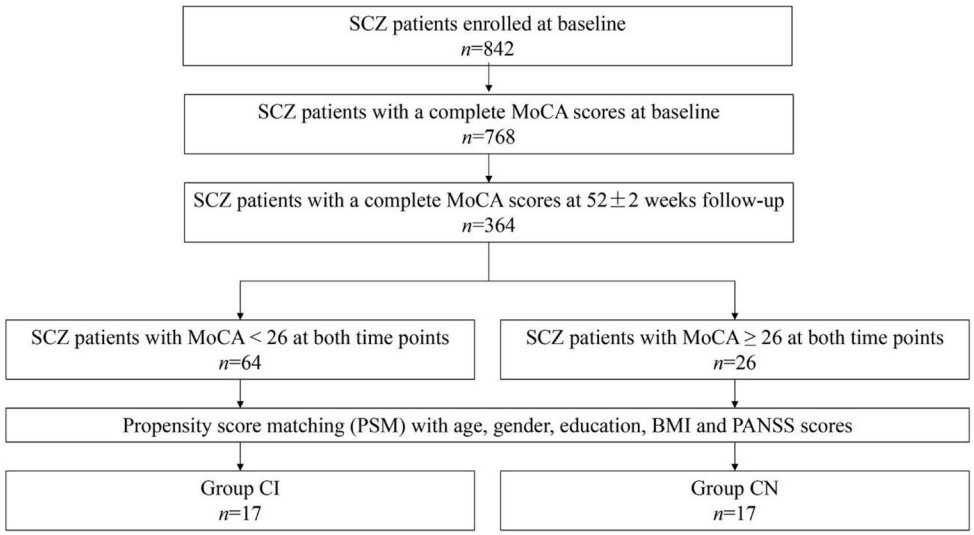

FIGURE 1
Study flow diagram. A total of 34 SCZ patients were screened from SALT-C in the present study.

TABLE 1 Demographics and clinical characteristics of participants.

Characteristics	CI ($n = 17$)	CN ($n = 17$)	HC ($n = 20$)
Age (years)	54.18 ± 13.97	49.53 ± 12.17	32.05 ± 4.62
Gender	F = 5, M = 12	F = 6, M = 11	F = 7, M = 13
Education (years)	10.00 ± 3.72	11.41 ± 3.12	16.2 ± 3.75
BMI (kg/m^2)	24.85 ± 3.76	23.53 ± 3.14	23.15 ± 3.17
PANSS	55.18 ± 10.51	56.59 ± 13.00	N/A
Smoker (*n*, %)	5 (29.4%)	4 (23.5%)	3 (15%)
MoCA	16.88 ± 4.47	27.59 ± 1.23	27.95 ± 1.23
Types of antipsychotic drugs			
Quetiapine (*n*, %)	1 (5.9%)	3 (17.6%)	N/A
Olanzapine (*n*, %)	8 (47.1%)	7 (41.2%)	N/A
Risperidone (*n*, %)	1 (5.9%)	3 (17.6%)	N/A
Aripiprazole (*n*, %)	6 (35.3%)	9 (52.9%)	N/A
Clozapine (*n*, %)	7 (41.2%)	0	N/A
Amisulpride (*n*, %)	1 (5.9%)	0	N/A

CI, SCZ patients with cognitive impairment; CN, SCZ patients with cognitive normal; HC, health control. BMI, body mass index; PANSS, the total score of Positive and Negative Syndrome Scale; MoCA, the total score of Montreal Cognitive Assessment Scale. No PANSS score and drug are used in the healthy control group (N/A). Values are expressed as mean ± standard deviation.

the dissatisfying model of CN *vs*. CI, the univariate analyses were involved for further reference, and 7 differentially expressed metabolites were screened successfully (Table 2). Among them, Imidazolepropionic acid, Erythronic acid, Homoserine, and Aspartic acid also displayed significant differences in the previous comparison of three groups, which showed great potential as biomarkers for the development of cognitive dysfunction.

ROC curve analysis

ROC curve analysis was carried out to evaluate the diagnostic capability of differentially expressed metabolites. In order to obtain a potentially simplified biomarker panel, the binary logistic regression analysis was applied to the four differentially expressed metabolites. As depicted in Figure 4A, the CI could be accurately distinguished from HC or CN by the biomarker panel (Imidazolepropionic acid, Erythronic acid, Homoserine, and Aspartic acid), in which the AUC were 0.974 and 0.841, respectively. Consistently, the biomarker panel also could distinguish CN from HC (AUC = 0.791).

Metabolic pathway analysis

Pathway analysis performed on the 46 differentially expressed metabolites by KEGG database, and a total of 35 pathways were enriched (Figure 4B). Based on *p*-value and pathway impact scores, the top three pathways were highlighted, referring to Alanine, aspartate and glutamate metabolism ($p < 0.0001$, impact = 0.57), D-glutamine and D-glutamate metabolism ($p = 0.000467$, impact = 0.33), and Citrate cycle (TCA cycle) ($p = 0.000172$, impact = 0.25).

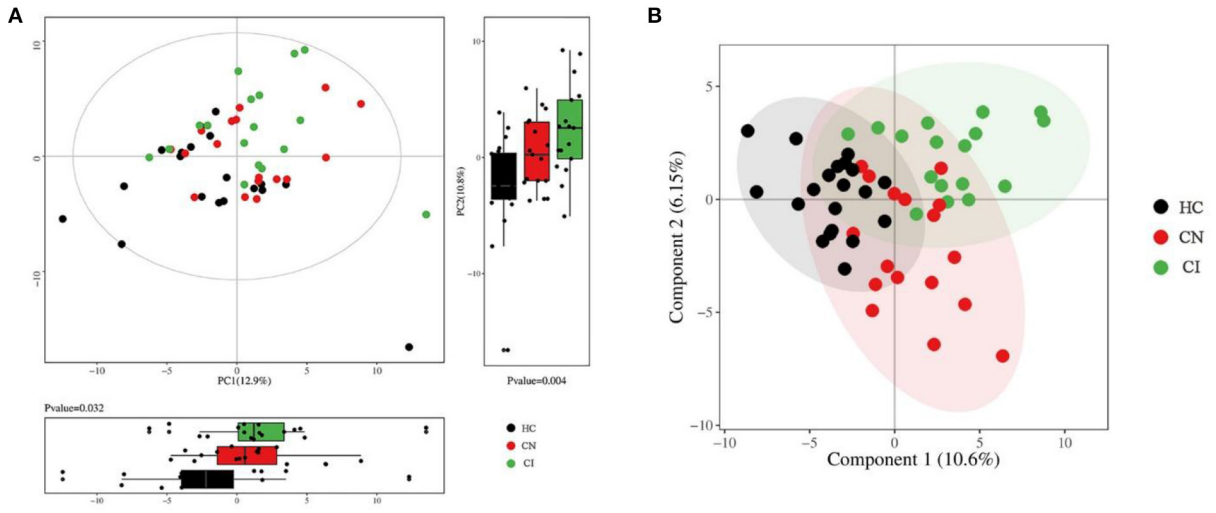

FIGURE 2
Multivariate statistical analysis of plasma metabolomics. **(A)** Overview of PCA score plots with principal component boxplot obtained from all samples. **(B)** Overview of PLS-DA score plots obtained from all samples.

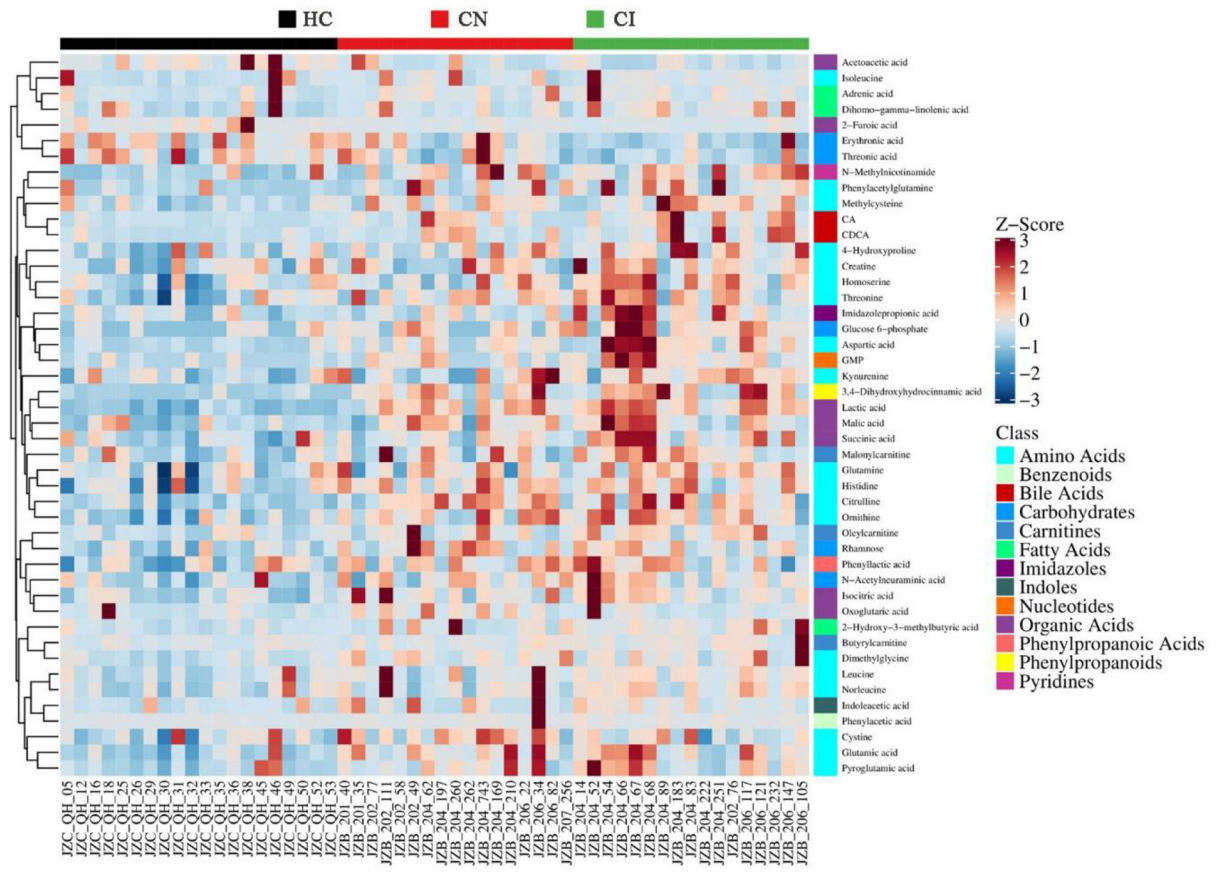

FIGURE 3
Heat map of the 46 differentially expressed metabolites between HC, CN and CI groups. The blue band indicates a decreased level of metabolite, and the red band indicates an increased level of metabolite.

TABLE 2 List of differentially expressed metabolites between CN and CI.

Metabolite	Class	CN (mean ± SD)	CI(mean ± SD)	Value of *p*
Imidazolepropionic acid	Imidazoles	0.7649 ± 0.0127	0.7914 ± 0.0335	1.45E-02
Erythronic acid	Carbohydrates	2.1629 ± 2.0511	1.0533 ± 1.6254	1.91E-02
Homoserine	Amino acids	6.0262 ± 2.1035	7.7902 ± 2.3761	2.61E-02
Glycolic acid	Organic acids	6.0910 ± 2.5021	4.2823 ± 2.2919	3.53E-02
Azelaic acid	Fatty acids	0.3199 ± 0.2840	0.1452 ± 0.1681	4.26E-02
Aspartic acid	Amino acids	6.2202 ± 4.3561	14.4624 ± 12.5125	4.57E-02
Malonic acid	Organic acids	2.9702 ± 0.1890	2.8591 ± 0.1186	4.83E-02

Mean represents the average relative abundance of metabolites in different groups. SD represents standard deviation. T-test was adopted for univariate analysis, and $p < 0.05$ is considered significant.

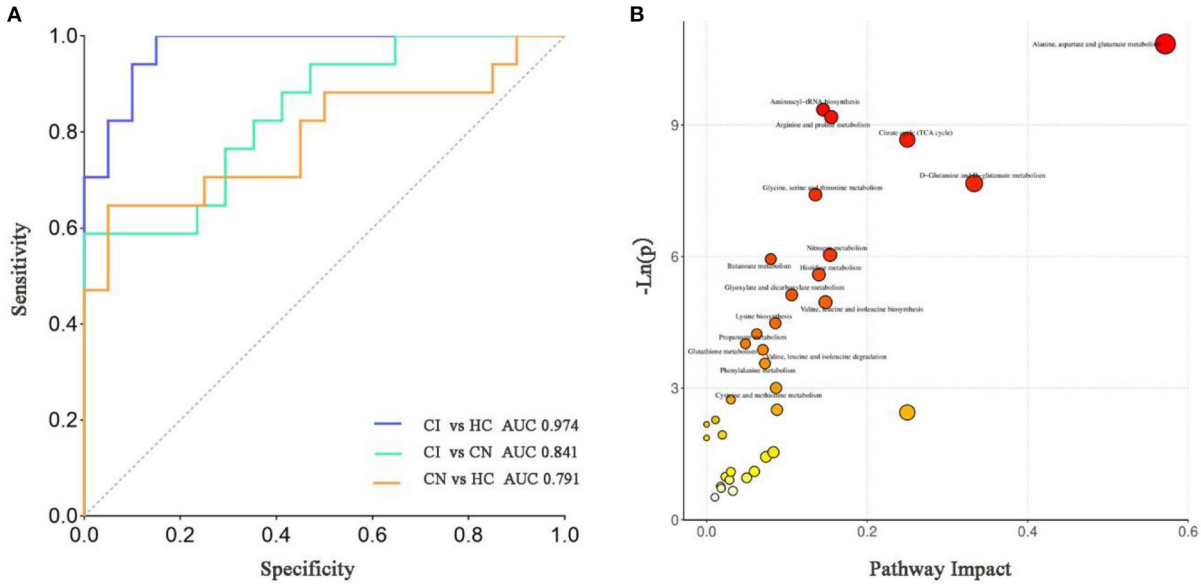

FIGURE 4
(A) The ROC diagnostic analysis of biomarker panel (Imidazolepropionic acid, Erythronic acid, Homoserine, and Aspartic acid) for different groups. **(B)** Pathway analysis bubble plot by the Hsa set in KEGG. The x-axis is the pathway impact, indicating the importance of differentially expressed metabolites in metabolic pathways. The y-axis is the negative logarithm of *p*-value. The size of pathway symbols represents the statistical significance level of pathway analysis. The color of pathway symbols represents the impact factor. Large sizes and dark colors represent central pathway enrichment and high pathway impact values, respectively.

Discussion

Metabolomics has certain reliability in exploring plasma metabolites, through which the previous studies have demonstrated the metabolic variation in SCZ (11, 18). To our knowledge, plasma metabolite of cognitive function has been rarely reported, which, however, is a potentially stable and reliable predictor of long-term outcomes in SCZ. In this study, we explored the characteristic of plasma metabolism in SCZ with cognitive impairment based on UPLC–MS/MS combined with both univariate statistical methods and multivariate statistical methods. According the results of metabolic profiling, we identified 46 differentially expressed metabolites between HC, CN, and CI groups, and 7 differentially expressed metabolites in comparison between CN and CI groups. These results verify the advantages of metabolomics in searching differentially expressed metabolites.

Different kinds of plasma metabolites were identified in SCZ with cognitive impairment. In comparisons of three groups (46, HC *vs.* CN *vs.* CI) and two groups (7, CN *vs.* CI), four plasma metabolites appeared repeatedly, that the Imidazolepropionic acid, Homoserine, and Aspartic acid were significantly increased in CI, and Erythronic acid significantly decreased in CI. ROC analysis demonstrated a good discriminative power of the biomarker panel formed by the four metabolites in cognitive function in SCZ.

Among the four metabolites, aspartic acid is reported to be strongly associated with psychiatric disorders (19, 20), which has previously been demonstrated in the metabolomics study of SCZ (11). The study using LC/MS/MS-based approach found that aspartic acid can serve as a good biomarker to distinguish SCZ patients from healthy controls. Despite the absence of consideration of cognitive symptoms in the previous study, the consistent screening results validated the feasibility of aspartic acid as a biomarker in SCZ. However, the previous study showed lower aspartic acid levels in SCZ patients (11), which is inconsistent with our result, where the plasma aspartic acid level was higher in SCZ patients, especially in SCZ patients with cognitive impairment. It can result from by two factors. First, the uptake of aspartic acid by the brain from plasma has been demonstrated when plasma level remains high for relatively long periods (21). While high content of aspartic acid can be a toxin, which induces hyperexcitability of neurons, eliciting degeneration of neurons with excess, which will ultimately develop to cognitive impairment (22). Another, current research has suggested the accelerative role of aspartate metabolism in the inflammatory responses (23). Related studies generally have pointed out that the pathogenesis of cognitive impairment in SCZ is closely related to the abnormal immune system (24). As mentioned above, aspartic acid has the potential to recognize SCZ and the cognitive function of SCZ, but requiring further studies.

Additionally, aspartic acid can be converted into homoserine by a two-reduction step of the terminal carboxyl group. A previous study found both the increased metabolites in the serum of patients with Alzheimer's disease and Parkinson's disease, showing a tandem variation (25). Not surprisingly, our results also indicated the increased homoserine in SCZ with cognitive impairment, as with aspartic acid. In addition, to our knowledge, imidazolepropionic acid and erythronic acid have not been found in previous metabolomics studies of mental disorders, of which the underlying mechanism requires to be further studied. Anyway, our results illustrate that the down-regulation of erythronic acid and the up-regulation of imidazolepropionic acid, homoserine, and aspartic acid may be the causes of cognition decline of CI.

In addition to the screened differentially expressed metabolites, abnormal metabolic pathways also serve as a key point reflecting the pathology of cognitive impairment. The results of pathway analysis indicated the association of cognitive performance of SCZ with Alanine, aspartate and glutamate metabolism, D-glutamine and D-glutamate metabolism, as well as Citrate cycle (TCA cycle). Among them, Alanine, aspartate and glutamate metabolism, D-glutamine and D-glutamate metabolism suggest a critical role of glutamate and glutamine in this study. Previous studies have consistently suggested that the occurrence and development of SCZ is closely related to glutamate, forming the glutamatergic dysfunction hypothesis of SCZ (26). In the hypothesis, the dysregulation of γ-aminobutyric acid (GABA) level and N-methyl-D-aspartate (NMDA) receptor elicit the abnormality of brain glutamate concentration, which are considered the core of the pathogenic trigger of SCZ and symptoms (e.g., cognitive impairment) (27, 28). The abnormal elevated blood glutamate levels have been found in SCZ (29, 30) and the glutamate is related to cognitive function in SCZ (31), which is consistent with our results (Supplementary Table 1) and confirmed to the glutamatergic dysfunction hypothesis. However, the variations of glutamatergic metabolite levels in SCZ, covering its association with SCZ at different levels of cognitive function, remain ambiguous and require further investigation (28, 31).

In this study, the other important pathway, Citrate cycle (TCA cycle) was found promoted in SCZ and associated with cognitive impairment in SCZ, which support the possibility that abnormalities in energy metabolism contribute to SCZ (32). To be specific, the hypothesis holds that TCA cycle affects inflammatory response and induces blood-brain barrier damage, which lead to the occurrence and development of SCZ and the core symptoms (33–35). Indeed, it has been found that the TCA cycle was abnormal in SCZ compared with HC, which is consistent with our results (33). While the correlation between TCA cycle and the cognitive level of SCZ still requires further study.

Finally, our study has points of strengths and limitations that warrant discussion. The primary strength is that the propensity score matching in the real-world setting compensates for the lack of a randomized-controlled study. Another evident strength points to the performance of UPLC–MS/MS-based metabolite profiling, which is in high selectivity, reliability, and sensitivity. However, the major limitation of this study is the relatively limited sample size, that only 64 or 26 of the 364 patients were consistent low or high MoCA, possibly resulting from the long interval between baseline and follow-up (52 ± 2 weeks) in our study, compared with 48 ± 32.2 days in the previous study, where the validity of MoCA in SCZ and the stability of cognitive function in SCZ have been confirmed (36). The time interval is a key factor for future research to consider. Second, although we used PSM to balance out some differences in CI and CN, there still leave some between-group differences and within-group differences failing to be ruled out (e.g., drugs, individual underlying diseases, and individual dietary differences). A more rigorous experimental design could be considered in future studies. Additionally, our study is a preliminary exploratory study that requires further external validation.

Conclusion

In summary, we demonstrated that the SCZ with cognitive impairment had a significant imbalance of metabolites.

According the results of metabolic profiling, we identified 46 differentially expressed metabolites between HC, CN and CI groups, and 7 differentially expressed metabolites compared to CN and CI groups. Four differential metabolites (imidazolepropionic acid, Homoserine, and Aspartic acid) were repeatedly identified in both screenings, by which the formed biomarker panel could be accurately discriminate the SCZ with cognitive impairment from matched patients and healthy subjects. The metabolomics based on UPLC–MS/MS method provide an objective reference for clinical treatment strategies.

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 Institutional Review Board of Shanghai Mental Health Center affiliated with Shanghai Jiao Tong University School of Medicine. The patients/participants provided their written informed consent to participate in this study.

Author contributions

YJ: data analysis and manuscript writing. XS: identification and quantification of plasma metabolites. XS, HL, and WY: conception and design of the study. JH: ethical review. MH, LZ, and NZ: project implementation and data collection. HL and WY: critical reading of the manuscript. YS, SY, JH, HL, and WY: overall supervision of the project. All authors contributed to the manuscript and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2022.950602/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The overview of identified metabolite classification classes. **(A)** Pie plot showing proportion of identified metabolite classes in all samples. **(B)** Stacked bar chart showing relative abundance of each metabolite classes in different groups.

SUPPLEMENTARY FIGURE 2
The OPLS-DA model building and validation. **(A–C)** OPLS-DA score plots, and **(D–F)** permutation plot between every two groups (HC vs. CI, HC vs. CN, and CN vs. CI).

SUPPLEMENTARY TABLE 1
List of differentially expressed metabolites between HC, CN and CI groups.

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