ENVIRONMENTAL RISK FACTORS IN AUTISM SPECTRUM DISORDER

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PUBLISHED IN: Frontiers in Psychiatry

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-83250-142-9
DOI 10.3389/978-2-83250-142-9

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ENVIRONMENTAL RISK FACTORS IN AUTISM SPECTRUM DISORDER

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Citation: Fukunaga, K., Matsuzaki, H., eds. (2022). Environmental Risk Factors in Autism Spectrum Disorder. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-83250-142-9

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frontiers | Frontiers in Psychiatry

TYPE Editorial
PUBLISHED 23 August 2022
DOI 10.3389/fpsyt.2022.978489

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Autism, a section of the journal Frontiers in Psychiatry

RECEIVED 26 June 2022
ACCEPTED 11 August 2022
PUBLISHED 23 August 2022

CITATION
Matsuzaki H and Fukunaga K (2022) Editorial: Environmental risk factors in autism spectrum disorder. *Front. Psychiatry* 13:978489. doi: 10.3389/fpsyt.2022.978489

Editorial: Environmental risk factors in autism spectrum disorder

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KEYWORDS

autism spectrum disorder, prenatal environment, inflammation, maternal immune activation (MIA), polygenic risk score

Editorial on the Research Topic
Environmental risk factors in autism spectrum disorder

Autism spectrum disorder (ASD) is a neurodevelopmental disease defined by social impairments and repetitive behaviors. While ASD are highly heritable, several environmental risk factors (e.g., maternal infection exposure to drugs or toxicants, and brain inflammation) have also been suggested, although the underlying causes remain controversial. To shed more light on this, our Research Topic presents a broad range of insights to define the causal environmental factors in animal models and patients, and to answer the questions of how and what neuronal circuits are involved in each symptom including social impairments and repetitive behaviors. Specifically, this Research Topic included functional assessments how environmental factors correlate to ASD risk by using clinical study and animal models. This Editorial introduce following articles in the special issue on the environmental risk factors in ASD.

Firstly, three clinical study reports discussing some environment factors associated with ASD and their psychometric properties were introduced. Abelson et al. conducted a nested matched case-control study of children with/without ASD, and compared the use of antimicrobial therapy during the 3 months before conception or during pregnancy between mothers of cases and controls and used multivariate conditional logistic regression models to assess the independent association between maternal use of antimicrobials during pregnancy and the risk of ASD in their offspring. They concluded that the reduced risk of ASD associated with prenatal antimicrobials use only in the Jewish population suggest the involvement of other ethnic differences in healthcare services utilization in this association. Iwabuchi et al. investigated the associations between mothers' metabolic conditions, leptin concentrations in umbilical cord serum, and autistic symptoms among 762 children from an ongoing cohort study, and identified

the umbilical cord leptin levels were associated with pre-pregnancy overweight, diabetes mellitus and SRS-2 scores in children. Although associations between maternal metabolic factors and autistic symptoms were not significant, these results imply that prenatal pro-inflammatory environments affected by maternal metabolic conditions may contribute to the development of autistic symptoms in children. Yoshikawa et al. were focusing on adverse childhood experiences (ACEs). In general, individuals with ASD have an increased risk of ACEs than typically developed (TD) children. They investigated the relationship between ACEs and microstructural integrity on frontal lobe-related white matter tracts using diffusion tensor imaging in 63 individuals with ASD and 38 TD participants, and suggested that an exposure to ACEs is associated with abnormality in the frontal lobe-related white matter in ASD.

Secondly, two animal model reports discussing some environment factors associated with ASD-like behaviors and brain phenotype were introduced. Kotajima-Murakami et al. examined whether exposure to the GABAA receptor antagonist picrotoxin causes ASD-like pathophysiology in offspring by conducting behavioral tests from the juvenile period to adulthood and performing gene expression analyses in mature mouse brains. They found that male mice exposed prenatally to picrotoxin exhibited a reduction of the social interaction in both adolescence and adulthood and showed a strong correlation between social interaction and enrichment of the "odorant binding" pathway gene module by using weighted gene co-expression network analysis (Kotajima-Murakami et al.). Their findings suggest that exposure to a GABAA receptor inhibitor during the embryonic period induces ASD-like behavior, and impairments in odorant function may contribute to social deficits in offspring. In other hands, it has been documented that the neuropeptide oxytocin (OXT) ameliorates core symptoms in patients with ASD. Matsuo et al. have recently reported that chronic administration of intranasal OXT reversed social and learning impairments in prenatally valproic acid (VPA)-exposed rats (1). They explored here molecular alterations in the hippocampus of rats and the effects of chronic administration of intranasal OXT, and clarified molecular profiling in the hippocampus related to ASD and improvement by chronic treatment with OXT (Matsuo et al.).

Finally, four review articles provide an interesting perspective focusing on maternal immune activation (MIA) resulting from bacterial or viral infection during pregnancy. Two of four are also focusing on role of interleukin-17A (IL-17A) on ASD etiology. Fujitani et al. examined the signaling pathways in both immunological and neurological contexts that may contribute to the improvement of autism spectrum disorder symptoms associated with maternal blocking of IL-17A and adult exposure to IL-17A, and suggested IL-17A antibodies may have ability to prevent ASD. Carter et al. focused on maternal immune activation (MIA) rodent models of ASD and reviewed the animal and human-based evidence indicating that IL-17A may mediate the observed effects of MIA on neurodevelopmental outcomes in the offspring. As severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection during pregnancy is a potent stimulator of the maternal immune response, authors state that this underscores the importance of monitoring neurodevelopmental outcomes in children exposed to SARS-CoV-2-induced MIA during gestation. Sato et al. reviewed evidence of the mechanisms by which environmental factors are related to ASD from three factor including prenatal drug exposure, parental aging, and MIA. Eve et al. reviewed articles to address the unexplored role that neuronal cell adhesion molecules may play in mediating inflammatory cascades that underpin neuroinflammation in ASD, primarily focusing on the Notch, nuclear factor-κB (NF-κB), and mitogen-activated protein kinase (MAPK) cascades.

To address the therapeutic approaches in ASD, we aim to define the causal environmental factors in animal models and patients. The goal of this Research Topic is to discover the novel therapeutic targets by definition of the common pathways and common environmental risks in human and animal models. As combination of environmental and genetic factors are believed to contribute to ASD pathogenesis, the perspective arisen from the articles may contribute to the development of preventive and therapeutic interventions for ASD. However, further research is needed to determine whether these risk factors are specific to ASD. Since the readers are now interesting in the polygenic risk score related to ASD, we expected submission focused on the connection between the polygenetic risk score and environmental risk. However, there was no such paper this time unfortunately. Further technological developments will deepen our understanding of the role of environmental factors in ASD etiology.

Author contributions

HM prepared the manuscript. All authors contributed to the article and approved the submitted version.

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

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frontiers in Psychiatry

BRIEF RESEARCH REPORT
published: 19 November 2021
doi: 10.3389/fpsyt.2021.771232

Association Between Antenatal Antimicrobial Therapy and Autism Spectrum Disorder—A Nested Case-Control Study

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OPEN ACCESS

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Specialty section:
This article was submitted to Autism, a section of the journal Frontiers in Psychiatry

Received: 06 September 2021
Accepted: 22 October 2021
Published: 19 November 2021

Citation:
Abelson N, Meiri G, Solomon S, Flusser H, Michaelovski A, Dinstein I and Menashe I (2021) Association Between Antenatal Antimicrobial Therapy and Autism Spectrum Disorder—A Nested Case-Control Study. Front. Psychiatry 12:771232. doi: 10.3389/fpsyt.2021.771232

Background: Multiple prenatal factors have been associated with autism spectrum disorder (ASD) risk. However, current data about the association between antimicrobial use during pregnancy and ASD is limited.

Methods: A nested matched case-control study of children with ASD (cases), and children without ASD or other psychiatric or genetic disorders (controls). We compared the use of antimicrobial therapy during the 3 months before conception or during pregnancy between mothers of cases and controls and used multivariate conditional logistic regression models to assess the independent association between maternal use of antimicrobials during pregnancy and the risk of ASD in their offspring.

Results: More than half of the mothers in the study (54.1%) used antimicrobial drugs during the 3 months before conception or during pregnancy. Rates of antimicrobial use were lower for mothers of children with ASD compared to mothers of controls (49.0 vs. 55.1%, respectively; $p = 0.02$), especially during the third trimester of pregnancy (18.8 vs. 22.9%, respectively; $p = 0.03$), and for the use of penicillins (15.7 vs. 19.7%, respectively; $p = 0.06$). These case–control differences suggest that antimicrobial administration during pregnancy was associated with a reduced risk of ASD in the offspring (aOR = 0.75, 95% CI = 0.61–0.92). Interestingly, this association was seen only among Jewish but not for the Bedouin mothers (aOR = 0.62, 95% CI = 0.48–0.79 and aOR = 1.21, 95% CI = 0.82–1.79).

Conclusions: The reduced risk of ASD associated with prenatal antimicrobials use only in the Jewish population suggest the involvement of other ethnic differences in healthcare services utilization in this association.

Keywords: Autism spectrum disorder (ASD), prenatal, antimicrobial drugs, public health, ethnic disparities

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental condition, characterized by deficits in social communication and interaction and accompanied by the presence of restrictive, repetitive behaviors, interests, or activities (1, 2). ASD is a major public health issue, with healthcare expenditure on children with ASD being nine times higher than that for children with normal development, and three times higher than that for children with intellectual disabilities (3). Of particular concern is the remarkable rise in the prevalence of ASD in the past few decades; for example, in the USA, ASD rates rose from approximately 1 in 150 children at the beginning of this century (4) to 1 in 54 children under the age of eight by 2016 (5). It is likely that the dramatic increase in ASD prevalence stems from a better awareness of the condition among physicians and parents, accompanied by changes in ASD diagnostic criteria. However, the contribution to ASD prevalence of new risk factors or changes in lifestyle habits cannot be excluded (3, 6–8).

It is commonly accepted that the prenatal period plays a significant role in ASD susceptibility, and a wide range of prenatal risk factors have been associated with ASD (6–13). One of these factors is maternal infection during pregnancy, with some studies reporting a stronger association with infections requiring hospitalization and with multiple infections during pregnancy (7, 14–18). Other studies, including some on animal models, have suggested that maternal immune activation rather than the infection itself is the cause of the elevated risk of ASD (7, 19, 20). In parallel, an association between maternal fever and ASD has been demonstrated, with a stronger association with ASD for a longer duration of fever (21, 22). In this regard, the use anti-pyretic treatment during pregnancy has been suggested to have a protective effect on ASD risk (6, 7, 13, 23). Consequently, it is not clear whether the reported association between prenatal infection and ASD is due to exposure to an infectious organism, the immune response associated with the resulting infection, the corresponding medical treatment, or a combination of these factors (6, 7).

The treatment of an infection during pregnancy usually includes treatment with antimicrobials, which account for ∼80% of all medications prescribed to pregnant women (24). It is estimated that 19–49% of pregnant women receive antimicrobial agents during pregnancy and that this percentage will continue to increase (24–29). The use of antimicrobial therapy during pregnancy has been associated with various unwanted outcomes in the offspring, including cerebral palsy, epilepsy, immune system alterations, childhood asthma, changes in gut microbiota, obesity, and functional impairments (24). The effect of antimicrobial use during pregnancy on ASD risk is not yet clear: while some studies have reported that the use of antimicrobials during pregnancy is associated with an increased risk of ASD (17, 21, 30–34), other studies did not find such association (35). Therefore, the objective of this study was to investigate the association between the use of antimicrobials during pregnancy and the risk of ASD in the offspring.

METHODS

Study Design

We conducted a nested case-control study of children who were born at the Soroka University Medical Center (SUMC), Beer-Sheva, between the years 2008 and 2016 and who were members of Clalit, the largest Health Maintenance Organization (HMO) in Israel. SUMC is the only tertiary hospital in southern Israel and is associated with the Clalit HMO. Cases were defined as children who were diagnosed with ASD at SUMC and who were enrolled in the database of the National Autism Research Center of Israel (NARCI), which is a repository of comprehensive medical data pertaining to those children and their families (36, 37). Controls were children with no diagnosis of ASD, or other psychiatric or genetic disorders, who were randomly sampled from the computerized database of the SUMC and were individually matched to the case group at a ratio of 5:1 according to age, sex, and ethnic origin (Jewish or Bedouin). Exclusion criteria included incomplete maternal records and children with other neurodevelopmental or psychiatric disorders.

TABLE 1 | Demographic and clinical characteristics of mothers of children with ASDs and matched non-ASDs controls*.

Variable	ASD** *N* = 451	Non-ASD** *N* = 2,255	*P*-value**
Demographic characteristics			
Age [mean years (±SD)]	29.48 (± 5.9)	29.02 (± 5.47)	0.11
Socioeconomic status [median (IQR)]	**8 (3–11)**	**6 (0–10)**	**<0.01**
Chronic medical conditions			
Obesity [*N* (%)]	**45 (10.0%)**	**134 (5.9%)**	**<0.01**
Any psychiatric [*N* (%)]	12 (2.7%)	39 (1.7%)	0.20
Depression	8 (1.8%)	28 (1.2%)	0.37
Other psychiatric	5 (1.1%)	9 (0.4%)	0.08
Epilepsy [*N* (%)]	**10 (2.2%)**	**13 (0.6%)**	**<0.01**
Asthma [*N* (%)]	38 (8.4%)	161 (7.1%)	0.41
Atopic dermatitis [*N* (%)]	15 (3.3%)	94 (4.2%)	0.49
Diabetes mellitus [*N* (%)]	23 (5.1%)	75 (3.3%)	0.12
Any hypertension [*N* (%)]	**24 (5.3%)**	**73 (3.2%)**	**0.02**
Thyroid [*N* (%)]	26 (5.8%)	146 (6.5%)	0.60
Hyperlipidemia [*N* (%)]	5 (1.1%)	16 (0.7%)	0.37
Rheumatic [*N* (%)]	13 (2.9%)	52 (2.3%)	0.41
Chronic medications			
Antiepileptic [*N* (%)]	7 (1.6%)	24 (1.1%)	0.42
Psychiatric any [*N* (%)]	11 (2.4%)	63 (2.8%)	0.63
SSRI	8 (1.8%)	30 (1.3%)	0.48
Asthmatic [*N* (%)]	21 (4.7%)	86 (3.8%)	0.48
Inhaled adrenergic [*N* (%)]	**19 (4.2%)**	**55 (2.4%)**	**0.04**
Anti-hyperglycemic [*N* (%)]	**9 (2.0%)**	**15 (0.7%)**	**<0.01**
Antihypertensive [*N* (%)]	8 (1.7%)	18 (0.8%)	0.06
Antacids [*N* (%)]	46 (10.2%)	193 (8.6%)	0.30
Glucocorticosteroids [*N* (%)]	14 (3.1%)	61 (2.7%)	0.75

*Cases and controls were matched according to sex, date of birth, and ethnic origin.
**Bold text indicates significant case-control differences at $\alpha < 0.05$.

Data Acquisition

Medical and sociodemographic data for both cases and controls were obtained from the SUMC computerized database, which contains electronic records of all medical treatments given at the hospital as well as medical data from the outpatient clinics of the Clalit HMO. For this study, we extracted complete pregnancy and birth records for all children in the study. In addition, we obtained basic sociodemographic data for the mothers as well as data regarding chronic conditions and regularly prescribed medications that were recorded prior to childbirth. Data about antimicrobial use prior to (up to 3 months before conception) and during the pregnancy included antimicrobial type, which was encoded in the database according to the Anatomical Therapeutic Chemical (ATC) Classification. The method of administration was classified to topical use (cream or ointment), mucosal (cream, ointment, or a suppository for use orally, rectally, or vaginally) or systemic (oral, intramuscular, or intravenous). The trimester in which the drug was given was calculated using the date of the prescription and the week of the child's delivery. Unfortunately, the medical indication associated with each drug prescription was not available in SUMC database, since physicians are not required to document this information in the computerized system. Antimicrobial treatments with a prevalence of <1% were aggregated with other variables of the same category or were removed from the analyses. All antimicrobial treatments included in this study are listed in **Supplementary Table 1**.

Statistical Analysis

We used standard univariate statistics to compare sociodemographic and medical variables between cases and controls. Variables with statistically significant case-control differences ($p < 0.05$) were included in multivariable conditional logistic regression models that were used to assess the association between the type of antimicrobial therapy with the risk of ASD. We also performed a sensitivity analysis by testing the association between prenatal antimicrobial and ASD in the offspring separately for the Jewish and Bedouin mothers, since these two ethnic groups are known to differ significantly in their access to medical care and their ASD prevalence (38). We used the Breslow-Day test (39) to assess the homogeneity of the odds ratios (ORs) between these two ethnic groups. The statistical analysis was performed using the IBM SPSS Statistics software, version 23.0, and R studio, version 1.1.456 (R Foundation for Statistical Computing version 3.4.4). This study was approved by the SUMC ethics committee (SOR 222-14).

RESULTS

Overall, 451 cases and 2,255 controls were included in this study. Of these 2,706 children, 81% were males and 71% were of Jewish origin. **Table 1** presents the basic demographic and clinical characteristics of mothers of cases and controls. Mothers of cases were of higher socioeconomic status than mothers of controls (median socioeconomic level of 8 vs. 6, $p \leq 0.01$) and

TABLE 2 | Antimicrobials taken by mothers of children with ASDs and matched non-ASDs controls*.

Variable		All $N = 2,706$	ASD $N = 451$	Non-ASD $N = 2,255$	*P*-value
Any antimicrobial [*N* (%)]		**1,463 (54.1%)**	**221 (49.0%)**	**1,242 (55.1%)**	**0.02**
Antimicrobial period [*N* (%)]	3 Months before conception	700 (25.9%)	107 (23.7%)	593 (26.3%)	0.28
	Trimester 1	762 (28.2%)	118 (26.2%)	644 (28.6%)	0.33
	Trimester 2	760 (28.1%)	123 (27.3%)	637 (28.2%)	0.72
	Trimester 3	**619 (22.9%)**	**85 (18.8%)**	**534 (23.7%)**	**0.03**
Antimicrobial type [*N* (%)]	Any mucosal	423 (15.6%)	71 (15.7%)	352 (15.6%)	1
	Any topical	674 (24.9%)	99 (22%)	575 (25.5%)	0.13
	Any systemic	1,072 (39.6%)	163 (36.1%)	909 (40.3%)	0.11
	Systemic Beta-lactam	983 (36.3%)	150 (33.3%)	833 (36.9%)	0.15
	Penicillin	514 (19.0%)	71 (15.7%)	443 (19.7%)	0.06
	Cephalosporin	647 (23.9%)	101 (22.4%)	546 (24.2%)	0.44
	Macrolide	51 (1.9%)	4 (0.9%)	47 (2.1%)	0.13
	Antifungal	644 (23.8%)	103 (22.8%)	541 (24%)	0.64
	Mucosal	398 (14.7%)	67 (14.9%)	331 (14.7%)	0.98
	Topical	450 (16.6%)	65 (14.4%)	385 (17.1%)	0.19
	Systemic	**15 (0.6%)**	**6 (1.3%)**	**9 (0.4%)**	**0.03**
	Aminoglycoside topical	94 (3.5%)	12 (2.7%)	82 (3.6%)	0.37
	Antiviral topical	50 (1.8%)	7 (1.6%)	43 (1.9%)	0.75
	Chloramphenicol topical	103 (3.8%)	16 (3.6%)	87 (3.9%)	0.86
	Other topical	46 (1.7%)	10 (2.2%)	36 (1.6%)	0.46

Cases and controls were matched according to sex, date of birth, and ethnic origin.
Bold text indicates significant case-control differences at $\alpha < 0.05$.

had higher rates of obesity (10.0 vs. 5.9%; $p \leq 0.01$), epilepsy (2.2 vs. 0.6%; $p \leq 0.01$), and hypertension (5.3 vs. 3.2%; $p = 0.02$). Mothers of cases also had statistically significantly higher rates of certain chronic medication prescriptions, specifically, inhaled adrenergic (4.2% in ASD vs. 2.4% in non-ASD; $p = 0.04$) and anti-hyperglycemic (2% in ASD vs. 0.7% in non-ASD; $p \leq 0.01$) medications compared to mothers of controls.

Rates of antimicrobial prescriptions during the preconception and pregnancy periods are shown in **Table 2.** More than half of the women in the study (54.1%) were prescribed an antimicrobial treatment during the preconception or pregnancy periods. Mothers of children with ASD had lower rates of antimicrobial drug prescriptions prior to or during pregnancy than mothers of non-ASD children (49.0 vs. 55.1%, respectively; $p = 0.02$), with the largest difference between these groups seen in the third trimester (18.8 vs. 23.7%; $p = 0.03$). The most prevalent antimicrobial treatments were beta lactam drugs (penicillins and cephalosporins; $N = 983$), followed by antifungal medications ($N = 644$), with these two types of drugs accounting for over 75% of all administered antimicrobial agents. Notably, there was a marginally insignificant difference in the prescription of penicillins between the groups, with mothers of cases having lower rates of penicillin prescriptions during pregnancy than mothers of controls (15.7 vs. 19.7%; $p = 0.06$). In addition, mothers of cases had significantly higher rates of systemic antifungal drugs prescriptions than mothers of controls (1.3 vs. 0.4%; $p = 0.03$). There were no other significant differences in prescription rates of specific antimicrobial drugs between the study groups.

We then used multivariate conditional logistic regression models to assess the independent association of antimicrobial agents with ASD risk. Each such model included a particular antimicrobial drug together with demographic, socioeconomic, and clinical variables associated with ASD, as defined in **Table 1**. The results of these multivariate models are presented in **Table 3**. Overall, prescription of any antimicrobial treatment during pregnancy was associated with a protective effect on ASD risk (aOR = 0.75; 95%CI = 0.61–0.92). This association was driven mainly by drugs used in the third trimester of pregnancy (aOR = 0.73; 95%CI = 0.57–0.95) and by systemically administered medications (aOR = 0.79; 95%CI = 0.64–0.98). Notably, while penicillins and macrolides were significantly associated with a protective effect on the risk of ASD (aOR = 0.71; 95%CI = 0.54–0.94 and aOR = 0.34; 95%CI = 0.12–0.97, respectively), systemically administered antifungal medications during pregnancy were significantly associated with an increased risk of ASD in the offspring (aOR = 3.85; 95%CI = 1.34–11.01). However, the number of pregnant women treated with systemic antifungals in this sample was low ($N = 15$).

Finally, we performed a sensitivity analysis exploring the association between antimicrobial drug prescriptions during pregnancy and risk of ASD in the offspring of Jewish and Bedouin mothers, two ethnic groups that differ in their sociodemographic and clinical characteristics (**Supplementary Table 2**). The results of this analysis are presented in **Table 4**. Interestingly, a significant difference was seen in the risk of ASD associated with antimicrobial drug prescription during pregnancy in these two populations (Breslow-Day p-value < 0.01). Specifically, any antimicrobial drug prescription during pregnancy was associated with a reduced risk of ASD in the offspring of Jewish mothers (aOR = 0.61; 95%CI = 0.48–0.79) but not of Bedouin mothers (aOR = 1.18; 95%CI = 0.79–1.75). This ethnic difference in ASD risk was mainly driven by any systemic antimicrobial drugs ($aOR_{Jewish} = 0.62$; 95%CI = 0.47–0.81 and $aOR_{Bedouin} = 1.31$; 95%CI = 0.89–1.92, respectively) and primarily by penicillins ($aOR_{Jewish} = 0.55$; 95%CI = 0.38–0.78 and $aOR_{Bedouin} = 1.18$; 95%CI = 0.73–1.90, respectively).

TABLE 3 | Crude and adjusted ORs for ASD of different types of antimicrobial agents in a multivariate model including background characteristics.

Antimicrobial agent	Crude OR (95% CI)	aOR[#] (95% CI)
Any antimicrobial ($N = 1,463$)	0.79 (0.65–0.97)*	0.75 (0.61–0.92)**
3 months pre-conception ($N = 710$)	0.88 (0.7–1.12)	0.8 (0.63–1.02)
Trimester 1 ($N = 771$)	0.89 (0.71–1.12)	0.86 (0.68–1.09)
Trimester 2 ($N = 767$)	0.97 (0.77–1.22)	0.93 (0.74–1.18)
Trimester 3 ($N = 621$)	0.75 (0.58–0.97)*	0.73 (0.57–0.95)*
Any systemic antimicrobial ($n = 1,072$)	1.03 (0.78–1.37)	0.79 (0.64–0.98)*
Any mucosal antimicrobial ($n = 423$)	0.83 (0.65–1.06)	1.03 (0.78–1.38)
Any topical antimicrobial ($n = 674$)	0.85 (0.69–1.04)	0.8 (0.62–1.02)
Systemic Beta-lactam ($n = 983$)	0.86 (0.69–1.06)	0.8 (0.65–1.02)
Penicillin ($n = 514$)	0.78 (0.59–1.02)	0.71 (0.54–0.94)*
Cephalosporin ($n = 647$)	0.91 (0.72–1.16)	0.86 (0.67–1.10)
Macrolide ($N = 51$)	0.41 (0.15–1.16)	0.34 (0.12–0.97)*
Antifungal ($N = 644$)	0.95 (0.74–1.2)	0.93 (0.73–1.19)
Mucosal ($n = 398$)	1.04 (0.78–1.38)	1.03 (0.77–1.38)
Topical ($n = 450$)	0.83 (0.62–1.1)	0.8 (0.6–1.07)
Systemic ($n = 15$)	3.28 (1.17–9.23)	3.83 (1.34–11.01)*
Aminoglycoside topical ($n = 94$)	0.73 (0.4–1.35)	0.72 (0.39–1.34)
Antiviral topical ($n = 50$)	0.83 (0.37–1.85)	0.7 (0.31–1.59)
Chloramphenicol topical ($n = 103$)	0.93 (0.54–1.6)	0.98 (0.57–1.71)
Other topical ($n = 46$)	1.39 (0.69–2.8)	1.29 (0.63–2.68)

*Significant at $\alpha = 0.05$.

**Significant at $\alpha = 0.01$.

[#]Adjusted ORs to the following variables: age, low socioeconomic status, obesity, epilepsy, hypertension, anti-hyperglycemic medication, and inhaled adrenergic medication.

DISCUSSION

The results of this study suggest that the administration of antimicrobial drugs during gestation is associated with a reduced risk of ASD in the offspring of Jewish mothers living in southern Israel. Furthermore, we show that this association is driven primarily by the administration of antimicrobials, particularly penicillins, during the third trimester of pregnancy. To the best of our knowledge, no other study has documented such a trend. Other studies that explored the association between antimicrobial use during pregnancy and ASD risk reported a positive or null association between these variables (16, 21, 40–42). The discrepancy between these studies and our results of a lower risk of ASD associated with prenatal use of antimicrobials

TABLE 4 | Adjusted ORs for ASD of different types of antimicrobial agents in a multivariate model including background characteristics, stratified by ethnicity.

Antimicrobial agent	Jews aOR[#] (95% CI) (*N* = 1,914)	Bedouins aOR[#] (95% CI) (*N* = 792)	Breslow day *P*-value
Any antimicrobial (*N* = 1,463)	**0.61 (0.48–0.79)*** **	**1.18 (0.79–1.75)**	**<0.01**
3 months pre-conception (*N* = 710)	0.86 (0.64–1.14)	0.7 (0.43–1.12)	0.59
Trimester 1 (*N* = 771)	0.76 (0.57–1.01)	1.03 (0.67–1.58)	0.22
Trimester 2 ((*N* = 767)	0.87 (0.66–1.15)	1.08 (0.70–1.67)	0.17
Trimester 3 (*N* = 621)	0.66 (0.48–0.91)**	1 (0.63–1.59)	0.13
Any systemic antimicrobial (*n* = 1,072)	**0.62 (0.47–0.8)*** **	**1.31 (0.89–1.92)**	**<0.01**
Any mucosal antimicrobial (*n* = 423)	1 (0.72–1.40)	1.21 (0.68–2.13)	0.52
Any topical antimicrobial (*n* = 674)	0.77 (0.58–1.03)	0.78 (0.47–1.3)	0.79
Systemic Beta–lactam (*N* = 983)	**0.65 (0.49–0.85)****	**1.23 (0.83–1.81)**	**0.01**
Penicillin (*N* = 514)	0.55 (0.38–0.78)***	1.18 (0.73–1.90)	0.02
Cephalosporin ((*N* = 647)	0.75 (0.55–1.03)	1.05 (0.69–1.62)	0.20
Macrolide (*N* = 51)	0.2 (0.05–0.86)*	1.07 (0.23–5.06)	0.15
Antifungal (*N* = 644)	0.92 (0.69–1.22)	1.04 (0.63–1.71)	0.63
Mucosal (*N* = 398)	0.97 (0.69–1.37)	1.31 (0.74–2.32)	0.36
Topical (*N* = 450)	0.8 (0.58–1.12)	0.79 (0.42–1.47)	0.99
Systemic (*N* = 15)	4 (1.07–14.94)*	3.48 (0.56–21.47)	0.99
Aminoglycoside topical (*n* = 94)	0.54 (0.24–1.21)	1.25 (0.46–3.41)	0.23
Antiviral topical (*n* = 50)	0.59 (0.22–1.55)	0.49 (0.07–3.39)	0.75
Chloramphenicol topical (*n* = 103)	0.89 (0.46–1.74)	1.14 (0.39–3.32)	0.40
Other topical (*n* = 46)	1.7 (0.79–3.65)	#N/A	0.08

*Significant at $\alpha = 0.05$.
**Significant at $\alpha = 0.01$.
***Significant at $\alpha = 0.001$.
[#]Adjusted ORs to the following variables: age, low socioeconomic status, obesity, epilepsy, hypertension, anti-hyperglycemic medication, and inhaled adrenergic medication.

could have several biological and/or clinical explanations, as described below.

Several hypotheses have been proposed regarding the association between antimicrobial therapy during pregnancy and risk of ASD. One of the main hypotheses, which may be relevant to this study, is related to the effect of antimicrobial treatment on the composition of the gut microbiota of the mother and consequently on that of her fetus. Alterations in the gut microbial flora may alter the functioning of the gut–brain axis in the developing fetus and lead to abnormalities in brain developments associated with ASD (6, 24, 31, 35, 43). Generally, alterations in the gut microbiota have been shown to be associated with an increased risk of ASD (43–45). Nevertheless, it is also possible that antimicrobials may confer a protective effect (like that observed in our study) by reducing the effect of harmful microbial species in the gut of either the fetus or the mother. In addition, antimicrobial administration during pregnancy may weaken the activation of the maternal immune system, which has been suggested as a factor causing an elevated risk of ASD (7, 19, 20). Another possible explanation is that microbial treatment shortens the illness of the mother and, therefore, reduces the negative effects of maternal fever on the risk of ASD (13, 19, 22, 23). Lastly, antimicrobial therapy during pregnancy may convey a protective effect through the treatment of secondary "silent" [asymptomatic (46)] infections that may influence the neurodevelopment of the fetus. These theories are supported by the protective effect of systemically administered antimicrobials (i.e., penicillins and macrolides) that target a broad range of bacteria.

The observation that a protective effect of prenatal antimicrobial intake on ASD risk was shown only for the Jewish mothers in this study is most probably related to cultural and/or environmental differences between Jewish and Bedouin people living in Israel. In this context, the prescription of antimicrobials may be regarded as a proxy for the utilization of healthcare services. The differences in the protective effect of antimicrobial therapy on ASD risk between the Bedouin and Jewish populations in this study may simply be related to differences in the quantity and/or quality of prenatal medical care between these two ethnic groups (47, 48). This association was observed mainly in the third trimester, concurrent with the requirement for more frequent prenatal visits during this trimester. An additional factor that must be taken into consideration is the practice of prescribing antimicrobial therapy prior to confirmation of a viral or bacterial etiology of the patient's illness. This over-prescription of antimicrobials, often driven by patients themselves seeking prompt medical care, rather than adopting a wait-and-see approach, may confound the relationship between antimicrobial therapy and risk of ASD in those mothers who seek increased medical care, particularly since those mothers may also be more likely to engage in other protective behaviors that, in turn, would positively affect the

neurodevelopmental outcomes in their offspring. It is important to note that the protective effects, or associated protective effects, of antimicrobial use were not found in other studies, which may have studied different populations with similar utilizations of health services.

Notably, in contrast to all the other results in this study, systemic antifungal medications were associated with an increased risk of ASD. While this association may be due to the severity of the infection rather than to the iatrogenic effects of the prescribed antimicrobials (49, 50), this finding should be regarded with caution since only 15 women (0.6%) in our sample were prescribed this medication.

Among the strengths of this study is its design, namely, data was collected through the use of a clinical database rather than through retrospective questionnaires, thereby greatly increasing the internal validity of the data. In addition—as facilitated by the NARCI database—our sample size was relatively large compared to other studies on children with ASD, and we were able to include many known risk factors within the analyses. Lastly, in an attempt to mitigate potential limitations, clinical data on antimicrobial prescriptions were acquired from many different types of medical services via the SUMC database, including general practitioner visits, specialist clinics, emergency department visits, and hospitalization summaries.

Nevertheless, our findings should be considered in the context of the following study limitations. Although large, the sample size was not sufficiently large for analyses to include more than a few subcategories of antimicrobial agents. Secondly, the data about prenatal antimicrobial administration did not include information about the dosage, indicated illness for the prescription nor to the compliance with these prescriptions. Differences in prescription compliance between mothers of ASD and non-ASD offspring could have affected our results, but there are no indications for us to believe that such differences do indeed exist. Thirdly, we did not have data about the medical indications for the antimicrobial prescriptions, which limited further analyses on this equally important factor. Lastly, the study was conducted on data from mothers living in a specific geographical location and enrolled in a single HMO. Therefore, generalization of the study findings to other populations is limited. These limitations call for further research, which may help to better understand the association between antimicrobial use and ASD risk. Nonetheless, this study did reveal the important finding that prescription of antimicrobials, in the 3 months before conception and during pregnancy, do not convey any added risk of ASD in offspring.

CONCLUSIONS

The results of our study shed some light on the possible positive effects of shared factors associated with antimicrobial treatment, such as high utilization of healthcare services during pregnancy, on the risk of ASD in the offspring of the treated mothers. Follow-up studies are required to establish the true nature of this association, as it may have significant clinical and scientific implications.

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 protocol of this study was reviewed and approved by the Ethic Committee of Soroka University Medical Center. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

NA conducted the data analysis. NA and SS drafted the manuscript. GM, HF, AM, and ID assisted in data collection and interpretation. NA and IM conceptualized the manuscript. IM coordinated the work and supervised the data analysis. All authors are responsible for the reported research and have reviewed and approved the final manuscript as submitted.

FUNDING

This study was partially funded by a grant from the Israeli Science Foundation (Grant No. 527/15).

ACKNOWLEDGMENTS

We thank Mrs. Inez Mureinik for critical reviewing and editing of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.771232/full#supplementary-material

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frontiers in Psychiatry

ORIGINAL RESEARCH
published: 03 January 2022
doi: 10.3389/fpsyt.2021.823260

Adverse Childhood Experience Is Associated With Disrupted White Matter Integrity in Autism Spectrum Disorder: A Diffusion Tensor Imaging Study

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Autism, a section of the journal Frontiers in Psychiatry

Received: 27 November 2021
Accepted: 08 December 2021
Published: 03 January 2022

Citation:
Yoshikawa H, Kitamura S, Matsuoka K, Takahashi M, Ishida R, Kishimoto N, Yasuno F, Yasuda Y, Hashimoto R, Miyasaka T, Kichikawa K, Kishimoto T and Makinodan M (2022) Adverse Childhood Experience Is Associated With Disrupted White Matter Integrity in Autism Spectrum Disorder: A Diffusion Tensor Imaging Study. Front. Psychiatry 12:823260. doi: 10.3389/fpsyt.2021.823260

Individuals with autism spectrum disorder (ASD) have an increased risk of adverse childhood experiences (ACEs) than typically developed (TD) children. Since multiple lines of studies have suggested that ACEs are related to myelination in the frontal lobe, an exposure to ACEs can be associated with white matter microstructural disruption in the frontal lobe, which may be implicated in subsequential psychological deficits after the adulthood. In this study, we investigated the relationship between ACEs and microstructural integrity on frontal lobe-related white matter tracts using diffusion tensor imaging in 63 individuals with ASD and 38 TD participants. Using a tractography-based analysis, we delineated the uncinate fasciculus (UF), dorsal cingulum (Ci), and anterior thalamic radiation (ATR), which are involved in the neural pathology of ASD, and estimated each diffusion parameter. Compared to the TD participants, individuals with ASD displayed significantly lower fractional anisotropy (FA) and higher radial diffusivity (RD) in the left ATR. Then, ASD individuals exposed to severe ACEs displayed higher RD than those exposed to mild ACEs and TD participants in the left ATR. Moreover, the severity of ACEs, particularly neglect, correlated with lower FA and higher RD in the left UF and ATR in individuals with ASD, which was not observed in TD participants. These results suggest that an exposure to ACEs is associated with abnormality in the frontal lobe-related white matter in ASD.

Keywords: autism spectrum disorder, adverse childhood experiences, white matter, cingulum, uncinate fasciculus, anterior thalamic radiation

INTRODUCTION

Autism spectrum disorder (ASD) is a major neurodevelopmental disorder characterized by impaired social communication and restricted repetitive behaviors (1). These characteristics often make it difficult to establish appropriate interpersonal relationships in daily and social life. Individuals with ASD demonstrate atypical structural and functional brain patterns than their typically developed (TD) counterparts and altered network connectivity is involved in the core and other related symptoms of ASD (2). In particular, abnormalities of the neural pathways connected to the frontal lobe are reportedly associated with the pathophysiology of ASD (3–6). Diffusion tensor imaging (DTI) studies have reported on white matter microstructural alteration in the frontal lobe-related white matter bundles in ASD, such as the corpus callosum, uncinate fasciculus (UF), arcuate fasciculus, anterior thalamic radiation (ATR), and cingulum (Ci) (7, 8).

Poor social ability can be associated with an increased risk of exposure to adverse life experiences in ASD. Children with developmental disabilities, including ASD, are likely to experience maltreatment, bullying, and maladaptation in the local community and social life (9). Thus, these adverse childhood experiences (ACEs) reportedly cause poor self-esteem and motivation, thus resulting in subsequent psychiatric comorbidities, such as depression, anxiety, and substance abuse in adults with ASD (10).

Researchers have reported on the relationship between ACE exposure and white matter microstructural disruption. In animal studies, juvenile stress such as social isolation or traumatic stress induced hypomyelination in the prefrontal cortex of mice (11–13). In human studies, an exposure to ACEs influenced white matter microstructural abnormalities in the anterior cingulate cortex, ventromedial prefrontal cortex, corpus callosum, corona radiata, inferior longitudinal fasciculus, and inferior occipitofrontal fasciculus (7, 14–16). While an exposure to neglect in childhood is associated with deteriorated frontal white matter microstructure (6), parental verbal abuse is related to white matter microstructural abnormality of the left arcuate fasciculus (17). Thus, serious maltreatment in early life stages is associated with white matter microstructural abnormalities. However, the mechanism by which an exposure to ACEs influences abnormal white matter microstructure in individuals with ASD has not been completely elucidated.

The relationship between ACEs and white matter microstructural disruption is of clinical importance for considering the pathological basis of ASD. In this study, we compared the association between ACEs and white matter microstructural disruption in individuals with ASD and TD participants. We focused on the UF, Ci, and ATR, which are white matter tracts connected to the frontal lobe. The UF is a hook-shaped bundle of nerves that connects the prefrontal cortex to the medial temporal region (18, 19). It is involved in visual and emotional memory, processing, and decision making (19–21). Regarding the Ci, the dorsal Ci connecting the anterior to the posterior cingulate cortex was explored in this study because it has been involved in emotion and executive control, which was important for ASD characteristics (8, 22). The ATR connects the anterior thalamic nuclei to the prefrontal cortex and is involved in executive function, the planning of complex behaviors, and emotional regulation (23, 24). Previous reports demonstrating the relationship between ACEs and white matter microstructural disruption of these tracts led us to perform this study (8, 25). We hypothesized that individuals with ASD exposed to serious ACEs display more severe white matter microstructural disruption than those exposed to mild ACEs and TD participants.

METHODS

Participants

We enrolled 63 age- and intelligence quotient (IQ)-matched individuals with ASD and 38 TD participants. The full-scale IQ of the participants was estimated using similarities and symbol search subsets of the Wechsler Adult Intelligence Scale, third edition (26). To match the IQ between individuals with ASD and TD participants, those with IQ <80 and >120 were excluded from the study. Individuals with ASD were recruited from the outpatient service of the Department of Psychiatry, Nara Medical University Hospital and affiliated psychiatric clinics in Japan. They were diagnosed by two trained psychiatrists based on the criteria of the Diagnostic and Statistical Manual-5 and the Japanese version of the Autism Diagnostic Observation Schedule, second edition (27), and autistic traits were also examined by the Autism-Spectrum Quotient Japanese version (AQ-J) (28). Twenty individuals with ASD had neuropsychiatric comorbidities, including major depressive disorder ($n = 6$), attention deficit hyperactivity disorder ($n = 4$), adjustment disorder ($n = 4$), anxiety disorder ($n = 3$), avoidant personality disorder ($n = 2$), alcohol use disorder ($n = 1$), epilepsy ($n = 1$), schizophrenia ($n = 1$), bipolar disorder ($n = 1$), panic disorder ($n = 1$), obsessive compulsive disorder ($n = 1$), oppositional defiant disorder ($n = 1$), and learning deficits ($n = 1$). TD participants were recruited from the public offering of the students, hospital, and school staff of the Nara Medical University. They did not have a history of psychiatric, neurological, or developmental disorders, and were requested to complete the Mini-International Neuropsychiatric Interview to exclude their current or past psychiatric history. Moreover, we evaluated them using the AQ-J, and a score <32 was used as the enrollment requirement. Thirty-four individuals with ASD were prescribed the following psychotropic medications during their participation: antidepressants ($n = 21$), hypnotic agents ($n = 16$), antipsychotics ($n = 13$), anti-anxiety agents ($n = 12$), anti-epileptic agents ($n = 3$), atomoxetine ($n = 2$), and anti-manic agents ($n = 1$). None of the TD participants had a history of psychotropic medications. Structural abnormalities of the brain were excluded in both groups, as determined by T1-weighted magnetic resonance imaging (MRI). We assessed the severity of ACEs using the Japanese version of the Child Abuse and Trauma Scale (CATS) (29). The CATS is a 38-item instrument that retrospectively evaluates adverse childhood experiences (30). Each item is measured on a five-point scale ranging from 0 to 4 and is divided into five major factors of adverse childhood experiences as follows: neglect or negative

home atmosphere, sexual abuse, punishment, emotional abuse, and others. In addition, the sum of the scores provides the total score. There was no cut-off point on the scale, and we measured the median of the total CATS score in individuals with ASD. Considering the median score was 37, individuals with ASD and total CATS score ≥38 were defined as experiencing higher ACEs (ASD with high CATS). In contrast, those with scores ≤37 were defined as experiencing lower ACEs (ASD with low CATS) to explore the relationship between the severity of ACEs and microstructural white matter alteration. This study was approved by the Institutional Review Board of Nara Medical University, and all analyses were performed in accordance with relevant guidelines and regulations. Written informed consent was obtained from all individuals prior to their participation in the study.

MRI Data Acquisition

All participants underwent brain MRI using a 3-Tesla clinical scanner equipped with a 32 phased-array head coil (Magnetom Verio; Siemens, Erlangen, Germany). The participants were scanned with a three-dimensional T1-weighted gradient echo sequence (repetition time [TR] = 1,900 ms; echo time [TE] = 2.54 ms; field of view [FOV] = 256 × 256 mm; acquisition matrix = 256 × 256; and 208 contiguous axial slices of 1 mm thickness). We acquired DT images with an echo-planar imaging sequence using a GeneRalized Autocalibrating Partially Parallel Acquisition factor of two. The imaging parameters were as follows: TR = 14,100 ms, TE = 81 ms, FOV = 256 × 256 mm, acquisition matrix = 128 × 128, 79 contiguous axial slices of 2 mm thickness, b = 1,000 s/mm^2, and 30-axis encoding.

Image Processing

DTI was performed using the ExploreDTI software (https://www.exploredti.com/). It included corrections for head motion and eddy current-induced geometric distortions of raw diffusion-weighted data (31). We estimated the diffusion tensor using a non-linear least squares approach (32). We delineated the UF, Ci, and ATR using deterministic tractography. In tractography, the region of interest was set according to a previous study (**Figure 1**) (33). In each of the delineated fiber tracts, we calculated three diffusion parameters as follows: fractional anisotropy (FA), mean diffusivity (MD), and radial diffusivity (RD). To assess the head motion, we evaluated the root mean square (RMS) deviation of absolute intervolume displacement with respect to the b = 0 images from intra-participant registration parameters using the rmsdiff tool in FSL (34). The average displacement distance between each consecutive pair of 31 volumes was calculated for each participant.

Statistical Analyses

For continuous variables in the demographic data, group comparisons were performed using unpaired two-tailed *t*-tests and Mann–Whitney U tests for normally distributed and non-normally distributed data, respectively. For categorical variables, we performed Fisher's exact test to compare the groups. Group differences in the estimated diffusion parameters in the UF, Ci, and ATR were assessed using the analysis of covariance (ANCOVA) for years of education and the AQ-J as covariates between individuals with ASD and TD participants with Bonferroni correction to avoid type I errors due to multiplicity ($p < 0.0083$). Additionally, regarding the group differences for

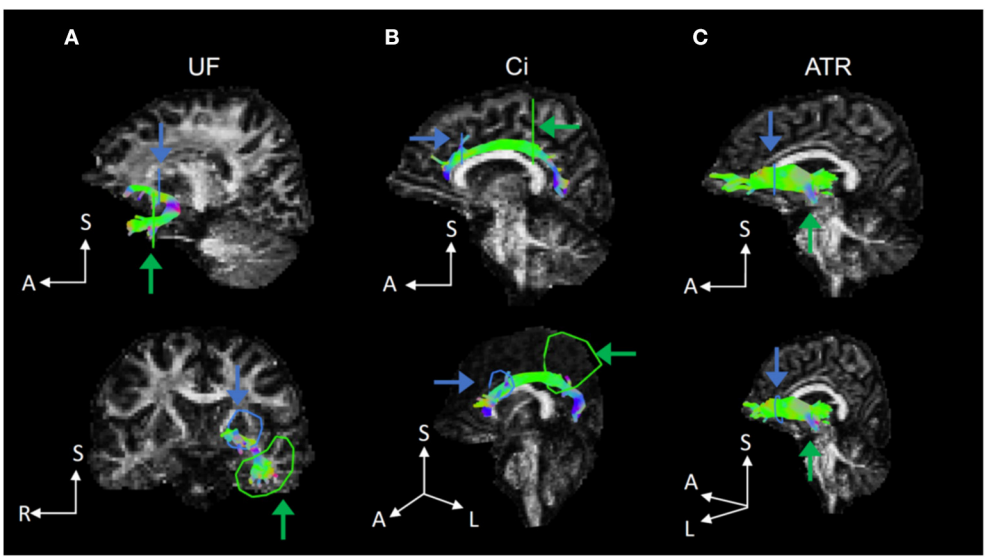

FIGURE 1 | Representative fiber tracts delineated by deterministic tractography for the UF, Ci, and ATR. **(A)** In the UF, we set a seed region of interest (ROI) in the white matter on a coronal plane at the tip of the inferior horn of the lateral ventricle and a target ROI in the white matter on a coronal plane at the tip of the frontal horn of the lateral ventricle. **(B)** In the Ci, we set a seed ROI in the white matter on a coronal plane on the top of the genu of the corpus callosum and a target ROI in the white matter on a coronal plane on the top of the splenium of the corpus callosum. **(C)** In the ATR, we set a seed ROI in the white matter on a coronal plane at the anterior limb of internal capsule and a target ROI in the white matter on a coronal plane at the anterior edge of pons. UF, uncinate fasciculus; Ci, cingulum; ATR, anterior thalamic radiation.

the comparison of ASD with low and high CATS score and TD participants, the estimated diffusion parameters in the UF, Ci, and ATR were assessed using the analysis of covariance (ANCOVA) for years of education and the AQ-J as covariates with Bonferroni correction to avoid type I errors due to multiplicity ($p < 0.0083$), additionally with post *hoc* Fisher's least significant difference test ($p < 0.05$) for exploring each of the group difference. To explore the relationships between the severity of ACEs and the extent of white matter microstructural alteration, we performed partial Spearman's rank correlation analyses. This helped us examine the relationships between CATS scores and estimated diffusion parameters in the UF, Ci, and ATR with years of education and the AQ-J as covariates in individuals with ASD and TD participants, respectively ($p < 0.05$, as significant). Normality assumptions for the statistical analysis were evaluated using the Shapiro–Wilk test for each dataset. The analyses were performed using SPSS version 27 (IBM Inc., Armonk, NY, USA).

RESULTS

Demographic Characteristics

Table 1 summarizes the demographic and clinical characteristics of the study participants. There were no significant differences in the age, IQ, sex, and handedness between the two groups ($p > 0.05$). In contrast, individuals with ASD displayed significantly lesser years of education and higher AQ-J scores than TD participants ($p < 0.05$). Individuals with ASD demonstrated significantly higher scores on each of the CATS scales than TD participants ($p < 0.05$). There were no significant differences in the age, IQ, years of education, sex, handedness, and AQ-J and ADOS-2 scores between those with ASD with high and low CATS scores (**Supplementary Table 1**). There was no significant difference in the extent of head motion based on the average RMS distance between individuals with ASD and TD participants (ASD: 1.4 ± 0.2 mm, TD: 1.4 ± 0.3 mm, U = 1,072, $p = 0.38$). Additionally, no significant difference of average RMS distance was shown between ASD individuals with low and high ASD (ASD individuals with low CATS: 1.4 ± 0.2 mm, TD: 1.4 ± 0.2 mm, U = 492, $p = 0.96$).

Group Comparisons of Each DTI Parameter of the Three Tracts Between Individuals With ASD and TD Participants

Table 2 outlines group comparisons of DTI parameters between individuals with ASD and TD participants. The ANCOVA revealed that the diagnosis exerted a statistically significant effect, such that individuals with ASD revealed lower FA ($F[2, 97] = 21.0$; $p < 0.001$) and higher RD ($F[2, 97] = 9.0$; $p = 0.003$) in the left ATR than TD participants. There were no significant differences in the DTI parameters in the UF and Ci between the groups ($p > 0.0083$).

TABLE 1 | Demographic characteristics of the study participants.

	ASD ($n = 63$)	TD ($n = 38$)	T or U or χ^2	*p*-value
Age (mean, SD)	27.3 (5.6)	27.8 (5.6)	1342.5	0.31
Duration of education (mean, SD)	15.0 (2.3)	16.2 (2.3)	1535	0.015*
IQ (mean, SD)	101.0 (12.0)	104.8 (9.6)	1438.5	0.09
Sex (male, %)	48 (76.2)	27 (71.1)	0.33	0.64
Handedness (right, %)	58 (92.1)	38 (100)	3.2	0.15
AQ-J (mean, SD)	31.4 (7.4)	18.4 (7.0)	253	<0.001*
ADOS-2 (mean, SD)	15.4 (3.1)	N/A	N/A	N/A
CATS (mean, SD)				
Total	44.0 (25.7)	20.8 (14.4)	455	< 0.001*
Punishment	10.1 (5.1)	7.8 (3.6)	860	0.018*
Sexual abuse	0.56 (1.3)	0.08 (0.4)	989	0.021*
Neglect	15.2 (9.7)	6.9 (6.8)	545	< 0.001*
Emotional abuse	11.0 (7.8)	3.8 (3.1)	459	< 0.001*
Others	7.1 (6.0)	2.2 (3.4)	463	< 0.001*

*ASD, autism spectrum disorder; TD, typically developed; IQ, Intelligence quotient; AQ-J, Autism-Spectrum Quotient - Japanese version; ADOS-2, Autism Diagnostic Observation Schedule second edition; CATS, Child Abuse and Trauma Scale; SD, standard deviation. *p < 0.083.*

Group Comparisons of Each DTI Parameter of the Three Tracts Among Individuals With ASD With High and Low CATS Scores and TD Participants

Figure 2 and **Supplementary Table 2** summarize the group comparisons of each DTI parameter among individuals with ASD having high and low CATS scores and TD participants. The ANCOVA revealed that the diagnosis exerted a statistically significant effect, such that individuals with ASD revealed lower FA ($p < 0.001$) and higher RD in the left ATR ($p = 0.002$). *Post hoc* comparisons revealed that those with ASD with high ($p < 0.001$) and low ($p < 0.001$) CATS scores demonstrated significantly lower FA in the left ATR than TD participants. In contrast, there was no significant difference in FA in the left ATR between individuals with ASD with high and low CATS scores ($p = 0.12$). Moreover, those with ASD and high CATS scores demonstrated significantly higher RD in the left ATR than those with low CATS scores ($p = 0.04$) and TD participants ($p < 0.001$). In addition, individuals with ASD and low CATS scores demonstrated significantly higher RD than TD participants ($p = 0.044$). There were no significant differences in MD in the left ATR among the groups ($p > 0.05$). Furthermore, we did not observe significant differences in the diffusion parameters in the right ATR ($p > 0.05$). Then, there were no significant diagnostic effect on the UF and Ci among the three groups ($p > 0.0083$).

Significant Association Between the Severity of CATS Scores in ASD and Diffusion Parameters in the UF and ATR

Figure 3 outline Spearman's partial correlation analyses between the CATS total scores and subscale scores in individuals with

TABLE 2 | Group comparisons of each diffusion parameter in individuals with ASD and TD participants.

		ASD	TD	*F*	*p*-value
UF	Right FA	0.410 (0.019)	0.420 (0.022)	2.2	0.14
	Left FA	0.409 (0.020)	0.417 (0.019)	0.64	0.43
	Right MD	0.720 (0.028)	0.725 (0.027)	0.27	0.61
	Left MD	0.744 (0.026)	0.752 (0.023)	0	0.99
	Right RD	0.547 (0.024)	0.546 (0.027)	1.1	0.29
	Left RD	0.565 (0.023)	0.566 (0.023)	0.14	0.71
Ci	Right FA	0.511 (0.035)	0.531 (0.029)	0.62	0.43
	Left FA	0.458 (0.043)	0.483 (0.036)	5.1	0.026
	Right MD	0.685 (0.031)	0.698 (0.022)	0.13	0.72
	Left MD	0.675 (0.030)	0.683 (0.026)	0.95	0.33
	Right RD	0.467 (0.031)	0.463 (0.029)	0.079	0.78
	Left RD	0.489 (0.029)	0.483 (0.030)	4.9	0.03
ATR	Right FA	0.417 (0.025)	0.423 (0.020)	0.046	0.83
	Left FA	0.418 (0.018)	0.441 (0.020)	21.0	<0.001*
	Right MD	0.688 (0.023)	0.689 (0.024)	1.2	0.27
	Left MD	0.688 (0.031)	0.689 (0.024)	0.13	0.72
	Right RD	0.521 (0.023)	0.519 (0.023)	0.75	0.39
	Left RD	0.521 (0.019)	0.514 (0.021)	9.0	0.003*

*ASD, autism spectrum disorder; TD, typically developed; UF, uncinate fasciculus; Ci, Cingulum; ATR, anterior thalamic radiation; FA, fractional anisotropy; MD, mean diffusivity; RD, radial diffusivity. *p < 0.05.*

ASD and TD participants. The CATS total score was negatively correlated with FA in the left UF ($p = 0.043$) and positively correlated with RD in the left ATR ($p = 0.034$) in individuals with ASD. There were no significant correlations between the CATS total score and each diffusion parameter in the UF and ATR in TD participants ($p > 0.05$). While the neglect subscale score was negatively correlated with FA in the left UF ($p = 0.02$) and ATR ($p = 0.023$), it was positively correlated with RD in the left ATR ($p = 0.037$) in individuals with ASD. Moreover, the emotional abuse subscale score was positively correlated with RD in the left ATR in individuals with ASD ($p = 0.044$). There were no significant correlations between each of the CATS subscale scores and each diffusion parameter in the UF and ATR in TD participants ($p > 0.05$).

DISCUSSION

In the present study, individuals with ASD demonstrated significantly lower FA and higher RD in the left ATR than TD participants, consistent with the findings of previous studies (8, 35, 36). In consideration of ACEs, those with ASD and high CATS scores revealed significantly lower FA and higher RD in the left ATR than TD participants. Moreover, they demonstrated significantly higher RD in the left ATR than those with low CATS scores. ACE severity was correlated with white matter microstructural alterations in the left UF and ATR, of which neglect and demonstrated a significant correlation with the left UF and ATR, in addition emotional abuse indicated a significant correlation with the left ATR. In other words, neglect and emotional abuse were clinically important for white matter development in ASD.

In the DTI parameter, FA reflects less restricted water diffusion of the white matter tract, and reduced FA is associated with disrupted fiber tracts (37). RD reportedly reflects diffusivity perpendicular to axonal fibers, and increased RD is associated with the disruption of the myelin sheath (38–40). Furthermore, decreased FA and increased RD can be considered to reflect the disruption of compacted myelin sheath structure of neural fibers (38, 41). Our findings suggested that individuals with ASD displayed pronounced disruption of compacted myeline sheath in the frontal-related fiber tracts than TD participants, consistent with the findings of previous reports (42). Moreover, ACEs were related to the severity of disrupted myeline sheath in individuals with ASD but not in TD participants. A previous animal study reported that Fmr1 knockout mice, as a possible model of ASD, exhibited excessive sensitivity to environmental changes and synaptic connectivity (43). Therefore, individuals with ASD may be more vulnerable to ACE exposure, in relation to white matter deficits than TD participants.

Individuals with ASD exposed to both severe and mild ACE demonstrated more white matter microstructural disruption than TD participants in the left ATR. In other words, those with ASD fundamentally demonstrated white matter disruption regardless of exposure to ACEs. Moreover, individuals with ASD and exposed to severe ACEs demonstrated worse white matter microstructural abnormality than those exposed to mild ACEs in the left ATR. Therefore, the ATR may be susceptible to ACE exposure, and its effect can be

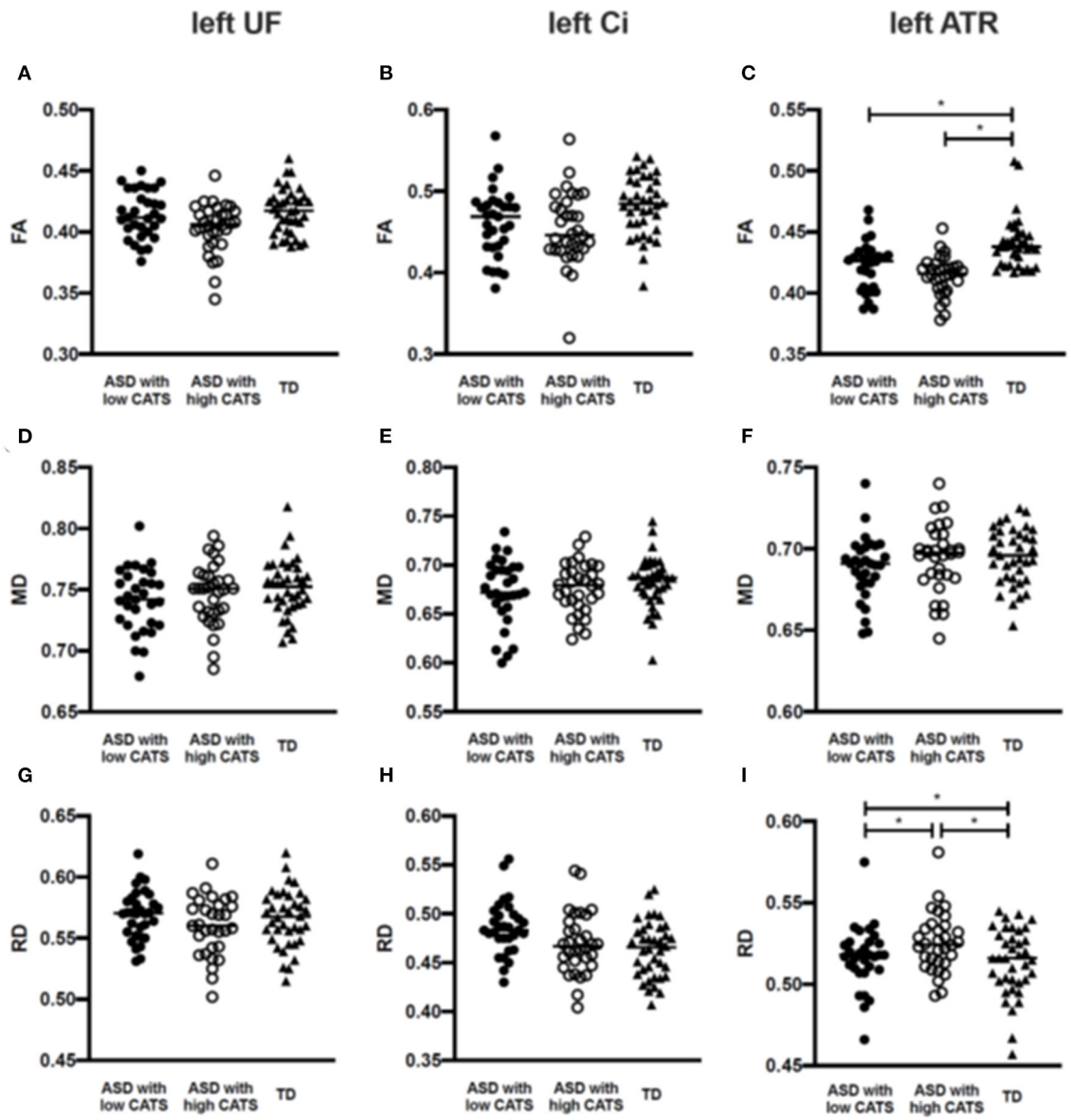

FIGURE 2 | Group comparisons of the estimated diffusion parameters among individuals with ASD with low and high CATS scores and TD participants. **(A–C)** Individuals with ASD with low and high CATS scores display significantly lower FA than TD participants. No significant differences are shown between individuals with ASD with low and high CATS scores. **(D–F)** There are no significant differences of MD among individuals with ASD and TD participants. **(G–I)** Individuals with ASD with low and high CATS scores display significantly higher RD than TD participants. Those with ASD and high CATS scores also display higher RD than those with low CATS scores. *$p<0.05$. ASD, autism spectrum disorder; TD, typically developed; CATS, Child Abuse and Trauma Scale; FA, fractional anisotropy; RD, radial diffusivity; ATR, anterior thalamic radiation.

associated with white matter microstructural disruption in ASD. Individuals with ASD and exposed to severe ACEs may be at an increased risk of psychiatric comorbidities, such as depression, anxiety disorder, and posttraumatic stress disorder (3, 10, 44). The ATR dysconnectivity, likely representing cortico-thalamic network dysfunction, can associate with cognitive dysfunction and emotional dysregulation, thereby resulting in psychological symptoms in adults with ASD.

An exposure to neglect in early life stages is associated with white matter abnormality in the prefrontal region (8), and interestingly, it is of note that mouse models of early life neglect show hypomyelination in the prefrontal cortex (11, 12). Our findings demonstrated significant correlations between the severity of ACEs, particularly neglect, and deteriorated DTI parameters in the left UF and ATR. Similar to the ATR, the UF is reportedly associated with psychiatric disorders (6, 45). An exposure to neglect in childhood has been associated with

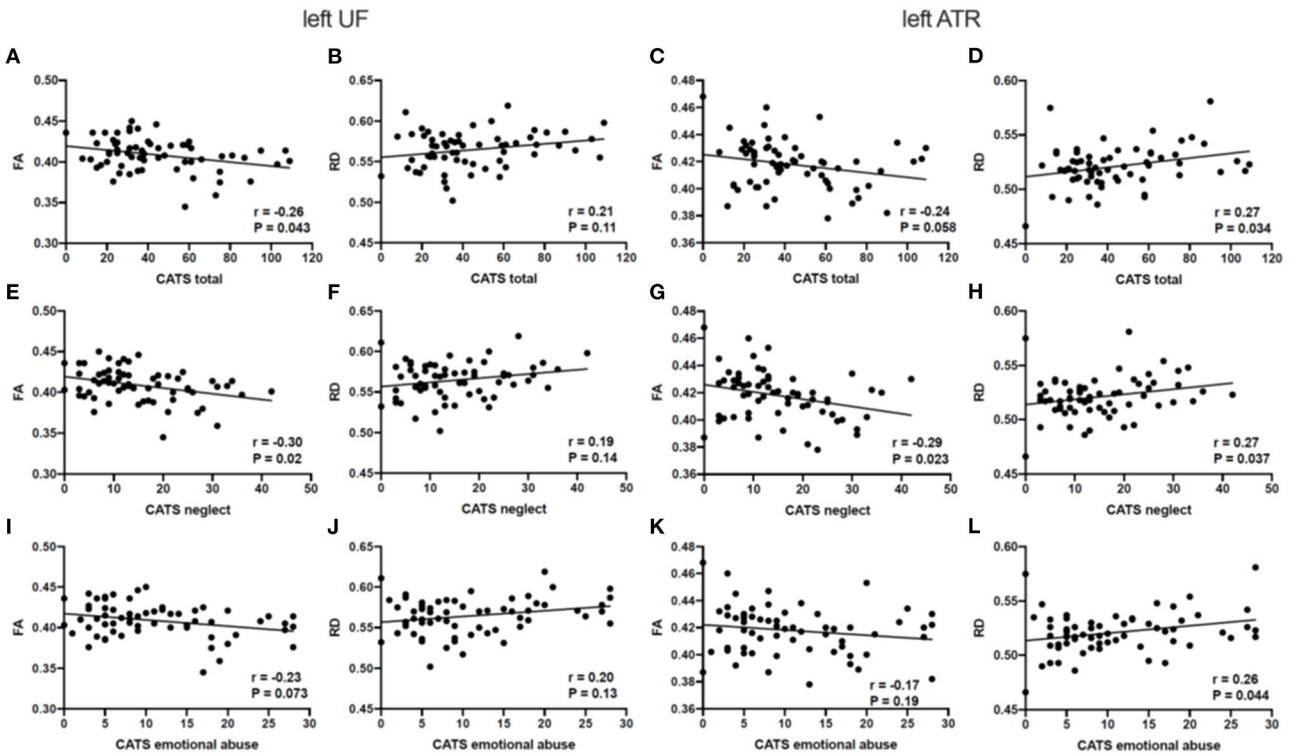

FIGURE 3 | Relationships between the estimated diffusion parameters and each CATS score in ASD. **(A–D)** RD in the left ATR is significantly correlated with the total CATS score in ASD. **(E–H)** FA in the left UF and ATR, and RD in the left ATR display significant correlations with the neglect CATS subscale scores in ASD. **(I–L)** RD in the left ATR is significantly correlated with the emotional abuse CATS subscale scores in ASD. $p < 0.05$ as statistically significant. CATS, Child Abuse and Trauma Scale; FA, fractional anisotropy; RD, radial diffusivity; UF, uncinate fasciculus; ATR. anterior thalamic radiation.

psychiatric comorbidities in adulthood. Then, previous reports demonstrated that an exposure to emotional abuse was also involved in white matter abnormality (46, 47), which were consistent with our results. Our findings suggested that the aforementioned exposure was associated with white matter microstructural abnormalities in the UF and ATR, which may be involved in emotional dysregulation and irregular decision-making and the subsequent appearance of psychological symptoms in ASD. Nonetheless, there were no significant correlations between the severity of ACEs and each DTI parameter in TD participants. Therefore, the susceptibility to ACEs differs between individuals with ASD and their TD counterparts. Moreover, individuals with ASD may present with affected frontal lobe-related white matter on exposure to ACEs.

Our samples demonstrated the laterality of abnormal white matter microstructure and its association with the severity of ACEs in ASD. This laterality has been previously reported in studies on ASD and was consistent with our results (16). A previous study reported that white matter microstructures were dominantly impaired on the left side in the UF and ATR in individuals with ASD exposed to ACEs (42).

This study had several limitations. First, since our work was a cross-sectional study, causal relationships between an exposure to ACEs and white matter microstructural abnormality have not been fully elucidated. Second, the CATS is a self-assessment questionnaire for adverse life events; thus, it might have introduced recall bias in the participants, thereby influencing our findings. Considering the CATS was validated in a previous study, we compared the severity of ACEs among our samples (48). Third, we could not deny the possibility that abnormalities in networks other than the UF, Ci, and ATR were related to ACE exposure. Additionally, some participants with ASD in this study had psychiatric comorbidities, which were related to white matter abnormalities. Future studies are warranted to address these issues.

In conclusion, an exposure to ACEs is more likely to be associated with white matter microstructural disruption in the frontal lobe-related white matter tracts in individuals with ASD than TD participants. Of the ACE types, neglect can be of critical importance for white matter disruption in ASD. Our findings suggested the importance of a comprehensive growth environment based on the consideration of ASD characteristics, which may assist in appropriate neuronal development in ASD patients.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Nara Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HY, SK, and MM wrote the manuscript and undertook the analysis and interpretation of imaging data. MM contributed to the conception, design of this research, and interpretation of the results. KM undertook the analysis and contributed to the interpretation of the results. MT, FY, and TK contributed to the interpretation of the results. TM and KK contributed to the acquisition of MRI data. RI and NK performed psychological evaluation. YY and RH supervised the ADOS-2. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Japanese Society for the Promotion of Science KAKENHI (Grant Numbers: 19K17116 to SK; 16H06403, 16H06400, 16H02666, and 16H05377 to MM), PRIME, AMED under Grant Number JP21gm6310015, AMED under Grant Number 21wm04250XXs0101, and AMED under Grant Number 21uk1024002s0201.

ACKNOWLEDGMENTS

We are grateful for the cooperation and patience of the patients who made this study possible.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.823260/full#supplementary-material

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

frontiers in Psychiatry

ORIGINAL RESEARCH
published: 03 February 2022
doi: 10.3389/fpsyt.2022.821354

Exposure to $GABA_A$ Receptor Antagonist Picrotoxin in Pregnant Mice Causes Autism-Like Behaviors and Aberrant Gene Expression in Offspring

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OPEN ACCESS

Edited by:
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Reviewed by:
Yukio Ago, Hiroshima University, Japan
Shiro Tochitani, Suzuka University of Medical Science, Japan

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Specialty section:
This article was submitted to Autism, a section of the journal Frontiers in Psychiatry

Received: *24 November 2021*
Accepted: *11 January 2022*
Published: *03 February 2022*

Citation:
Kotajima-Murakami H, Hagihara H, Sato A, Hagino Y, Tanaka M, Katoh Y, Nishito Y, Takamatsu Y, Uchino S, Miyakawa T and Ikeda K (2022) Exposure to $GABA_A$ Receptor Antagonist Picrotoxin in Pregnant Mice Causes Autism-Like Behaviors and Aberrant Gene Expression in Offspring. Front. Psychiatry 13:821354. doi: 10.3389/fpsyt.2022.821354

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that is characterized by impairments in social interaction and restricted/repetitive behaviors. The neurotransmitter γ-aminobutyric acid (GABA) through $GABA_A$ receptor signaling in the immature brain plays a key role in the development of neuronal circuits. Excitatory/inhibitory imbalance in the mature brain has been investigated as a pathophysiological mechanism of ASD. However, whether and how disturbances of GABA signaling in embryos that are caused by $GABA_A$ receptor inhibitors cause ASD-like pathophysiology are poorly understood. The present study examined whether exposure to the $GABA_A$ receptor antagonist picrotoxin causes ASD-like pathophysiology in offspring by conducting behavioral tests from the juvenile period to adulthood and performing gene expression analyses in mature mouse brains. Here, we found that male mice that were prenatally exposed to picrotoxin exhibited a reduction of active interaction time in the social interaction test in both adolescence and adulthood. The gene expression analyses showed that picrotoxin-exposed male mice exhibited a significant increase in the gene expression of odorant receptors. Weighted gene co-expression network analysis showed a strong correlation between social interaction and enrichment of the "odorant binding" pathway gene module. Our findings suggest that exposure to a $GABA_A$ receptor inhibitor during the embryonic period induces ASD-like behavior, and impairments in odorant function may contribute to social deficits in offspring.

Keywords: autism spectrum disorder, picrotoxin, $GABA_A$ receptor, social interaction, odorant binding, gene expression, microarray, WGCNA

INTRODUCTION

Autism spectrum disorder (ASD) is categorized as a neurodevelopmental disorder in the *Diagnostic and Statistical Manual of Mental Disorders*, 5th edition (1). Although ASD has several peripheral symptoms (e.g., aberrant sensitization and clumsiness of movement), characteristics of ASD are divided into two main categories: impairments in social interaction and communication and restricted and repetitive patterns of behaviors and interests (1). Symptoms of ASD are usually diagnosed during early childhood and remain during an individual's life. The ratio of the prevalence of ASD in males and females is ∼4:1 (2). Genetic and environmental causes of ASD have been investigated (3, 4), but the pathophysiology of ASD has not yet been thoroughly defined.

γ-Aminobutyric acid (GABA) is an inhibitory neurotransmitter in the mature brain that hyperpolarizes a membrane through the influx of chloride ions *via* $GABA_A$ receptor channels (5). $GABA_A$ receptor activation induces depolarizing membrane responses in the immature central nervous system (CNS), and GABA is important in the development of neuronal circuits, neurogenesis, and synapse formation (5, 6). Secreted GABA increased cell proliferation in the ventricular zone through $GABA_A$ receptor activation in mouse fetuses (7). Spontaneous Ca^{2+} oscillations, which are required for normal neuronal migration, are blocked or their frequency is reduced by the $GABA_A$ receptor blocker bicuculine in the cerebral cortex in newborn rats (8). Neuroblast migration in the hippocampus is impaired by treatment with antagonists of $GABA_A$ receptors and *N*-methyl-D-aspartate (NMDA) receptors, and $GABA_A$ receptor antagonism is more efficient than NMDA receptor antagonism in reducing cell migration (9). Previous studies showed that perinatal and postnatal $GABA_A$ receptor antagonist treatment led to aberrant behaviors in males. Bicuculine treatment during the neonatal period causes aberrant anxiety-like behavior in mature male mice and rats but not in females (10, 11). Male rats that were exposed to picrotoxin as embryos exhibited aberrant heterotypical sexual behaviors compared with control rats (12–14). These findings suggest that GABA signaling through $GABA_A$ receptors plays a key role in development of the immature CNS, and the inhibition of GABA signaling during developmental periods causes abnormal behaviors in male offspring.

Tochitani et al. recently reported that prenatal treatment with $GABA_A$ receptor agonists or antagonists altered social behaviors and locomotor activity in male offspring (15). They also reported that picrotoxin treatment from embryonic day 10–12 caused a rapid loss of interest in a familiar mouse, decreased locomotor activity, and decreased rearing (15). This study showed that disturbances of $GABA_A$ receptor signaling by picrotoxin administration during the embryonic period caused pathophysiological neurodevelopmental abnormalities, including ASD-like symptoms (15). However, unclear are whether and how picrotoxin affects body maturation and behaviors from adolescence to adulthood and gene expression in the mouse brain and whether there are correlations between such behavioral alterations and gene expression.

The present study investigated the effects of prenatal exposure to the $GABA_A$ receptor antagonist picrotoxin on body maturation and performance in several behavioral tests, including motor function, social interaction, pain responsiveness, self-grooming, and anxiety-like behavior. We then performed a comprehensive gene expression analysis using microarrays in the whole brain to explore the effects of picrotoxin on ASD-like pathophysiology. We also compared gene expression in the whole brain between picrotoxin-exposed mice and VPA-exposed mice (i.e., an established animal model of ASD). We analyzed data using BaseSpace and weighted gene co-expression network analysis (WGCNA). Here, we present evidence that offspring that are exposed to picrotoxin during the embryonic period exhibit impairments in social interaction in both adolescence and adulthood and that performance in the social interaction is strongly correlated with the odorant pathway in the WGCNA. Our results support the hypothesis that disturbances of $GABA_A$ receptor signaling during the embryonic period contributes to the pathophysiology of ASD.

MATERIALS AND METHODS

Mice and Picrotoxin Administration

Pregnant C57BL/6J mice were purchased from CLEA (Tokyo, Japan) on gestation day 6 and housed individually. All of the mice were housed on a 12/12 h light/dark cycle (lights on 8:00 a.m. to 8:00 p.m.) and had *ad libitum* access to food and water. Temperature was maintained at 23.0 ± 1.0°C. Picrotoxin was dissolved in saline. Pregnant female mice received a single intraperitoneal injection of 5 mg/kg picrotoxin (Sigma-Aldrich, St. Louis, MO, USA) on gestation day 12.5. The dose of picrotoxin was based on a previous study that reported that the 5.0 mg/kg dose twice daily did not cause malformation or infant death in offspring (16). We also tested 2.5 and 5.0 mg/kg doses in pregnant female mice and observed impairments in social interaction in both adolescence and adulthood only in offspring that were exposed to the 5.0 mg/kg dose (**Supplementary Figure 1**). Thus, in the present study, we administered 5.0 mg/kg picrotoxin in pregnant mice. Picrotoxin was injected only once on embryonic day 12.5 to avoid possible negative effects of repeated administration in pregnant mice and to avoid the possibility of causing a cleft palate in offspring (17). We chose embryonic day 12.5 for administration to compare gene expression between picrotoxin-exposed mice and mice that were prenatally exposed to VPA on embryonic day 12.5 (i.e., an established animal model of ASD) (18). Control pregnant mice were injected with saline. The volume of injection was 10 ml/kg. All pregnant mice were returned to their home cages immediately after the injection, and their conditions were observed carefully. Eleven pregnant mice were used in this experiment. Five pregnant mice were used as the control group. Six pregnant mice were used as the picrotoxin group. Five minutes after the picrotoxin injection, the movements of pregnant mice were slow, and they mostly lied down on the floor. After ∼60 min, the state of pregnant mice became normal (i.e., washing their faces and walking in the cage as usual). On average, 6–10 pups were obtained from picrotoxin- and saline-treated pregnant female mice. As in our

previous study (18), the pups were culled to eight animals per litter on postnatal day 4 (P4). For saline-exposed pups, for litters with <8 pups, pups were transferred from litters that had more than nine on P4. We did not observe postnatal mortality, malformation, or stunted pups. The day of birth was defined as day 0. The pups were weaned on P25, and mice of either sex were housed separately. Three to five offspring were housed in one home cage. All of the animal experiments were performed in accordance with the Guidelines for the Care of Laboratory Animals of the Tokyo Metropolitan Institute of Medical Science, and the housing conditions were approved by the Institutional Animal Care and Use Committee (approval no. 12-43).

Postnatal Body Maturation and Behavioral Analyses

The body weights and eye opening of the mice were monitored to assess postnatal body maturation. Body weight was recorded on P7, P9, P11, P14, P21, and P25. Eye opening was observed once daily from P12 to P18. The eye-opening score was the following: 0 = both eyes closed, 1 = one eye open, and 2 = both eyes open. All of the behavioral tests were performed from 9:00 a.m. to 6:00 p.m. The mice were given 60 min to habituate to the behavioral test room before the start of each test. Motor function during from P7 to P25 was assessed by the negative-geotaxis, righting reflex, cliff avoidance, and hanging wire tests. The social interaction test was conducted in both adolescence (5–6 weeks of age) and adulthood (10–11 weeks of age). The mice underwent the hot plate test (6–7 weeks of age), grooming test (7–8 weeks of age), open field test (8–9 weeks of age), and elevated plus maze test (9–10 weeks of age).

Negative Geotaxis Test

Negative geotaxis was tested on P7, P9, and P11. Each mouse was placed on a board that was tilted at 40°, facing downward. We assessed the latency to turn 180° (i.e., the tip of the nose faced upward). The cutoff time was a maximum of 20 s.

Righting Reflex Test

The righting reflex was tested on P7, P9, and P11. Each mouse was placed in the supine position, and the latency to return to the prone position was assessed. The cutoff time was a maximum of 15 s.

Cliff Avoidance Test

Cliff avoidance was tested on P7, P9, and P11. Each mouse was set on a desk at a height of 1 m, with its nose positioned outward at the edge of the desk. The latency to avoid the cliff was assessed. The cutoff time was a maximum of 20 s.

Hanging Wire Test

The hanging wire test (O'Hara & Co., Tokyo, Japan) was conducted on P25. The mice were placed on a grid wire surface (150 × 150 mm, divided into 10 mm grid squares), and the plane was inverted. The latency to fall was recorded. The cutoff time was a maximum of 600 s.

Social Interaction Test

The social interaction test was conducted during both adolescence (5–6 weeks of age) and adulthood (10–11 weeks of age) as previously described (18, 19). For habituation, each mouse was left alone in its home cage in a sound-attenuating chamber for 15 min. One unfamiliar C57BL/6J mouse of the same sex and age was then introduced to the cage. The behavior of the test mouse was video-recorded for 10 min and blindly scored for active social interaction, consisting of sniffing, allo-grooming, mounting, and following. One mouse that went out of its home cage during the 15 min habituation period was excluded from the analysis. Body weight was also recorded when each mouse performed the social interaction test. The number of mice per group was the following: 5–6 weeks of age (n = 18 control male mice, n = 26 picrotoxin-exposed male mice, n = 22 control female mice, n = 22 picrotoxin-exposed female mice) and 10–11 weeks of age (n = 18 control male mice, n = 26 picrotoxin-exposed male mice, n = 22 control female mice, n = 21 picrotoxin-exposed female mice).

Hot Plate Test

The hot plate test (Muromachi, Tokyo, Japan) was conducted at 6–7 weeks of age. Each mouse was set on a hot plate (55.0 ± 0.5°C), and the latency to flicking, jumping, and licking its paws was recorded.

Grooming Test

The grooming test was conducted at 7–8 weeks of age and consisted of a 2-day sequence. The first day was the habituation phase and the tested mice were placed in the experimental room for 60 min in their home cage. After 60 min, the mice were placed in a sound-attenuating chamber, and their movements were recorded for 30 min. In this phase, the mice were habituated to the experimental room and apparatus. The second day of the grooming test was the recording day. As on the first day, the mice were habituated to the experimental room, and their movements were recorded in a sound-attenuating chamber for 30 min. Grooming involved wiping the face, nose, ears, and head with forepaws and licking the body other than the face. We counted the number and seconds of grooming for 30 min.

Open Field Test

The open field test was conducted at 8–9 weeks of age. The apparatus (Muromachi) consisted of an open field (500 × 500 × 500 mm). Each mouse was placed in the center of the open field and allowed to explore it for 20 min under dim light. Behaviors were automatically recorded by a video tracking system (Muromachi).

Elevated Plus Maze Test

The elevated plus maze test (Muromachi) was conducted at 9–10 weeks of age. The apparatus consisted of two closed arms (300 × 60 mm, with 150-mm-high walls) and two open arms (297 × 54 mm). The apparatus was raised 40 cm above the floor. A video tracking system (Muromachi) automatically recorded behaviors.

RNA Extraction From Whole Brains

We conducted brain collection and RNA extraction according to a previous study (18). After the end of the social interaction test (10–11 weeks of age), whole brains were collected. We examined the whole brain in the present study because the precise brain regions that are associated with ASD have not yet been

clearly defined. Total RNA was extracted from the whole brain and homogenized in Ambion TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) using a homogenizer. RNA was isolated using chloroform and precipitated using isopropyl alcohol. The quality of RNA was assessed with Nanodrop 1000 (Thermo Fisher Scientific). All of the RNA samples had an $A_{260/280}$ ratio between 2.01 and 2.02 and $A_{230/260}$ ratio between 2.26 and 2.31.

Analyses of Whole-Genome Gene Expression

We conducted whole-genome gene expression using a microarray analysis according to a previous study (18). cRNA targets were synthesized and hybridized using the Whole Mouse Genome Microarray according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA). The array slides were scanned using a SureScan Microarray Scanner (Agilent Technologies, Santa Clara, CA, USA). Before analyzing gene expression, microarray data were normalized and sorted using GeneSpring 14.5 software (Agilent Technologies, Santa Clara, CA, USA). Each sample was normalized by a 75% percentile shift. Compromised probes were removed. Each group comparison was performed using *t*-tests ($p < 0.05$). Each group consisted of five male mice (control mice and picrotoxin-exposed mice). Gene ontology, pathway enrichment analysis, and comparisons with curated studies were conducted using BaseSpace (Illumine, San Diego, CA, USA; https://login.illumina.com/platform-services-manager/?rURL=https://accounts.public.basespace.illumina.com/b/authentication/login.nb;jsessionid=69E78F49406EDD33C6DE7D3C1A2F8FD8&clientId=NBR-Public#/) (20).

Weighted Gene Co-expression Network Analysis

The WGCNA was performed using the WGCNA package in R software and conducted for step-by-step block-wise network construction and module detection using the package implemented in R version 3.5.2 with the code provided by Langfelder and Horvath (21, 22). To focus on differentially expressed genes, genes with $p < 0.05$ were used for the WGCNA. In accordance with WGCNA default preprocessing steps, any obvious outliers in our sample were checked with an average linkage hierarchical cluster analysis of expression levels. Pearson correlation coefficients for all transcript pairs were then calculated to determine connection strengths between two transcripts. The connection strength between transcript m and transcript n was defined as $\alpha_{mn} = [\text{correlation (m, n)}]$, where the β value is set as the weighting coefficient. Power of beta = 30 was chosen based on the scale-free topology criterion (the linear regression model fitting index, R^2, was ∼0.9).

Statistical Analysis

The results of the behavioral tests were analyzed using Prism 9.2.0 software (GraphPad, San Diego, CA, USA). The data were analyzed using unpaired *t*-tests, the Mann-Whitney *U*-test, and two-way repeated-measures analysis of variance (ANOVA) followed by the Bonferroni *post-hoc* test. All of the data are presented as mean ± standard error of the mean (SEM). Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effects of Prenatal Exposure to Picrotoxin on Postnatal Development and Motor Function

No significant difference in body weight was found between control and picrotoxin-exposed male mice from P7 to P25 (**Figure 1A**). The two-way repeated-measures ANOVA showed no significant main effect of picrotoxin treatment [$F_{(1,42)} = 0.281$, $p = 0.599$; $n = 18$ control mice, $n = 26$ picrotoxin-exposed mice] and no picrotoxin treatment × day of testing interaction [$F_{(5,210)} = 1.035$, $p = 0.398$] but a significant effect of day of testing [$F_{(1.916,80.46)} = 2{,}034$, $p < 0.0001$]. Eye-opening scores in picrotoxin-exposed male mice tended to be low compared with control male mice on P13 ($U = 106.500$, $p = 0.059$; $n = 18$ control mice, $n = 22$ picrotoxin-exposed mice; **Figure 1B**), with no significant difference between control and picrotoxin-exposed mice on P12 or P14-18 ($p > 0.9999$). In the negative-geotaxis test, the two-way repeated-measures ANOVA showed no main effect of picrotoxin treatment [$F_{(1,42)} = 0.298$, $p = 0.588$], no picrotoxin treatment × postnatal day interaction [$F_{(2,84)} = 0.805$, $p = 0.450$; $n = 18$ control mice, $n = 26$ picrotoxin-exposed mice; **Figure 2A**], and a significant effect of day of testing [$F_{(1.970,82.73)} = 5.361$, $p = 0.007$]. In the righting reflex test, the two-way repeated-measures ANOVA showed a trend toward an effect of picrotoxin treatment [$F_{(1,42)} = 3.216$, $p = 0.080$], no picrotoxin treatment × day of testing interaction [$F_{(2,84)} = 1.415$, $p = 0.249$; $n = 18$ control mice, $n = 26$ picrotoxin-exposed mice; **Figure 2B**], and a significant effect of day of testing [$F_{(1.424,59.80)} = 39.04$, $p < 0.0001$]. In the cliff avoidance test, the two-way repeated-measures ANOVA showed a main effect of picrotoxin treatment [$F_{(1,42)} = 12.10$, $p = 0.001$], a trend toward a picrotoxin treatment × day of testing interaction [$F_{(2,84)} = 3.080$, $p = 0.051$; $n = 18$ control mice, $n = 26$ picrotoxin-exposed mice; **Figure 2C**], and a significant effect of day of testing [$F_{(1.612,67.72)} = 10.71$, $p = 0.0003$]. The Bonferroni *post-hoc* test revealed a significant difference on P7 ($p = 0.005$). The Bonferroni *post-hoc* test did not reveal a significant difference on P9 ($p = 0.401$) or P11 ($p = 0.248$). No difference in the latency to fall was found between control and picrotoxin-exposed male mice on P25 [$t_{(42)} = 0.836$, $p = 0.408$; $n = 18$ control mice, $n = 26$ picrotoxin-exposed mice; **Figure 2D**]. The results of these tests indicated that picrotoxin-exposed male mice exhibited a delay in body maturation and poor motor performance compared with control male mice.

Effects of Prenatal Exposure to Picrotoxin on Affective-Like Behaviors

We assessed the effects of picrotoxin on social interaction during both 5–6 weeks of age (adolescence) and 10–11 weeks of age (adulthood). In adolescence, picrotoxin-exposed male mice exhibited a decrease in active interaction time compared with control male mice [$t_{(42)} = 3.378$, $p = 0.002$; $n = 18$ control

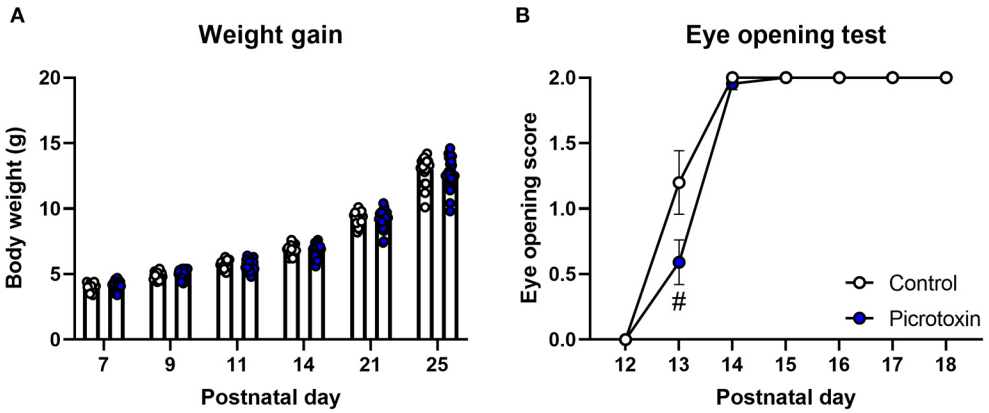

FIGURE 1 | Body maturation and eye-opening scores. **(A)** No significant difference in body weight was found between control and picrotoxin-exposed male mice. **(B)** A trend toward a difference in eye opening was observed between control and picrotoxin-exposed male mice on P13. The data are expressed as mean ± SEM. $^{\#}p < 0.1$ [two-way repeated-measures ANOVA in **(A)**, Mann-Whitney *U*-test in **(B)**].

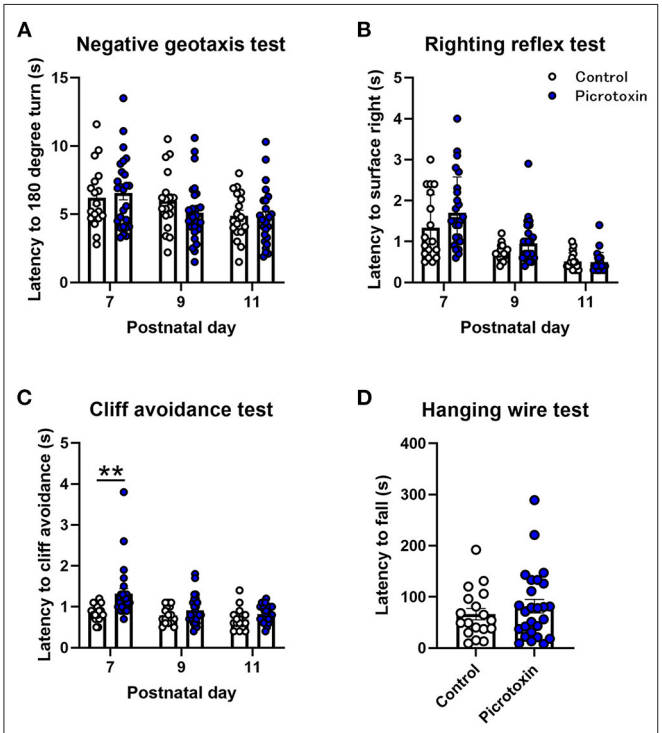

FIGURE 2 | Motor function tests. **(A)** No significant difference in the negative-geotaxis test was found between control and picrotoxin-exposed male mice. **(B)** No significant difference in the righting reflex test was found between control and picrotoxin-exposed male mice. **(C)** Picrotoxin-exposed male mice exhibited a significantly longer latency to cliff avoidance. **(D)** No significant difference in the hanging-wire test was found between control and picrotoxin-exposed mice. The data expressed as mean ± SEM. $^{**}p < 0.01$ (two-way repeated-measures ANOVA).

mice, $n = 26$ picrotoxin-exposed mice; **Figure 3A**]. In adulthood, picrotoxin-exposed male mice exhibited a decrease in active interaction time compared with control male mice [$t_{(42)} = 2.723$, $p = 0.009$; $n = 18$ control mice, $n = 26$ picrotoxin-exposed mice; **Figure 3A**]. No significant difference in the latency to flicking, jumping, and licking paws in the hot plate test was found between the control and picrotoxin-exposed male mice at 6–7 weeks of age [$t_{(42)} = 0.547$, $p = 0.587$; $n = 18$ control mice, $n = 26$ picrotoxin-exposed mice; **Supplementary Figure 2A**]. Patients with ASD typically engage in repetitive behaviors (1). The self-grooming test has been used to monitor repetitive behaviors in mouse models of ASD (23). Picrotoxin-exposed male mice exhibited a significant difference in the total number of self-grooming episodes in the grooming test compared with control male mice [$t_{(35)} = 2.221$, $p = 0.032$; $n = 20$ control mice, $n = 17$ picrotoxin-exposed mice; **Figure 3B**]. No significant difference in the total time of self-grooming in the grooming test was found between control and picrotoxin-exposed male mice [$t_{(35)} = -1.350$, $p = 0.186$; $n = 20$ control mice, $n = 17$ picrotoxin-exposed mice; **Supplementary Figure 1B**]. No significant difference in the total distance traveled in the open field test was found between control and picrotoxin-exposed male mice [$t_{(42)} = 0.412$, $p = 0.682$; $n = 18$ control mice, $n = 26$ picrotoxin-exposed mice; **Figure 3C**]. No significant difference in the time spent in the peripheral area was found between control and picrotoxin-exposed male mice [$t_{(42)} = 0.214$, $p = 0.832$; $n = 18$ control mice, $n = 26$ picrotoxin-exposed mice; **Figure 3D**]. Picrotoxin-exposed male mice exhibited an increase in the number of turning episodes compared with control male mice [$t_{(42)} = -2.218$, $p = 0.032$; $n = 18$ control mice, $n = 26$ picrotoxin-exposed mice; **Figure 3E**]. No significant difference in the time spent on the open arms of the elevated plus maze was found between control and picrotoxin-exposed male mice [$t_{(42)} = 0.253$, $p = 0.801$; $n = 18$ control mice, $n = 26$ picrotoxin-exposed mice; **Figure 3F**]. Body weight was recorded at both 5–6 and 10–11 weeks of age in the social interaction test. No significant difference was found between control and picrotoxin-exposed male mice in either adolescence or adulthood (**Supplementary Results** and **Supplementary Table 1**). After the social interaction test at 10–11 weeks of age, we collected the whole mouse brain and recorded

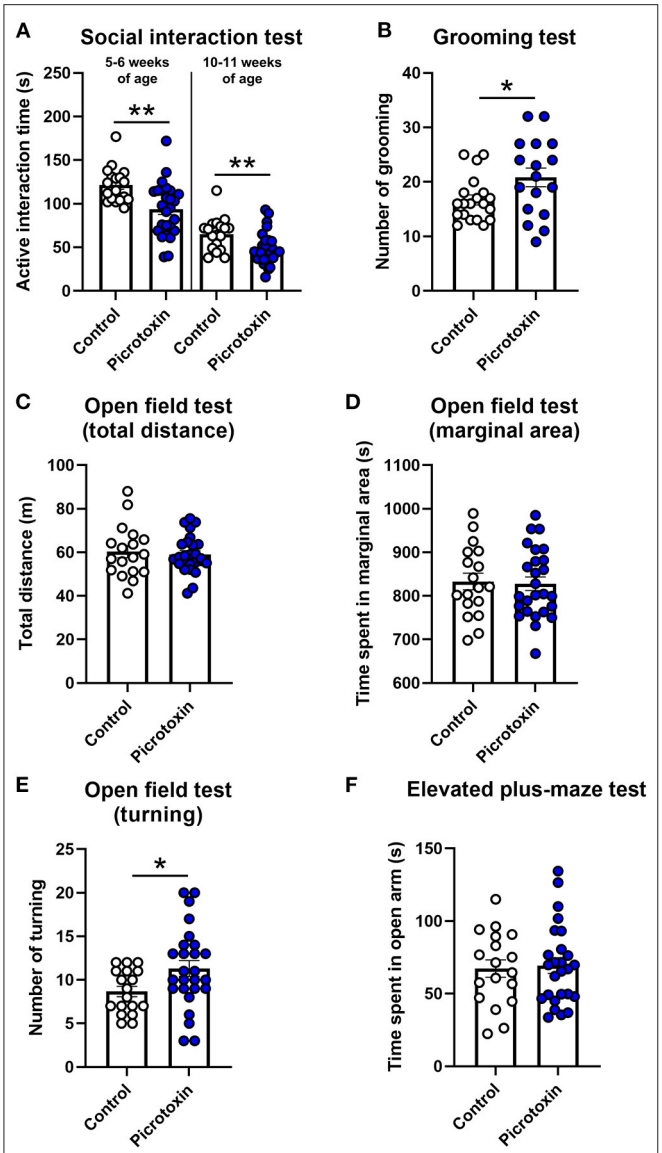

FIGURE 3 | Social interaction, hot plate, grooming, open field, and elevated plus maze tests. **(A)** Social interaction test. Picrotoxin-exposed male mice exhibited a decrease in active social interaction time compared with control male mice at 5–6 and 10–11 weeks of age. **(B)** Grooming test. Picrotoxin-exposed male mice exhibited an increase in the number of grooming episodes compared with control male mice. **(C,D)** Open field test. No significant difference was found between control and picrotoxin-exposed male mice in the total distance traveled **(C)** or time spent in the peripheral area **(D)**. **(E)** Open field test (turning behavior). Picrotoxin-exposed male mice exhibited an increase in the number of turning episodes compared with control male mice. **(F)** Elevated plus maze test. No significant difference in the time spent on the open arms was found between control and picrotoxin-exposed male mice. The data are expressed as mean ± SEM. $^{**}p < 0.01$, $^{*}p < 0.05$ (unpaired *t*-test).

brain weights to confirm the effects of picrotoxin treatment. No significant difference in brain weight was found between control and picrotoxin-exposed male mice (**Supplementary Results** and **Supplementary Table 1**).

TABLE 1 | Number of genes whose expression was altered by picrotoxin treatment.

Group	Total altered genes	Up regulated genes	Down regulated genes
Picro/Control	465	438	27

Picro, picrotoxin treatment.

We also analyzed body maturation, motor function, social interaction, behavior in the hot plate test, grooming behavior, activity in the open field test, and anxiety-like behavior in the elevated plus maze test in control female mice and picrotoxin-exposed female mice. No significant difference was found between control and picrotoxin-exposed female mice in the social interaction test. All behavioral data in female mice are presented in the **Supplementary Results** and **Supplementary Table 2**.

Effects of Picrotoxin Exposure *in utero* on Gene Expression

Picrotoxin-exposed male mice ($n = 5$) exhibited the differential expression of 465 genes (438 upregulated genes, 27 downregulated genes) compared with control male mice ($n = 5$; **Table 1**). To further analyze the functional significance of these genes, enrichment pathway analysis was performed for each upregulated and downregulated gene using BaseSpace. The enrichment pathways for each regulated gene are described in **Table 2**, **Supplementary Tables 4, 5**. The top five pathways for upregulated genes were odorant binding, neuropeptide receptor activity, positive regulation of neutrophil migration, coenzyme A (CoA)-ligase activity, and acid-thiol ligase activity (**Table 2**). The top five pathways for downregulated genes were protein N-terminus binding, regulation of myeloid cell differentiation, carbohydrate binding, regulation of hemopoiesis, and negative regulation of hemopoiesis (**Table 2**). We also identified common genes whose expression was altered in whole brains between picrotoxin-exposed male mice and VPA-exposed male mice [which have been used as an animal model of ASD and were reported in our previous study (18)]. The common genes whose expression was altered were *Camk1d*, *Platr26*, *Zfp599*, *Fyb*, and *Cdc7* (**Table 3**). These genes were upregulated in both picrotoxin-exposed male mice and VPA-exposed male mice. Of these altered genes, *Fyb* expression was recovered by rapamycin treatment in VPA-exposed mice (18). These results suggest that exposure to picrotoxin *in utero* alters gene expression in the offspring brain, and the genes whose expression is altered are common to an existing mouse model of ASD.

Weighted Gene Co-expression Network Analysis

We next constructed a WGCNA to explore relationships between behavioral and gene traits in picrotoxin-exposed mice. The dendrogram represents a single tight-clustering branch of each sample (**Figure 4**). Gene expression in the whole brain in both control and picrotoxin-exposed male mice was discriminated into two clusters, indicating that there were no outliers in the

TABLE 2 | Gene ontology pathway enrichment analysis of upregulated and downregulated genes.

Biogroups	Normalized score	*P*-value	Common genes
Up regulated genes			
Odorant binding	100	0.0002	12
Neuropeptide receptor activity	73	0.002	4
Positive regulation of neutrophil migration	70	0.0025	3
CoA-ligase activity	67	0.0032	2
Acid-thiol ligase activity	65	0.0038	2
Down regulated genes			
Protein-N-terminus binding	100	0.0003	3
Regulation of myeloid cell differentiation	94	0.0005	3
Carbohydrate binding	77	0.002	3
Regulation of hemopoiesis	77	0.0027	3
Native regulation of hemopoiesis	65	0.005	2

TABLE 3 | Genes whose expression was altered in picrotoxin-exposed and VPA-exposed mice.

Gene	Fold change, *P*-value (Picro)	Fold change, *P*-value (VPA)
Camk1d	1.3145 (0.0101)	1.4299 (9.80-E04)
Platr26	1.2708 (0.0165)	1.5662 (0.0133)
Zfp599	1.2675 (0.0454)	1.2655 (0.0262)
Fyb	1.2231 (0.0474)	2.7767 (4.88-E04)
Cdc7	1.2114 (0.0259)	1.3891 (0.0396)

Picro, picrotoxin; VPA, valproic acid.

samples. A total of four distinct gene modules were identified from the expression of 1,867 genes using a dynamic tree cutting algorithm (**Figure 5**). The brown, blue, turquoise, and gray gene modules included 18, 28, 1,776, and 45 genes, respectively. The 45 uncorrelated genes were grouped into in the gray module. To find modules of interest, correlations between the color modules and performance in the behavioral tests were computed (**Figure 6A**). Performance in the grooming test was excluded in this correlation analysis because we could not find a consistent result with regard to the number and time of self-grooming. Picrotoxin treatment significantly correlated with four modules (brown module: $r = -0.78$, $p = 0.008$; blue module: $r = 0.82$, $p = 0.004$; turquoise module: $r = 0.96$, $p = 1e\text{-}05$; gray module: $r = 0.88$, $p = 7e\text{-}04$). Performance in the social interaction test at 5–6 weeks of age was significantly associated with picrotoxin treatment (gray module: $r = -0.75$, $p = 0.01$). All four modules significantly correlated with performance in the social interaction test at 10–11 weeks of age. Among these four modules, the turquoise module showed the highest negative correlation (brown module: $r = 0.73$, $p = 0.02$; blue module: $r = -0.77$, $p = 0.11$; turquoise module: $r = -0.91$, $p = 2e\text{-}04$; gray module: $r = -0.79$, $p = 0.006$). The number of turning episodes in the open field test was significantly associated with the blue module ($r = 0.85$, $p = 0.002$). The enrichment pathways for each module are listed in **Supplementary Tables 6–9**. The top three pathways for each module are described in **Figure 6B**. The turquoise module that was most associated with performance in the social interaction test at 10–11 weeks of age was enriched for the odorant binding and tissue differentiation and development pathways (**Figure 6B**, **Supplementary Table 6**). The blue module that was most associated with performance in the open field test at 8–9 weeks of age was enriched for synapse-associated pathways (**Figure 6B**, **Supplementary Table 7**).

DISCUSSION

In the present study, we found that prenatal exposure to picrotoxin in mice on day 12.5 of gestation had long-term and selective effects on postnatal behaviors and gene expression in male offspring. In the behavioral tests, prenatal exposure to picrotoxin (i) tended to delay body maturation and motor function, (ii) induce impairments in social interaction in both adolescence and adulthood, and (iii) increase the number of turning episodes in the open field test. The gene expression analysis showed that picrotoxin-exposed mice exhibited alterations of the expression of 465 genes, including five genes that were in common with alterations in VPA-exposed male mice. The WGCNA showed that social interaction in adulthood had the strongest negative correlation with the turquoise gene module, and turning episodes in the open field test had a strongest positive correlation with the blue gene module. "Odorant binding" and "presynaptic membrane" were the top pathways for the turquoise and blue gene modules, respectively.

The pathophysiology of ASD has not been well-defined. Many hypotheses suggest causal explanations of ASD. An E/I imbalance in the mature brain is theorized to be a key pathophysiological mechanism of ASD (24–28). The influence of GABA during prenatal development is excitatory, and GABA/$GABA_A$ receptor signaling plays a key role in cellular processes during development. Previous studies reported that disturbances of GABA/$GABA_A$ receptor signaling affect postnatal behaviors in offspring. Perinatal exposure to picrotoxin in rat dams affected sexual behavior in male offspring by increasing the latency to the first mount and intromission (12). The neonatal blockade of $GABA_A$ receptors with bicuculline produced abnormal passive avoidance memory and increased brain-derived neurotrophic factor levels in the brain on P61-70 (11). Prenatal exposure to ethanol, a $GABA_A$ receptor agonist, dysregulated the vertical and horizontal cleavage planes of neural progenitors in the developing neocortex, and picrotoxin treatment prior to ethanol administration restored regulation of the cleavage plane to control levels (16). Tyzio et al. reported that a rat model of VPA-induced ASD and mouse model of Fragile X syndrome abolished the switch of GABA from excitatory to inhibitory transmission. Maternal pretreatment with bumetanide, an antagonist of Na-K-Cl cotransporter 1 chloride importers, before delivery improved this switch in GABA activity and aberrant ultrasonic vocalizations in offspring (29). Tochitani et al. recently reported that the modulation of $GABA_A$ receptors by agonists and antagonists did not cause

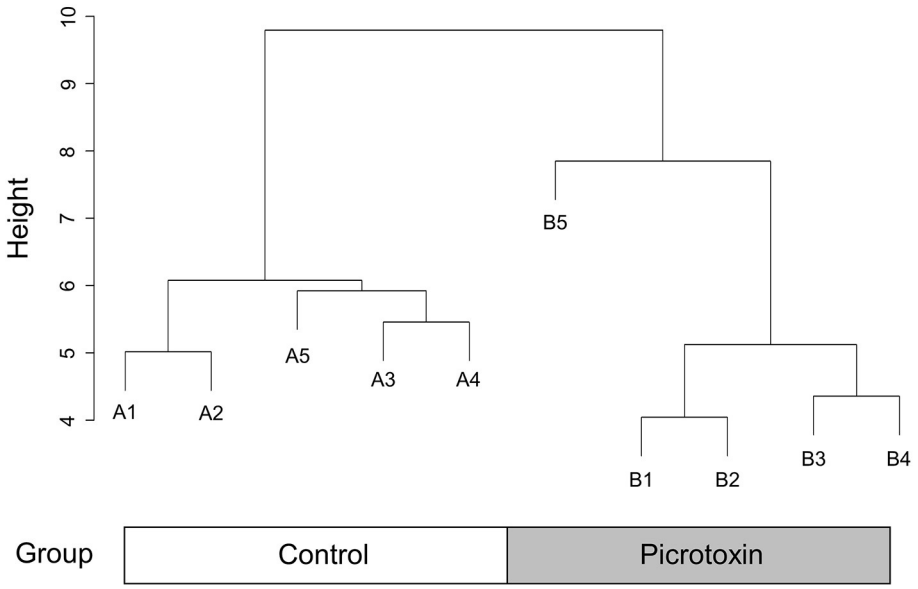

FIGURE 4 | Sample cluster dendrogram. Hierarchical clustering of samples of 1,867 genes in the mouse whole brain.

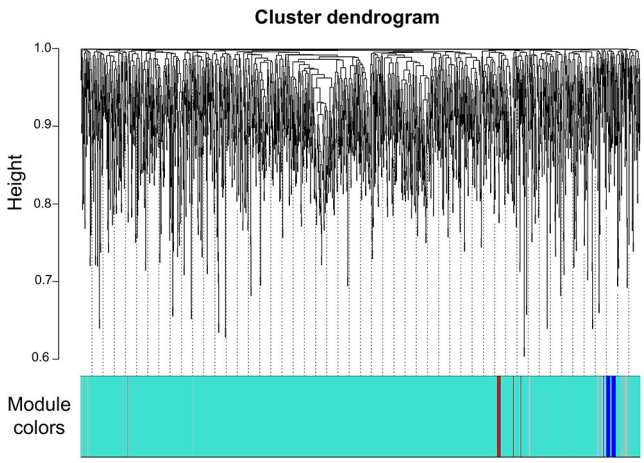

FIGURE 5 | Gene dendrogram. Gene dendrogram and clustered modules coded by four colors.

social deficits but resulted in the rapid loss of interest in a stranger mouse compared with control mice (15). The different findings between their study and the present study might be attributable to the treatment duration, dose, social interaction test that was conducted, or mouse strain. Nonetheless, the present results support the findings of Tochitani et al. and further our understanding of correlations between disturbances of $GABA_A$ receptor signaling during the embryonic period and the pathophysiology of ASD. Tochitani et al. also reported that the administration of 5.0 mg/kg picrotoxin twice daily on embryonic days 10–12 affected neuronal progenitor cells in the neocortex on embryonic day 13, decreased Tbr2-positive cells, increased Pax 6-positive cells, and decreased Doublecortin (Dcx; a marker for immature and migrating neurons)-positive layers (15). Picrotoxin administration during the embryonic period disturbs development of the neocortex and might contribute to the pathophysiology of ASD. In the present study, we found that prenatal $GABA_A$ receptor blockade with picrotoxin affected behavior in male offspring in both adolescence and adulthood and caused ASD-like behaviors, including deficits of social interaction.

Picrotoxin-exposed male mice exhibited alterations of genes, including 438 upregulated genes and 27 downregulated genes, compared with control male mice. We conducted gene ontology pathway enrichment analysis to understand the function of these genes. We found that genes whose expression was upregulated are related to odorant binding, neuropeptide receptor activity, neutrophil migration, and catalysis (**Table 2**). The downregulated genes are related to protein-N-terminus binding, myeloid cell differentiation, carbohydrate binding, and hemopoiesis (**Table 2**). Upregulated and downregulated genes with normalized scores of 100 were related to odorant binding and protein-N-terminus binding, respectively. A previous clinical study reported that patients with ASD exhibited aberrant olfactory responses, with a lower discrimination score and higher bias score in an olfactory test (30). One of the identified genes in the protein-N-terminus binding pathway was *Hesx1*, which is required to program human embryonic stem cell neural fate (31).

We also detected alterations of the expression of genes that were regulated in the same direction (i.e., upregulated) in picrotoxin-exposed male mice and VPA-exposed male mice.

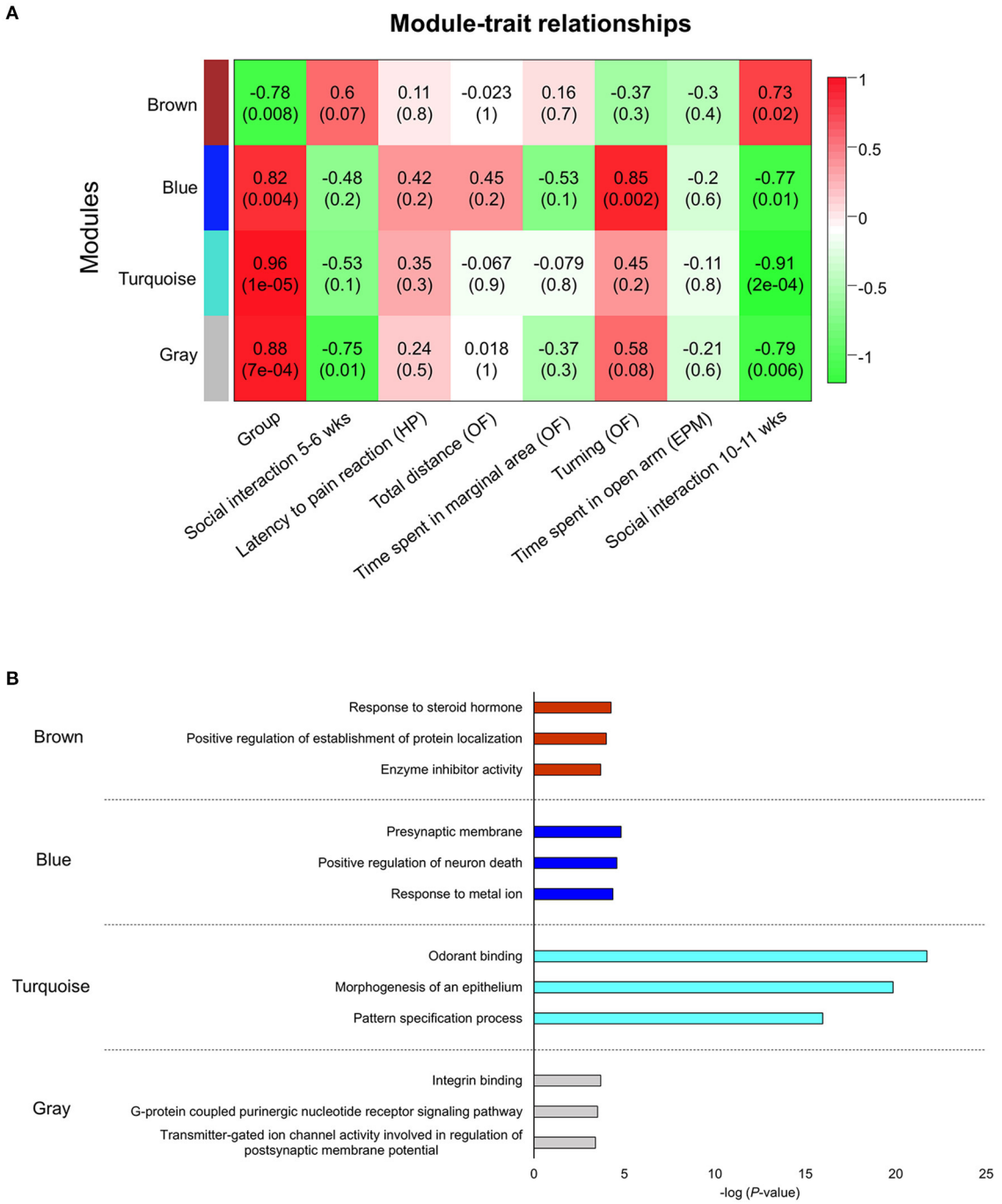

FIGURE 6 | Network construction of co-expressed genes that correlated with performance in the behavioral tests and gene ontology pathway enrichment analysis of each module. **(A)** Module trait correlation for treatment, social interaction (5–6 weeks of age), latency to flicking, jumping, licking paws (hot plate test), total distance traveled (open field test), time spent in the peripheral area (open field test), turning episodes (open field test), time spent on the open arms (elevated plus maze test), and social interaction (10–11 weeks of age). HP, hot plate test; OF, open field test; EPM, elevated plus maze test. **(B)** The blue module was the most correlated with turning episodes in the open field test. The turquoise module was the most correlated with social interaction (10–11 weeks of age).

Exposure to VPA has been used as an animal model of ASD (18, 32–34). We detected five genes whose expression was commonly altered between picrotoxin-exposed male mice and VPA-exposed male mice: *Camk1d*, *Platr26*, *Zfp599*, *Fyb*, and *Cdc7* (**Table 3**). *Camk1d* and *Cdc7* were reportedly associated with brain and embryonic development (35, 36). *Platr26* is a long non-coding RNA, and *Platr1-32* were suggested to be functionally integrated into the mouse embryonic stem cell gene expression program

(37). Zinc finger protein plays a key role in tissue development and differentiation (38). *De novo* deletions in the 19q.13.11 region that encompasses four KRAB-ZNFs, including *Zfp* 599, were identified in two unrelated cases of microcephaly (39). In our previous study (18), *Fyb* expression was recovered by the mammalian target of rapamycin complex 1 inhibitor rapamycin in VPA-exposed male mice. *Fyb* is broadly expressed in the mouse brain and strongly expressed in the olfactory bulb (40). Valproic acid is clinically used as an anti-epileptic drug and increases GABA release (41). We speculate that these five genes are key molecules in the pathophysiology of ASD-like behavior in mice, in which GABA signaling is disturbed during embryonic periods.

A WGCNA was used to detect correlations between co-expression genes and performance in various behavioral tests. We identified four modules based on the WGCNA. All four gene modules (brown, blue, turquoise, and gray) had a significant correlation with picrotoxin treatment (Group) and social interaction at 10–11 weeks of age, and the blue gene module had a significant correlation with turning episodes in the open field test. The turquoise module had the strongest correlation with performance in the social interaction test at 10–11 weeks of age. The top three enrichment pathways for the turquoise module were odorant binding, epithelium morphogenesis, and pattern specification process. Overall, the turquoise module included pathways that are related to tissue differentiation and development (**Figure 6B**, **Supplementary Table 6**). Interestingly, the most significantly enriched pathway was "odorant binding." Active interaction time in the social interaction test consisted of sniffing, allo-grooming, mounting, and following. The sniffing ratio was the most active social interaction behavior (**Supplementary Table 3**). Aberrant olfactory function may have altered social interaction in picrotoxin-exposed male mice. Patients with ASD also exhibit sensory issues, including aberrant olfactory function (42). A previous clinical study reported that patients with ASD exhibited aberrant odor awareness (43). A significant correlation was found between olfactory threshold and social problems, determined by the Child Behavior Checklist, in male children with ASD (44). The sniffing duration ratio correlated with the social affect component of the Autism Diagnostic Observation Schedule but not the restricted/repetitive behavior component (45). Impairments in social interaction in ASD are often caused by the misreading of emotional cues. Endevelt-Shapira et al. investigated social chemosignals in adult patients with ASD. When typically developing participants sniffed an undetectable scent of fear (i.e., skydiver sweat) or control sweat, they presented enhanced autonomic arousal responses (i.e., electrodermal activity) in response to the skydiver sweat compared with control sweat, whereas patients with ASD did not exhibit such changes in the autonomic arousal response (46). These clinical findings suggest that the olfactory system plays a key role in sociability in patients with ASD. Franco et al. reported that a reduction of lateral inhibition in the brain impaired odor discrimination and social behavior in a *Drosophila* model of Fragile X syndrome that exhibited ASD-like behaviors (47). *Scn1a*$^{+/-}$ mice also exhibit ASD-like behaviors, including social deficits and the avoidance of social odors (e.g., male urine odors), in a Y-maze olfactory choice test (48). Mitral cells in the olfactory bulb are generated on embryonic days 9–13. The olfactory tubercle receives a robust axonal projection from mitral cells that are generated on embryonic day 12 (49). The present study did not investigate specific changes in the olfactory bulb or olfactory cortex, but our findings suggest that picrotoxin administration during the embryonic period may affect development of the olfactory system and its connections with other brain regions. The blue module was directory correlated with turning behavior in the open field test at 8–9 weeks of age. The blue module included pathways that are related to synapses as the most enriched pathways (**Figure 6B**, **Supplementary Table 7**). The top network in the blue module was "presynaptic membrane." Presynaptic function has been investigated in neurodevelopmental disorders, including ASD (50). Patients with ASD and animal models of ASD exhibit different behaviors in a novel environment. Patients with ASD exhibited higher stress responses to novel stimuli compared with controls (51, 52). The ablation of metabotropic glutamate receptor 5 in mice resulted in synaptic deficiency and an increase in novelty-induced locomotion compared with wildtype mice (53). We speculate that the high number of turning episodes is an aberrant response to a novel environment. Patients with ASD often exhibit stimming behavior (e.g., hand-flapping, body rocking, and spinning in circles), also known as repetitive/restricted behavior, to manage their emotions and overwhelming sensory inputs (54). A previous study reported that prenatal zinc deficiency in mice, which is an animal model of ASD, resulted in a side preference of rotational behavior in a round arena (a 360° turn was considered a rotation) (55), but we did not observe differences between right and left turning episodes in the present study (P8, **Supplementary Table 10**).

In the present study, the detected pathways in the turquoise and blue modules are helpful for clarifying the mechanisms of ASD-like behaviors, which may aid the identification of possible treatment targets. The uncorrelated genes were assigned to the gray module and was thus normally excluded from further analysis. However, the gray module negatively correlated with performance in the social interaction test in both adolescence and adulthood in picrotoxin-exposed male mice. The key pathways that caused social deficits in both adolescence and adulthood were in the gray module. Social interaction at 10–11 weeks of age was the most correlated with the turquoise module. Social interaction at 5–6 weeks of age was not correlated with the turquoise module. We speculate that adults and adolescents may recruit different pathways that contribute to social behaviors. The present findings may help clarify differences in neural mechanisms that are involved in sociability between adults and adolescents.

We observed sex differences in picrotoxin-exposed mice. Exposure to picrotoxin during the embryonic period caused social deficits in males but not females. The ratio of males to females is higher in the ASD patient population (2). A previous study reported that treatment with the $GABA_A$ receptor inhibitor bicuculine during the neonatal period elicited aberrant anxiety-like behavior in male mice but not in female mice (10, 11). Tracosis et al. reported that the autistic male mice showed reduced the Hurst exponent in resting state fMRI in the medial

prefrontal cortex, indicating increased excitation but female mice did not show the reduced the Hurst exponent (56). Valproic acid-exposed female mice did not exhibit impairments in social interaction (33) but exhibited a decrease in Nissl-positive cells in the somatosensory cortex compared with male mice (57). Female mice also did not exhibit ASD-like behaviors despite having the Nlgn3/Cyfip1 risk allele (58). A recent study reported that low placenta levels of allopregnanolone (ALLO), a progesterone-derived $GABA_A$ receptor modulator, resulted in sex-dependent alterations of neurodevelopment and behavior in offspring (59). Male plKO mice that were derived from pregnant plKO mice that have specific placental ALLO reduction exhibited ASD-like behaviors, including social deficits, and an increase in cerebellar myelin proteins on P30, whereas female plKO mice did not exhibit these changes (59). Similar results were found in human preterm infants that were characterized by premature loss of the placenta (59). This study suggests that $GABA_A$ receptor function in female mice might not be disrupted by low ALLO levels or that female mice may recruit a compensatory mechanism in response to impairments in $GABA_A$ receptor function (59). Sex bias in ASD may be related to chromosomal and hormonal mechanisms, immune activation, or interactions among sex, genetics, and environmental factors (60). These previous studies and the present study suggest that female sex may be a protective factor against ASD, making males more susceptible to ASD. Further studies are warranted to verify possible sex differences in animal models of ASD.

We suggest that exposure to picrotoxin during the embryonic period contributes to the pathophysiology of ASD via $GABA_A$ receptor signaling. Picrotoxin also blocks homomeric glycine receptor (GlyR) subtypes (61, 62). Glycine receptors are ligand-gated chloride channels that are expressed in the brain and spinal cord (63). Pilorge et al. reported that male patients with ASD had a *de novo* missense mutation of *GLRA2*, which encodes the GlyRα2 subunit. These authors also found that *Glra2* knockout mice did not exhibit social behavior and spent less time exploring a novel object (64). In brain slices that were prepared on embryonic day 11, pretreatment with picrotoxin inhibited the response of neuronal progenitors to the application of taurine, which is a ligand of GlyRs and $GABA_A$ receptors (15). We did not investigate the specific mechanisms of action of picrotoxin during the embryonic period in the present study, but $GABA_A$ receptor or GlyR inhibition by picrotoxin is suggested to contribute to the pathophysiology of ASD.

The present study found ASD-like behaviors, gene aberrations, and correlations between gene expression and behavior in mice that were exposed to picrotoxin during the embryonic period. Our results provide a better understanding of the pathophysiology of ASD. Our study suggests that prenatal exposure to an $GABA_A$ receptor inhibitor induces ASD-like behaviors in offspring. The prenatal inhibition of $GABA_A$ signaling may a mechanism that contributes to ASD.

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.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Experimentation Ethics Committee of Tokyo Metropolitan Institute of Medical Science.

AUTHOR CONTRIBUTIONS

HK-M and KI designed the experiments and wrote the paper. HK-M performed the mouse behavioral testing and analyzed the behavioral data. HK-M and HH analyzed the gene expression data. HH conducted the WGCNA and wrote the methods of WGCNA. YN and YT performed the microarray analysis. HK-M and YH generated the picrotoxin mouse model of ASD and were responsible for breeding management. AS, MT, YK, SU, and TM participated in refinements of the experiments and discussion. All authors read and approved the final manuscript to be published.

FUNDING

This research was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, KAKENHI [17K15765, JP16H06276 (AdAMS)].

ACKNOWLEDGMENTS

We thank Michael Arends for editing the manuscript and Etsuko Kamegaya and Yukiko Matsushima for assistance with breeding mice.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2022.821354/full#supplementary-material

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frontiers in Psychiatry

ORIGINAL RESEARCH
published: 03 February 2022
doi: 10.3389/fpsyt.2021.816196

Associations Among Maternal Metabolic Conditions, Cord Serum Leptin Levels, and Autistic Symptoms in Children

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Introduction: Accumulating evidence has shown that maternal metabolic conditions, such as pre-pregnancy overweight, diabetes mellitus, and hypertensive disorders of pregnancy (HDP) are potential risk factors of autism spectrum disorder (ASD). However, it remains unclear how these maternal conditions lead to neurodevelopmental outcomes in the offspring, including autistic symptoms. Leptin, an adipokine that has pro-inflammatory effects and affects fetal neurodevelopment, is a candidate mediator of the association between maternal metabolic factors and an increased risk of ASD. However, whether prenatal exposure to leptin mediates the association between maternal metabolic conditions and autistic symptoms in children has not been investigated yet.

Methods: This study investigated the associations between mothers' metabolic conditions (pre-pregnancy overweight, diabetes mellitus during or before pregnancy, and HDP), leptin concentrations in umbilical cord serum, and autistic symptoms among 762 children from an ongoing cohort study, using generalized structural equation modeling. We used the Social Responsive Scale, Second Edition (SRS-2) at 8–9 years old to calculate total T-scores. Additionally, we used the T-scores for two subdomains: Social Communication and Interaction (SCI) and Restricted Interests and Repetitive Behavior (RRB).

Results: Umbilical cord leptin levels were associated with pre-pregnancy overweight [coefficient = 1.297, 95% confidence interval (CI) 1.081–1.556, $p = 0.005$] and diabetes mellitus (coefficient = 1.574, 95% CI 1.206–2.055, $p = 0.001$). Furthermore, leptin levels were significantly associated with SRS-2 total T-scores (coefficient = 1.002, 95% CI 1.000–1.004, $p = 0.023$), SCI scores (coefficient = 1.002, 95% CI 1.000–1.004, $p = 0.020$), and RRB scores (coefficient = 1.001, 95% CI 1.000–1.003, $p = 0.044$) in

OPEN ACCESS

Edited by:
Juehua Yu,
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Reviewed by:
Kai Shi,
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Daniel S. Tylee,
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Specialty section:
This article was submitted to Autism, a section of the journal Frontiers in Psychiatry

Received: *16 November 2021*
Accepted: *31 December 2021*
Published: *03 February 2022*

Citation:
Iwabuchi T, Takahashi N, Nishimura T, Rahman MS, Harada T, Okumura A, Kuwabara H, Takagai S, Nomura Y, Matsuzaki H, Ozaki N and Tsuchiya KJ (2022) Associations Among Maternal Metabolic Conditions, Cord Serum Leptin Levels, and Autistic Symptoms in Children. Front. Psychiatry 12:816196. doi: 10.3389/fpsyt.2021.816196

children. Associations between maternal metabolic factors and autistic symptoms were not significant.

Discussion: The present study uncovered an association between cord leptin levels and autistic symptoms in children, while maternal metabolic conditions did not have an evident direct influence on the outcome. These results imply that prenatal pro-inflammatory environments affected by maternal metabolic conditions may contribute to the development of autistic symptoms in children. The findings warrant further investigation into the role of leptin in the development of autistic symptoms.

Keywords: autism spectrum disorder, maternal metabolic conditions, overweight, diabetes mellitus, hypertensive disorders of pregnancy, leptin

INTRODUCTION

Autism spectrum disorder (ASD) is a highly prevalent neurodevelopmental condition, with >3% prevalence in Japan (1), characterized by difficulties in social communication and repetitive and restricted patterns in behaviors and interests (2). Autistic symptoms are observable across the general population and are not limited to the clinical population (3). Although ASD is heritable (4, 5), various environmental factors are plausibly associated with an increased risk of ASD (6, 7). Moreover, previous studies have demonstrated associations between prenatal or perinatal factors and autistic symptoms in children (8). Recently, several umbrella reviews have provided an overview of the important contributions of environmental factors, including obstetric, demographic, and neurotoxic factors, on ASD (9, 10). However, the biological mechanisms that link environmental factors and elevated autistic symptoms remain largely unexplored.

Previous studies have implicated maternal metabolic conditions before and during pregnancy, including diabetes mellitus (DM) (11–13), hypertensive disorder of pregnancy (HDP) (14–17), and pre-pregnancy overweight (18–20), with an increased risk of ASD. The global burden of such metabolic conditions (21–23) warrants studies on understanding the mechanisms by which maternal factors are associated with ASD pathogenesis. Several researchers have highlighted the role of maternal metabolic condition-induced inflammation in neurodevelopmental outcomes in children (24–26). Maternal pre-pregnancy overweight or obesity has been linked to *in utero* inflammatory environments, consequently affecting fetal neurodevelopment (27, 28). Animal studies have further elaborated that maternal DM enhances the production of pro-inflammatory cytokines or chemokines, which adversely affects fetal neurodevelopment (29, 30). Likewise, the association of HDP, especially preeclampsia, with enhanced inflammation has been supported by animal studies (31, 32). However, limited evidence from human longitudinal studies challenges the identification of causal pathways between maternal metabolic conditions and ASD.

Leptin, an adipokine (or adipocytokine), has pro-inflammatory effects and may mediate the link of maternal metabolic diseases to neurodevelopmental outcomes in children (33). Leptin, primarily secreted from the white adipose tissues, plays an important role not only in the regulation of food intake by acting on the hypothalamus (34, 35) but also in immunity and inflammation (36, 37). For example, leptin upregulates the secretion of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β (38–40). Conversely, other studies have suggested that leptin production is upregulated by TNF-α and IL-1β (41–43), possibly forming a pro-inflammatory feedback loop. The administration of inflammatory stimuli, such as lipopolysaccharide, enhances leptin production (44, 45), whereas leptin deficiency likely results in an immunosuppressive phenotype characterized by reduced levels of inflammatory cytokines (46, 47). Furthermore, animal studies have suggested that maternal systemic inflammation, induced by factors such as a high-fat diet, causes disrupted leptin signaling and affects neurodevelopment in children (48–50). Collectively, these findings indicate that leptin may contribute to strengthening *in utero* neuroinflammation induced by maternal metabolic conditions and consequently influence neurodevelopment in the offspring.

An association between leptin and ASD has also been suggested previously. A postmortem study found increased leptin levels in the anterior cingulate gyrus of individuals with ASD (51). As for peripheral markers, several cross-sectional studies have reported higher serum or plasma leptin levels in individuals with ASD compared to those in typically developing individuals (52–56). A longitudinal study reported that higher plasma leptin levels in early childhood (mean age of measurement, 18.4 months) were associated with an increased risk for later diagnosis of ASD (57). Another recent study suggested that children at 4–12 years of age who received an ASD diagnosis, compared to their typically developing counterparts, showed different trajectory patterns in peripheral leptin levels (58). However, most of these studies measured leptin levels in serum or plasma samples collected from children or adults and examined their associations with ASD. Associations between umbilical cord leptin levels and later ASD symptoms have been scarcely examined except for two recent studies (57, 59). These studies examined whether cord plasma leptin levels were associated with later ASD diagnosis or autistic symptoms, but found no significant associations between them. However, the relatively small sample sizes used in these studies restricts the generalizations of their findings.

Furthermore, no study has investigated whether leptin acts as a mediator of the link between maternal metabolic conditions and ASD-like behavioral characteristics in children.

Using a population-representative birth cohort (60), we aimed to examine the associations among maternal metabolic conditions (maternal diabetes, pre-pregnancy overweight, and HDP), leptin concentrations in umbilical cord serum, and later autistic symptoms in children. We hypothesized that the cord serum leptin levels mediate the link between maternal metabolic conditions and autistic symptoms. We employed path analysis to investigate (1) whether maternal metabolic conditions before or during pregnancy were associated with autistic symptoms in children of 8–9 years of age, and (2) if yes, whether the association is mediated by umbilical cord leptin levels.

METHODS

Participants

The present study used a subsample of the Hamamatsu Birth Cohort for Mother and Child (HBC) Study, which included 762 children and their 699 mothers (see the section Results for details). The HBC Study consisted of 1,138 mothers and their children ($n = 1{,}258$; 611 boys, 647 girls) born in Japan between December 2007 and June 2011. Our previous study described detailed recruitment procedures (60). The present study was conducted in accordance with the Declaration of Helsinki, and written informed consent was obtained from each mother for the participation of herself and her infant. The Hamamatsu University School of Medicine and the University Hospital Ethics Committee approved the study protocol (Ref. 18-166, 19-9, 20-82, 22-29, 24-67, 24-237, 25-143, 25-283, E14-062, E14-062-1, E14-062-3, 17-037, 17-037-3, 20-233).

Measurement

Autistic Symptoms

Using the Japanese version of the Social Responsive Scale, Second Edition (SRS-2) (61), we assessed autistic symptoms in children aged 8–9 years. The SRS-2 raw scores were evaluated based on the responses of a parent or caregiver to 65 items. We then converted the raw scores to T-scores normed for sex [mean = 50, standard deviation (SD) = 10]. We used the SRS-2 total T-scores as a proxy for autistic symptoms in children. Higher T-scores indicate higher ASD-like behavioral characteristics. Additionally, we used T-scores compatible with the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (2), consisting of the Social Communication and Interaction (SCI) and Restricted Interests and Repetitive Behavior (RRB) scores.

Leptin Levels in Umbilical Cord Serum

Umbilical cord blood samples were collected from mothers immediately after delivery *via* venipuncture of the umbilical vein. The samples were kept at room temperature for 30 min after collection and then centrifuged at 3,500 rpm for 10 min, from which serum was taken and divided into 200 μl aliquots, and stored at −80°C until analysis (60). Leptin concentrations in cord serum were measured using enzyme-linked immunosorbent assay kits by Skylight Biotech, Inc. (Akita, Japan), as described previously (62). Leptin levels in cord serum ranged 0.1–78.1 ng/mL, and participants with zero value for cord serum leptin (two children) were excluded from the analysis.

Maternal Metabolic Conditions: Pre-pregnancy Overweight, DM Before or During Pregnancy, and HDP

Based on the pre-pregnancy body mass index (BMI), mothers were categorized as overweight (BMI $\geq$ 25) and non-overweight (BMI $<$ 25). Clinical diagnoses of maternal DM before or during pregnancy and HDP were evaluated based on the electronic medical records. All of them were treated as dichotomous variables.

Covariates

We included children's sex, maternal age at delivery, educational attainment of mothers, household income at birth, gestational age at birth ($<$37 or $\geq$37 weeks), birth weight, maternal smoking status during pregnancy, and mode of feeding during 0–6 months of age (breastfeeding only, formula only, breastfeeding, and formula) in the model as possible confounders based on previous studies (57, 63).

Statistical Analysis

We conducted path analysis to investigate a series of associations between maternal metabolic conditions, leptin concentrations, and children's autistic symptoms. The data on leptin levels in cord serum and SRS-2 scores (i.e., total, SCI, and RRB scores) were not normally distributed (all $p < 0.001$, Shapiro-Wilk tests). Since the non-negative data (i.e., SRS-2 T-scores and leptin levels in cord serum) had positively skewed distributions, we employed a generalized structural equation modeling with gamma family and log link (64–67). The model was adjusted for the covariates mentioned above. We used the *gsem* command in Stata version 15.1 to perform the path analysis. First, we performed path analysis using SRS-2 total T-scores to indicate autistic symptoms (Model 1; **Figure 1A**). Second, if SRS-2 total scores were significantly associated with any variable, we investigated whether the umbilical cord leptin levels and maternal metabolic conditions were associated with autistic symptoms in two subdomains compatible with DSM-5 (i.e., SCI and RRB; Model 2; **Figure 1B**). When the *gsem* command is used, full-information maximum likelihood (FIML) estimation is not available to deal with missing data. Therefore, we conducted sensitivity analyses using the *sem* command with the FIML option, although non-normality was not considered. All participants in the HBC Study were included in these analyses.

RESULTS

Participant Characteristics

A total of 840 participants who completed the SRS-2 assessment were included in the analysis. Of them, 78 children were excluded due to the lack of data on cord blood leptin levels, leaving 762 children and 699 mothers included in the analysis. The participant characteristics are summarized in **Table 1**. Using Little's test with the *mcartest* command in Stata (68, 69), we

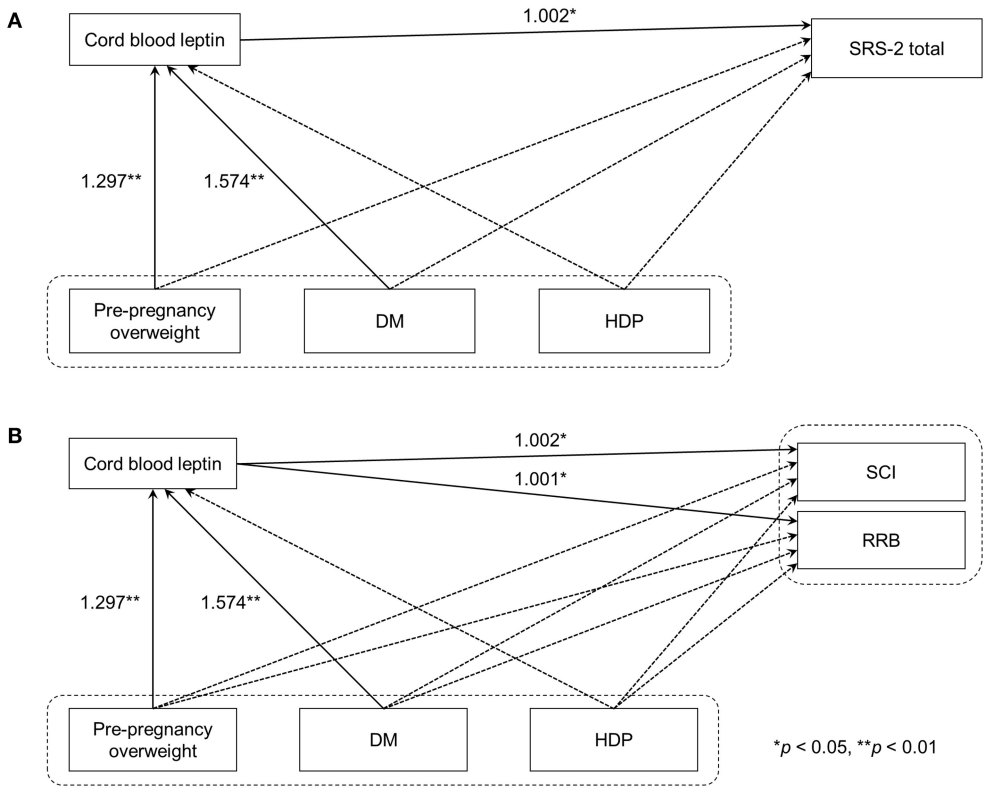

FIGURE 1 | Path analyses of maternal metabolic conditions, cord blood leptin concentrations, and autistic symptoms in children. **(A)** Model 1 used SRS-2 total T-scores as the indicator of autistic symptoms in children. **(B)** In Model 2, DSM-5 compatible T-scores were used instead. Solid line arrows and dashed line arrows indicate significant and non-significant associations between observed variables, respectively. Numbers represent exponentiated coefficients obtained from the analysis (*$p < 0.05$, **$p < 0.01$). DM, diabetes mellitus before or during pregnancy; HDP, hypertensive disorders of pregnancy; SRS-2, the Social Responsive Scale, Second Edition; DSM-5, the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders; SCI, Social Communication and Interaction; RRB, Restricted Interests and Repetitive Behavior.

confirmed that the missingness of the variables of interest included in the model was completely at random ($\chi^2 = 9.74$, $df = 16$, $p = 0.87$) and not dependent on covariates ($\chi^2 = 162.23$, $df = 160$, $p = 0.43$).

Associations Among Maternal Metabolic Conditions, Cord Blood Leptin, and Autistic Symptoms

As the coefficients were log-transformed, we present the exponentiated coefficients hereafter. In both Model 1 and Model 2, maternal pre-pregnancy overweight [Model 1, coefficient = 1.297, 95% confidence interval (CI) 1.081–1.556, $p = 0.005$; Model 2, coefficient = 1.297, 95% CI 1.081–1.556, $p = 0.005$] and DM (Model 1, coefficient = 1.574, 95% CI 1.206–2.055, $p = 0.001$; Model 2, coefficient = 1.574, 95% CI 1.206–2.055, $p = 0.001$) were significantly associated with increased levels of leptin in cord serum. In Model 1, we found that cord leptin concentrations were positively associated with SRS-2 total T-scores (coefficient = 1.002, 95% CI 1.000–1.004, $p = 0.023$; **Table 2**). This result indicates that a one-unit increase in cord serum leptin multiplies the SRS-2 total T-score by 1.002. As for the covariates, cord leptin levels were associated with children's sex (higher in female, coefficient = 0.549, 95% CI 0.493–0.612, $p < 0.001$), maternal age at delivery (coefficient = 0.984, 95% CI 0.973–0.995, $p = 0.005$), gestational age at birth (coefficient = 0.699, 95% CI 0.538–0.909, $p = 0.008$), and birth weight (coefficient = 1.001, 95% CI 1.000–1.001, $p < 0.001$) (see **Supplementary Table 1** for details). Moreover, the additional analysis (Model 2) revealed that cord serum leptin concentrations were significantly associated with both SCI scores (coefficient = 1.002, 95% CI 1.000–1.004, $p = 0.020$) and RRB scores (coefficient = 1.001, 95% CI 1.000–1.003, $p = 0.044$). We found no direct associations between maternal metabolic conditions (i.e., maternal pre-pregnancy overweight, DM, or HDP) and autistic symptoms in children (i.e., total, SCI, and RRB scores) (all $p > 0.05$; **Tables 2**, **3**). The sensitivity analyses confirmed that the associations mentioned above remained significant even when missing values were handled with FIML (**Tables 4**, **5**).

DISCUSSION

Using longitudinal data from a population-representative birth cohort in Japan, the present study examined associations among maternal metabolic conditions, umbilical cord serum leptin

TABLE 1 | Sample characteristics of participating children and their parents.

	n (%)
Child's sex	
Male	386 (50.7)
Female	376 (49.3)
Preterm birth	44 (5.8)
Mother's smoking status during pregnancy	55 (7.2)
Mode of feeding	
Breastfeeding only	424 (33.7)
Formula only	237 (18.8)
Breastfeeding and formula	597 (47.5)
Maternal pre-pregnancy overweight	75 (9.8)
Diabetes mellitus before or during pregnancy	8 (1.0)
Hypertensive disorders of pregnancy	87 (11.4)
	Mean (SD)
Leptin levels in cord serum (ng/mL)	4.5 (5.3)
Birth weight (g)	2945.5 (432.7)
Gestational age at birth (weeks)	39.0 (1.5)
Maternal age at birth (years)	31.9 (4.9)
Household income at birth (million JPY)	6.2 (2.8)
Mother's education (years)	14.0 (1.8)
SRS-2 T-scores	
Total	51.2 (7.3)
Social Communication and Interaction (SCI)	53.9 (9.0)
Restricted Interests and Repetitive Behavior (RRB)	49.6 (7.4)

SD, Standard Deviation; JPY, Japanese yen; SRS-2, the Social Responsiveness Scale, Second Edition.

TABLE 2 | Estimated coefficients and *p*-values in Model 1.

Outcome	Exposure	Coefficient (95% CI)[a]	*P*-value
SRS-2 total	Leptin level	**1.002 (1.000-1.004)**	**0.023**
	Pre-pregnancy overweight	1.000 (0.967-1.035)	0.956
	DM	0.997 (0.949-1.049)	0.934
	HDP	1.008 (0.977-1.039)	0.614
Leptin level	Pre-pregnancy overweight	**1.297 (1.081-1.556)**	**0.005**
	DM	**1.574 (1.206-2.055)**	**0.001**
	HDP	0.983 (0.832-1.163)	0.848

[a]*Coefficients are exponentiated.*
SRS-2, the Social Responsiveness Scale, Second Edition; DM, diabetes mellitus before or during pregnancy; HDP, hypertensive disorders of pregnancy. The bold values indicate statistical significance.

TABLE 3 | Estimated coefficients and *p*-values in Model 2.

Outcome	Exposure	Coefficient (95% CI)[a]	*P*-value
SCI	Leptin level	**1.002 (1.000-1.004)**	**0.020**
	Pre-pregnancy overweight	1.001 (0.963-1.041)	0.934
	DM	0.999 (0.942-1.059)	0.987
	HDP	1.008 (0.972-1.045)	0.654
RRB	Leptin level	**1.001 (1.000-1.003)**	**0.044**
	Pre-pregnancy overweight	1.021 (0.987-1.057)	0.215
	DM	1.023 (0.972-1.077)	0.370
	HDP	1.009 (0.977-1.041)	0.564
Leptin level	Pre-pregnancy overweight	**1.297 (1.081-1.556)**	**0.005**
	DM	**1.574 (1.206-2.055)**	**0.001**
	HDP	0.983 (0.832-1.163)	0.848

[a]*Coefficients are exponentiated.*
SCI, Social Communication and Interaction; RRB, Restricted Interests and Repetitive Behavior; DM, diabetes mellitus before or during pregnancy; HDP, hypertensive disorders of pregnancy. The bold values indicate statistical significance.

TABLE 4 | Result of the sensitivity analysis (Model 1).

Outcome	Exposure	Coefficient (95% CI)[a]	*P*-value
SRS-2 total	Leptin level	**0.086 (0.012-0.159)**	**0.022**
	Pre-pregnancy overweight	0.005 (−0.063-0.074)	0.883
	DM	−0.015 (−0.084-0.053)	0.656
	HDP	0.000 (−0.067-0.069)	0.983
Leptin level	Pre-pregnancy overweight	**0.083 (0.029-0.137)**	**0.003**
	DM	**0.165 (0.111-0.218)**	**<0.001**
	HDP	−0.027 (−0.080-0.024)	0.302

[a]*Standardized coefficients are shown.*
SRS-2, the Social Responsiveness Scale, Second Edition; DM, diabetes mellitus before or during pregnancy; HDP, hypertensive disorders of pregnancy. The bold values indicate statistical significance.

levels, and autistic symptoms in children aged 8–9 years. The results demonstrated that maternal DM and pre-pregnancy overweight were associated with leptin levels in cord serum. As expected, we found significant associations between cord serum leptin levels and increased autistic symptoms indexed by SRS-2 total T-scores. Additionally, the path analysis showed that cord serum leptin levels were associated with both impaired social communication (measured in SCI) and restricted interests and repetitive behavior (measured in RRB), indicating cord leptin level as a biological factor associated with the two subdomains of ASD symptoms in common. In contrast, we found no significant associations between maternal metabolic conditions (pre-pregnancy overweight, DM, and HDP) and autistic symptoms in children. This non-significant association is inconsistent with our hypothesis that maternal metabolic conditions are linked to later autistic symptoms.

To the best of our knowledge, the present study is the first to identify an association between leptin levels in umbilical cord serum and later autistic symptoms in children. Only two studies have examined associations between adipokine levels in cord plasma and ASD (57, 59), reporting no significant association of cord leptin levels with a later ASD diagnosis and autistic symptoms. There are some methodological differences between these studies and the present study. For example, Raghavan et al. (57) reported no significant association between leptin concentrations in cord plasma and ASD diagnosis [odds ratio (OR) = 0.90, 95% CI 0.66–1.24]. However, the study by Raghavan et al. did not investigate an association between leptin and autistic symptoms observed across the general population, and the number of children receiving ASD diagnosis was small in that study (39 out of 655 children with cord blood samples). The study by Joung et al. (59) examined associations

TABLE 5 | Result of the sensitivity analysis (Model 2).

Outcome	Exposure	Coefficient (95% CI)[a]	*P*-value
SCI	Leptin level	**0.094 (0.020-0.167)**	**0.012**
	Pre-pregnancy overweight	0.007 (−0.061-0.075)	0.839
	DM	−0.014 (−0.083-0.054)	0.677
	HDP	−0.001 (−0.069-0.067)	0.972
RRB	Leptin level	**0.082 (0.008-0.155)**	**0.028**
	Pre-pregnancy overweight	0.047 (−0.020-0.116)	0.171
	DM	0.017 (−0.050-0.086)	0.613
	HDP	0.006 (−0.061-0.075)	0.847
Leptin level	Pre-pregnancy overweight	**0.083 (0.029-0.137)**	**0.003**
	DM	**0.164 (0.110-0.218)**	**<0.001**
	HDP	−0.028 (−0.080-0.024)	0.290

[a]*Standardized coefficients are shown.*

SCI, Social Communication and Interaction; RRB, Restricted Interests and Repetitive Behavior; DM, diabetes mellitus before or during pregnancy; HDP, hypertensive disorders of pregnancy. The bold values indicate statistical significance.

between autistic symptoms assessed using SRS-2 and cord leptin levels, similar to the present study, but reported no significant association between them ($\beta = -0.20$, 95% CI −1.34–0.94). However, the sample size (295 children) was smaller than that in our study. Needless to mention that further investigations are needed, the present findings underscore the importance of cord leptin levels in altered neurodevelopment associated with later autistic symptoms.

Several biological pathways possibly link increased cord leptin levels and increased autistic symptoms at a later stage of development. As mentioned earlier, increased levels of leptin in cord blood may reflect pro-inflammatory prenatal environments (33). Recently, *in utero* inflammation has garnered attention as a potential risk for altered neurodevelopment, which may lead to various psychiatric disorders, including ASD (70). Because leptin can cross the blood-brain barrier (71), leptin levels in cord serum may reflect elevated leptin levels in the fetal brain and increased neuroinflammation modulated by leptin. Other possible pathways may involve mitochondrial dysfunctions and/or oxidative stress (72, 73), both of which have been associated with ASD (74, 75), possibly resulting from *in utero* leptin exposure. Multiple studies have reported several other important roles of leptin in neurodevelopment. For example, leptin-deficient animals showed alterations in neuronal and cortical development (76, 77) and myelination (78). A recent study demonstrated that leptin is also associated with the emergence of inhibitory function of GABAergic neurons (79). Given the repeated observations of altered cortical structures (80–82), reduced white matter integrity (83, 84), and excitatory/inhibitory imbalance (85, 86) in ASD, it seems plausible to assume that leptin levels at birth play an important role in ASD etiopathology.

Contrary to our hypothesis, we did not find any significant association between maternal metabolic conditions and autistic symptoms although maternal overweight/obesity and DM are relatively well-established risk factors for ASD (9). One possible explanation for this discrepancy is the difference in ethnic populations. In previous meta-analyses or systematic reviews, most of the included studies were conducted in Western countries; for example, in a recent meta-analysis on the association between maternal BMI and children's ASD included studies from the USA or European countries exclusively (20). However, Asian children, including Japanese children, have lower birth weights than their Western peers (87). Higher birth weight is generally associated with higher levels of cord blood leptin (88, 89); therefore, cord blood leptin concentrations may be lower in Japanese children than in children born in other countries. The present study demonstrated that maternal DM and overweight were associated with elevated leptin levels in cord blood, and that increased leptin levels were associated with increased autistic symptoms in children. For children with relatively higher birth weights and higher cord serum leptin levels at birth, the effects of maternal metabolic conditions on perinatal leptin levels may be more severe on later neurodevelopment. In contrast, children in countries with relatively lower birth weights and baseline leptin levels (e.g., children in Japan) may be less predisposed to an increased risk of ASD attributable to maternal metabolic conditions. Taken together, we speculate that the effects of maternal metabolic conditions on autistic symptoms are less prominent in Japanese children than in those of other ethnicities. Future studies are required to clarify the reasons for these inconsistent results.

The present study replicated the previous findings that maternal DM and pre-pregnancy overweight increased cord blood leptin concentrations (90–95). In adults, blood leptin levels correlate with BMI (96, 97), and hyperleptinemia and leptin resistance are prevalent in individuals with obesity or overweight (98, 99); this is also the case for pregnant women (100). Moreover, during pregnancy, leptin is supplied not only by maternal adipose tissues but also by the placenta (101). While it is still inconclusive whether metabolic conditions of mothers (such as obesity) upregulate or downregulate the placental production of leptin (102), DM and pre-pregnancy overweight likely cause an increase in cord serum leptin levels, thereby leading to altered neurodevelopment in offspring.

No significant association was observed between HDP and cord serum levels of leptin. This does not corroborate previous studies reporting significant associations between HDP and elevated leptin levels (103–105). However, most of these studies compared mothers with preeclampsia and those without hypertensive disorders. By definition, HDP consists of several clinical conditions, such as chronic hypertension and preeclampsia (106); such heterogeneity possibly resulted in this discrepancy. Further studies with larger sample sizes would help resolve this inconsistency.

The present study has several limitations. First, the sample size of the present study was relatively small, given that the number of mothers who had metabolic conditions, especially DM (only 1.0%), was very low. This may have affected the negative findings of the associations between these conditions and autistic symptoms. In addition, although we found a significant association between cord serum leptin levels and autistic symptoms, the effect size in the present study was small compared to other studies like Raghavan et al. (57),

which showed an association between early childhood plasma leptin levels and later ASD diagnosis (OR = 1.80, 95% CI = 1.25–2.60, $p = 0.002$). We believe the smaller effect size in our study compared to that in Raghavan et al.'s is primarily due to differences in the outcomes being measured (ASD symptoms vs. ASD diagnosis), but further replication with larger sample sizes is needed. Second, our analysis included only a subsample of the population-representative HBC Study. Although we confirmed that the missingness of outcome variables was completely at random and independent of the covariates, caution should be exercised when generalizing the present findings. Third, we considered maternal DM before or during pregnancy and HDP as categorical, although these diseases consisted of distinguishable clinical conditions; for example, gestational diabetes and pre-pregnancy DM can be differentiated, but these were considered as one condition in the present study. Future studies should investigate the effects of these conditions separately. Fourth, we did not confirm ASD diagnosis but relied on parental reports to evaluate autistic symptoms in children. Although SRS-2 is a validated and reliable measure, associations among maternal metabolic conditions, cord leptin levels, and clinical diagnosis of ASD in children must be further investigated.

The present study examined a series of associations among maternal metabolic conditions, umbilical cord serum levels of leptin, and autistic symptoms in children aged 8–9 years. Contrary to previous studies, maternal DM, pre-pregnancy overweight, and HDP were not associated with later autistic symptoms in children. However, maternal DM and pre-pregnancy overweight were found to be associated with increased leptin concentrations in cord serum and that, in turn, leptin levels were associated with autistic symptoms in total and in the DSM-5 compatible subdomains (namely, SCI and RRB). These findings suggest the importance of leptin in ASD etiology. Another important implication of the present study is that maternal metabolic conditions before or during pregnancy were not found directly associated with autistic symptoms in children, but leptin levels increased by those conditions might affect later neurodevelopment.

DATA AVAILABILITY STATEMENT

The data generated for this study is subject to the following licenses/restrictions: Privacy and confidentiality of participants. Requests to access these datasets should be directed to Kenji J. Tsuchiya, tsuchiya@hama-med.ac.jp.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Hamamatsu University School of Medicine and the University Hospital Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

TI and NT had full access to all the data used in the study and takes responsibility for the integrity of the data and accuracy of the data analysis. TI, NT, and KT conceptualized the study and drafted the manuscript. TI, NT, TN, and MR performed the statistical analyses. TN, TH, AO, and HM provided technical and material support. KT supervised the study. All authors conducted the data acquisition, contributed to critical revision of the manuscript and significantly to the study, and the creation of this manuscript.

FUNDING

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology in Japan (Grant Number 19K14175 to TI; Grant Numbers 19H03582, 21K19639, and 21KK0145 to KT), AMED (Grant Number JP21gk0110039h0003 to KT), and the National Institute of Mental Health (Grant Number NIMH R01 MH102729 to YN).

ACKNOWLEDGMENTS

We are grateful to the individuals who participated in the study. We would like to thank Ms. Chikako Nakayasu, Ms. Yuko Amma, and Ms. Haruka Suzuki for data collection, and Ms. Noriko Kodera and Ms. Emi Higashimoto for administration.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.816196/full#supplementary-material

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frontiers in Psychiatry

MINI REVIEW
published: 08 February 2022
doi: 10.3389/fpsyt.2022.836181

Maternal and Adult Interleukin-17A Exposure and Autism Spectrum Disorder

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Epidemiological evidence in humans has suggested that maternal infections and maternal autoimmune diseases are involved in the pathogenesis of autism spectrum disorder. Animal studies supporting human results have shown that maternal immune activation causes brain and behavioral alterations in offspring. Several underlying mechanisms, including interleukin-17A imbalance, have been identified. Apart from the pro-inflammatory effects of interleukin-17A, there is also evidence to support the idea that it activates neuronal function and defines cognitive behavior. In this review, we examined the signaling pathways in both immunological and neurological contexts that may contribute to the improvement of autism spectrum disorder symptoms associated with maternal blocking of interleukin-17A and adult exposure to interleukin-17A. We first describe the epidemiology of maternal immune activation then focus on molecular signaling of the interleukin-17 family regarding its physiological and pathological roles in the embryonic and adult brain. In the future, it may be possible to use interleukin-17 antibodies to prevent autism spectrum disorder.

Keywords: maternal immune activation (MIA), IL-17A, autism spectrum disorder (ASD), Th17 cell, γδT cells, embryonic brain development, psoriasis

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Autism, a section of the journal Frontiers in Psychiatry

Received: *15 December 2021*
Accepted: *14 January 2022*
Published: *08 February 2022*

Citation:
Fujitani M, Miyajima H, Otani Y and Liu X (2022) Maternal and Adult Interleukin-17A Exposure and Autism Spectrum Disorder. Front. Psychiatry 13:836181. doi: 10.3389/fpsyt.2022.836181

INTRODUCTION

Prenatal exposure to maternal immune activation (MIA) has been implicated as an environmental risk factor for autism spectrum disorder (ASD). The relationship between MIA and the pathogenesis of neurodevelopmental disorders including ASD has been discussed at length (1–7).

In the first part of this review, we describe the epidemiology of MIA, including maternal infection and maternal autoimmune diseases, as risk factors for ASD. Subsequently, among immunological factors, we focus on molecular signaling of the interleukin (IL)-17 family regarding its physiological and pathological roles in the embryonic and adult brain, based essentially on animal experiments.

MIA AND ASD

Abnormalities in the immune system have been widely observed in the brain and periphery of patients with ASD. Studies have shown that ASD is associated with chronic neuroinflammation, with increased activation of microglia and astrocytes and the production of cytokines and chemokines in the brain (8, 9).

Infections during pregnancy can cause prematurity or stillbirth, and pathogens can be vertically transmitted to the fetus, causing congenital infections and severe diseases, known as TORCH syndrome (*Toxoplasma gondii*, other, rubella virus, cytomegalovirus, herpes simplex virus) (10, 11). In addition to the threat from these pathogens, other clinical evidence suggests that ASD is increased in the offspring of pregnancies during seasonal outbreaks and epidemics of influenza, measles, epidemic parotitis, and polio (7). Moreover, animal studies have shown that MIA, including viral infection and mimicry, results in neurodevelopmental abnormalities in rodents and non-human primates similar to human ASD phenotypes (3, 12, 13). However, this relationship has not been elucidated, because a meta-analysis showed that the odds ratio (OR) of offspring with ASD is only 1.13 (95% confidence interval [CI] 1.03–1.23) (14).

Furthermore, chronic inflammatory and allergic conditions in pregnancy, such as autoimmune diseases (15) (OR 1.34, 95% CI 1.23–1.46) or asthma (OR 1.43, 95% CI 1.38–1.49) (16), are prominent risk factors for ASD (6). The correlation between asthma and ASD has been well-demonstrated (17, 18). Among autoimmune diseases, maternal psoriasis is also a significant risk factor for ASD (OR 1.39, 95% CI 1.00–1.95) (18). Maternal psoriasis has recently received attention because IL-17A is one of the most important cytokines in the pathogenesis of psoriasis (19, 20).

IL-17 SIGNALING

IL-17A (commonly known as IL-17) is a signature cytokine of a distinct $CD4^+$ T helper 17 (Th17) cell that is characterized by the expression of retinoic acid receptor-related orphan receptor gamma t (RORγt) and is activated by IL-23. IL-17A is most strongly implicated in human disease among the six IL-17 family members (IL-17A, IL-17B, IL-17C, IL-17D, IL-25(also known as IL-17E), and IL-17F). As shown in **Figure 1**, all the family members except IL-17D basically function as homodimers; however, IL-17A and IL-17F form a heterodimer (21). The IL-23/IL-17A signaling axis has been found to play a critical role in autoimmune diseases (21, 23, 24).

It has been revealed that IL-17A is produced by other cell populations, such as IL-17-producing $CD8^+$ T (Tc17) cells, γδT cells, natural killer T cells, natural Th17 cells, natural killer cells, group 3 innate lymphoid cells, neutrophils, and mast cells (21, 23, 24) (**Figure 1**).

The IL-17 receptor family is composed of five members (IL-17RA to IL-17RE), which are distinct subclasses of receptors characterized by an intracellular motif called SEFIR (SEF [similar expression to FGF receptor]/IL-17 receptor) (**Figure 1**) (21, 24). The initial event in IL-17R signaling is the recruitment of Act1, a multifunctional protein containing the SEFIR domain required for IL-17R-Act1 interaction. Act1 has E3 ubiquitin ligase activity and rapidly recruits and ubiquitinates tumor necrosis factor receptor-associated factor 6 (TRAF6), another E3 ubiquitin ligase (**Figure 1**). Like other receptors that recruit TRAF6, IL-17 triggers the activation of the canonical nuclear factor κB (NF-κB) cascade and pro-inflammatory and anti-microbial genes (21, 24). TRAF6 also promotes the activation of mitogen-activated protein kinase and activator protein 1 (AP1) pathways, and CCAAT/enhancer-binding protein (C/EBP) transcription factors (21, 24).

RELATIONSHIP BETWEEN IL-17 EXPOSURE AND ASD IN THE RODENT EMBRYONIC BRAIN

Little is known about the role of IL-17A in brain development under non-inflammatory conditions. Therefore, we obtained the RNA sequencing results from Mouse Genome Informatics and found that *Il-17a* and *Il-17f* were not or hardly expressed in the mouse embryonic brain (http://www.informatics.jax.org/). In contrast, IL-17 family receptors, *Il-17ra, rc, and rd*, are all expressed in the embryonic and adult mouse brain. These results were confirmed by Choi et al. in 2016, where they demonstrated that IL-17RA is expressed mainly in the cortical plate of the mouse embryonic brain (25).

Accumulating evidence supports a role for Th17 cells and their effector cytokine IL-17A in ASD (26) (**Figure 2A**). Polyinosinic:polycytidylic acid [poly (I:C)] is structurally similar to double-stranded RNA and is used to model the actions of extracellular double-stranded RNA, such as viral mimicry. Poly (I:C)-induced MIA in the gestating dam is relayed to the embryo via the placenta. Choi et al. (25) showed that increased Th17 cells in the placenta secrete IL-17A, which enters the fetal circulation. Since mouse blood brain barrier begins to form between E11 and E17 (27), circulated IL-17A can enter the brain and regulate development without local production of IL-17A (25). Another group also confirmed the upregulation of IL-17A in maternal blood and the postnatal offspring brain (28). In addition to poly (I:C), Lipopolysaccharide, major component of the outer membrane of Gram-negative bacteria, is also used to induce MIA as a bacterial septic shock model (29).

IL-17A acts directly on the mouse fetal brain on embryonic day (E) 14.5, resulting in an ASD-like phenotype, including abnormal behaviors in ultrasonic vocalization tests, social interaction tests, and marble burying tests (25). Direct injection of IL-17A into the fetal lateral ventricles on E14.5 resulted in phenocopied ASD-like behaviors and cortical disorganization in the offspring induced by poly (I:C)-evoked MIA (25). *Il-17ra* mRNA is detectable in the fetal brain on E14.5 and is upregulated by poly (I:C)-MIA in an IL-17A-dependent manner (25). Direct injection of IL-17A into the fetal brain on E14.5 resulted in thinning of the cortical plate on E18.5, which was not observed in MIA induction on E14.5 (25) (**Figure 2A**). Interestingly, Choi et al. found that poly (I:C)-induced MIA and IL-17A administration to the embryonic brain on E14.5 resulted in patch-like cortical dysplasia on E18.5 (25), which is similar to

Abbreviations: ASD, autism spectrum disorder; CI, confidence interval; CNS, central nervous system; E, embryonic day; EAE, experimental allergic encephalomyelitis; Ig, immunoglobulin; IL, interleukin; MIA, maternal immune activation; OR, odds ratio; S1DZ, primary somatosensory cortex dysgranular zone; SEFIR, similar expression to FGF receptor /IL-17 receptor; SFB, segmented filamentous bacteria; Th17, T helper 17; TRAF6, tumor necrosis factor receptor-associated factor 6.

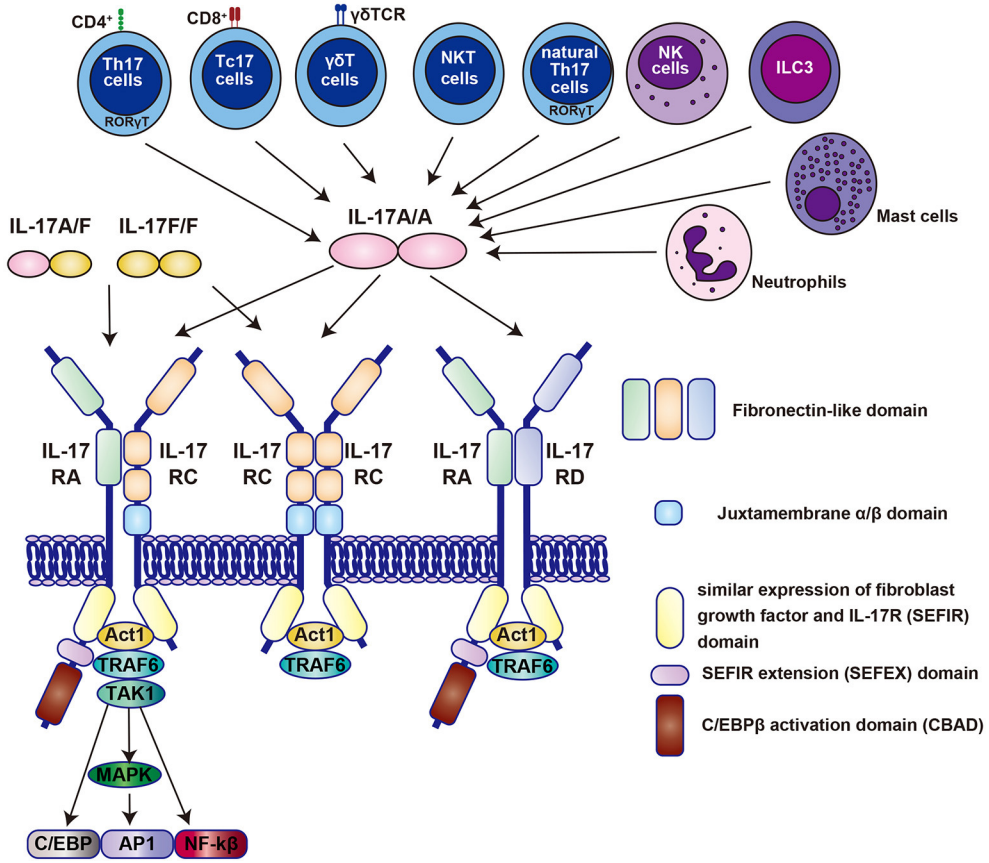

FIGURE 1 | The molecular binding system of the IL-17 family centered on IL-17A and its receptors. All the family members of the IL-17 family, except IL-17D function as homodimer, whereas IL-17A and IL-17F form a heterodimer (denoted as IL-17A/F) (21). All receptors also function as homodimers or heterodimers. Homodimers of IL-17A (denoted as IL-17A/A) selectively bind to specific IL-17RA/RC, RC/RC, or RA/RD receptor complexes. Contrastingly, IL-17A/F and IL-17F/F bind only to IL-17RA/RC and RC/RC receptor complexes. Each IL-17 receptor has an extracellular fibronectin-like domain that binds the ligand and an intracellular SEFIR (similar expression of fibroblast growth factor and IL-17R) domain that recruits molecules such as Act1 and TRAF6 (21). The IL-17 receptor family has been shown by Goepfert et al., to be structurally bent between the first and second fibronectin domains (22). IL, interleukin; Th17, T helper 17; Tc17, IL-17-producing $CD8^+$ T cells; NKT, natural killer T cells; NK; natural killer cells; ILC3, type 3 innate lymphoid cells; NF, nuclear factor; TRAF6, tumor necrosis factor receptor-associated factor 6; MAPK, mitogen-activated protein kinase; TAK1; transforming growth factor-β-activated kinase 1.

some human patients with ASD (30). Their group reproduced the results by another study (31); however, another group mentioned that they could not find any patches after MIA; therefore, the occurrence of cortical patches remains controversial (32).

Kim et al. showed that maternal microbiota, including segmented filamentous bacteria (SFB), promote IL-17A production in maternal gut Th17 cells (33). They treated MIA-evoked dams with vancomycin to kill SFB, and this treatment inhibited the ASD-phenotype in offspring, such as abnormal ultrasonic vocalization, repetitive behavior, or sociability, with decreased IL-17A production (**Figure 2A**). More recently, another group showed that the administration of IL-17A during the entire maternal period causes early and persistent cortical abnormalities and ASD-like phenotypes in male offspring (34). The offspring showed abnormal expression of synaptic and cell cycle genes, disrupted adult glia, inhibitory synapses, and abnormal behaviors (34). Moreover, IL-17A injection into the fetal brain on E14.5 resulted in microglial activation and altered localization (35) (**Figure 2A**). In addition, maternal overexpression of IL-17A induced abnormal behavior in offspring, and in parallel, elevated kynurenine levels in maternal serum and fetal plasma were observed. Moreover, maternal kynurenine-injected mice exhibited behavioral abnormalities similar to those observed in the offspring of *Il-17a*-overexpressed dams (36) (**Figure 2A**).

IL-17A EXPOSURE IN THE ADULT BRAIN OF RODENTS

Contrary to the analysis in the embryonic brain, the expression of IL-17A and its receptors in the adult central nervous system (CNS) has been intensively studied. Das Sarma et al. showed that IL-17RA is expressed in some cultured astrocytes (16.8%) and slightly in microglia (0.80%) (37), and Liu et al. (2014) showed that in the adult dentate gyrus, astrocytes mainly

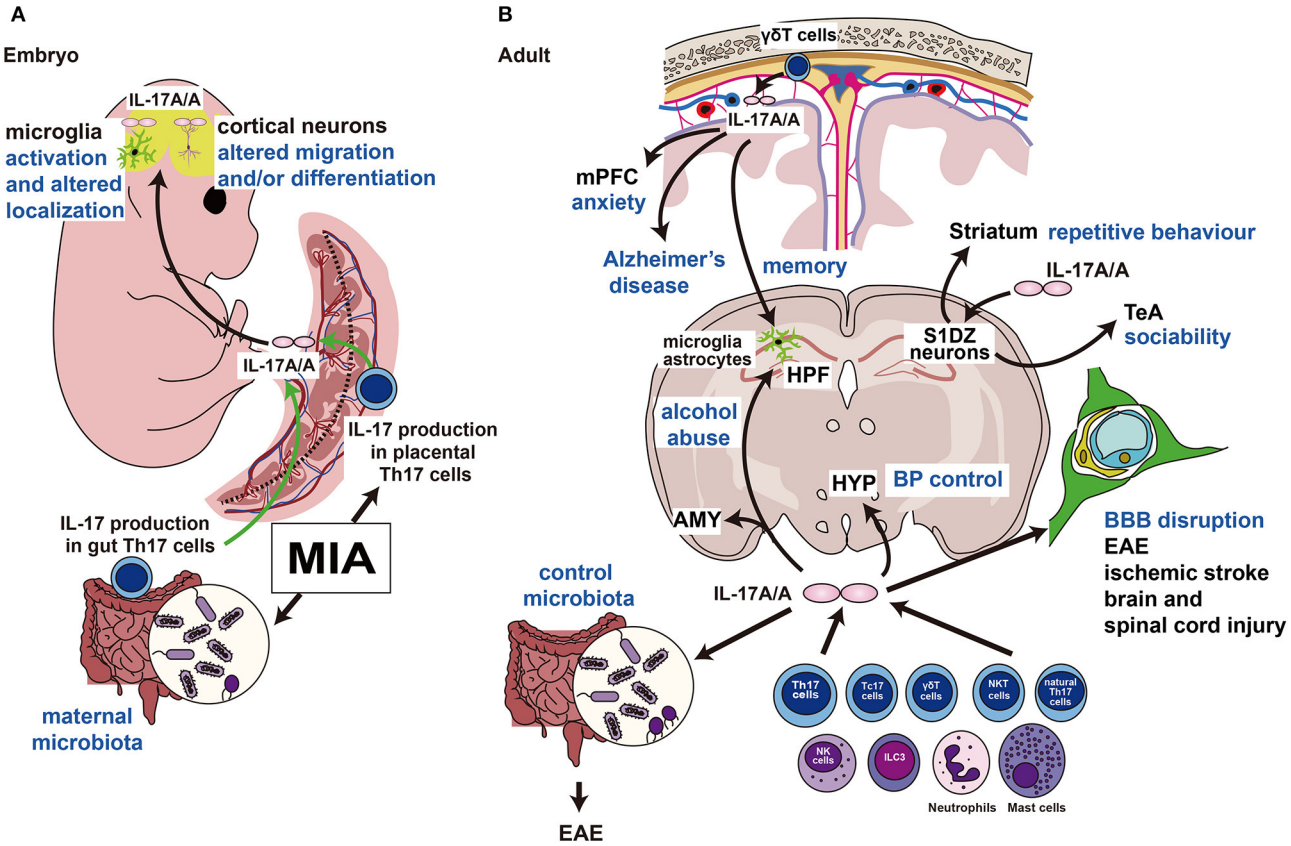

FIGURE 2 | Functions of IL-17A in the embryonic and adult brains. **(A)** MIA induces the production of IL-17A (IL-17A/A indicates the homodimer) in Th17 cells of the maternal intestine and placenta. IL-17A crosses the blood-placental barrier and affects embryonic brain development. **(B)** IL-17A is produced by γδT cells in the cranial meninges and regulates the mPFC, which controls emotion, and HPF, which regulates memory formation. This pathway may be activated in the Alzheimer's disease model. Direct injection of IL-17A into the S1DZ restores the ASD phenotypes of repetitive behavior or sociability. IL-17A also controls the gut microbiota, and its disruption causes autoimmunity. IL17A is involved in the destruction of the BBB in several neurological disorders. Intravenous injection of IL-17A affects the hypothalamus and alters systemic blood pressure. IL, interleukin; MIA, maternal immune activation; Th17, T helper 17; mPFC; medial prefrontal cortex; HPF, hippocampal formation; S1DZ, primary somatosensory cortex dysgranular zone; ASD, autism spectrum disorder; BBB, blood-brain barrier; HYP, hypothalamus; BP, blood pressure; EAE, experimental allergic encephalomyelitis; Tc17, IL-17-producing $CD8^+$ T cells; NKT, natural killer T cells; NK; natural killer cells; ILC3, type 3 innate lymphoid cells; AMY, amygdala; TeA, temporal association area.

express *Il17a* under physiological conditions (38). Their study revealed that IL-17A is a negative regulator of neurogenesis in the adult hippocampus, and *Il17a* knockout enhances synaptic function (38).

In addition to these published results, we obtained the RNA sequencing results from the Human Brain Atlas (https://www.proteinatlas.org/) and Brain RNA-Seq (https://www.brainrnaseq.org/), based on published papers (39, 40). According to these databases, *Il-17a* and *Il-17f* mRNA are rarely expressed in any cell type in the mouse brain; *Il-17ra* mRNA is mainly expressed in macrophages/microglia in small amounts in oligodendrocytes, neurons, and oligodendrocyte precursor cells and is almost absent in astrocytes and endothelial cells. In terms of tissue distribution, a small amount of *Il-17ra* mRNA was observed in the cerebral cortex. Since *Il-17rc* and *rd* mRNA are much more abundant in the pituitary gland, it is necessary to analyze the expression of each isoform of the IL-17 receptor.

Chen et al. used forward genetic methods to show that the *Caenorhabditis elegans* homolog of *Il-17a* functions as a neuromodulator in somatosensory neurons (41). Subsequently, Ribeiro et al. showed that IL-17A controls synaptic plasticity and short-term memory (42) (**Figure 2B**). Intriguingly, IL-17A is secreted by fetal-derived meningeal resident γδT cells and plays an important role in memory formation via glial cell production of brain-derived neurotrophic factor under physiological conditions (42). Furthermore, even under physiological conditions, IL-17A secreted from γδT cells and IL-17RA signaling in neurons of the medial prefrontal cortex controls anxiety-like behaviors, not sociability or memory (43). Alves De Lima et al. also found that the number of meningeal γδT cells increases after birth; therefore, depletion of IL-17A or γδT cells in the postnatal period may affect behavior (43) (**Figure 2B**).

Reed et al. showed the beneficial effects of IL-17A on social behavior disorders (44) (**Figure 2B**). They first detected

abnormalities in the neural circuits responsible for repetitive behavior and sociability examined using the marble burying test and social interaction test, respectively (31). The main focus of abnormal circuits in MIA offspring is the primary somatosensory cortex dysgranular zone (S1DZ). Interestingly, using optogenetics, it was reported that S1DZ neurons projecting to the temporal association cortex control sociability, and S1DZ neurons projecting to the striatum regulate repetitive behavior in MIA offspring (31). On the other hand, lipopolysaccharide administration can restore social behavioral deficits in MIA-exposed offspring. More interestingly, direct IL-17A delivery into the S1DZ can also restore disturbed social behavior even in monogenic ASD mouse models such as *Cntnap2* or *Fmr1* mutant mice (44). The authors concluded that the production of IL-17A during inflammation can ameliorate the expression of social behavior deficits by directly affecting neural activity in the brain (44).

In addition to ASD, IL-17A signaling has received strong attention for its pathophysiological functions in various neurological disorders (45–48) (**Figure 2B**). In particular, the importance of IL-17A has been strongly demonstrated in experimental allergic encephalomyelitis (EAE), a model of multiple sclerosis (45, 47, 48). In a recent study, *Il-17a/f*-deficient mice lost sensitivity to EAE, which correlates with changes in the gut microbiota (49). Another important aspect of IL-17A is the regulation of blood-brain barrier functions (50–52) (**Figure 2B**). Furthermore, the involvement of IL-17A signaling has been revealed in various experimental models of ischemic brain injury (53), traumatic brain injury (54), and spinal cord injury (55, 56) (**Figure 2B**).

In addition to the immunological disorders described above, emerging evidence suggests that IL-17A secreted by meningeal γδT cells regulates the pathogenesis of Alzheimer's disease (57, 58), IL-17A secreted by Th17 cells is involved in alcohol abuse (59), and IL-17A regulates blood pressure via the activation of paraventricular nucleus neurons (60) (**Figure 2B**).

RELATIONSHIP BETWEEN IL-17A EXPOSURE IN THE HUMAN BRAIN AND ASD

It has been reported that neurons, glia, and endothelial cells in the human cortex express receptors for IL-17 (61). However, no information on the specific expression of the IL-17 receptor isoform in the human brain has been reported. Therefore, we obtained the RNA sequencing results from the Human Protein Atlas (https://www.proteinatlas.org/) and Brain RNA-Seq (https://www.brainrnaseq.org/), based on a published paper (62). We found that *IL17A* is rarely expressed in the human brain as revealed by both databases. On the other hand, receptors for the IL-17 family, *IL-17RA* and *RC,* are both expressed in embryonic and adult brains as examined by both databases.

In some patients with ASD, IL-17A has been found at high levels in the blood and correlates with the severity of behavioral symptoms (63, 64). A genome-wide association study showed that copy number variation of the *IL17A* gene is a risk factor for ASD (65). However, the evidence indicated an indirect correlation. Therefore, to show a direct causal relationship between maternal IL-17A exposure and ASD, we propose the following clinical investigation.

First, psoriasis has received much attention in recent years, since maternal psoriasis is also a significant risk factor for ASD (18), and IL-17A is one of the most important cytokines in the pathogenesis of psoriasis (19, 20). Therefore, maternal psoriasis is a candidate disease to be investigated (**Figure 3**).

To modulate IL-17A signaling, three commercially available antibodies are currently available to treat humans: secukinumab (human monoclonal antibody to IL-17A, immunoglobulin [Ig]G1), ixekizumab (humanized monoclonal antibody to IL-17A, IgG4), and brodalumab (human monoclonal antibody to the IL-17 receptor, IgG2). Both secukinumab and ixekizumab are approved for psoriasis, psoriatic arthritis, and ankylosing spondylitis; brodalumab is only approved for the treatment of psoriasis (66). All subclasses of IgG (IgG1–IgG4) cross the human placenta (67), therefore, all candidate antibodies can block the abnormal upregulation of IL-17 signaling in the fetus. Since the human blood brain barrier also begins to form during pregnancy as well as in mice (68), candidate antibodies may enter the fetal brain after angiogenesis. Clinically, no complication with prenatal usage of secukinumab was reported (69).

If the incidence of ASD is reduced in the offspring of pregnant patients with psoriasis treated with IL-17-related antibodies, this would indicate a direct causal relationship between MIA and ASD via IL-17 signaling (**Figure 3**).

Lastly, considering the current pandemic situation, I can't avoid mentioning the topic of COVID-19. To the best our knowledge, no evidence that COVID-19 in pregnant mother could be the risk of ASD in offspring has been reported. However, some reports showed that IL-17A is involved in pathophysiology of COVID-19 infection (70), therefore long term observation will elucidate whether maternal COVID-19 infection may impact fetal brain development (71).

DISCUSSION

The pathophysiological mechanism of ASD or brain development caused by MIA or IL-17A exposure remains to be addressed. As mentioned in section Relationship Between IL-17A Exposure in the Human Brain and ASD, in humans, there is a lack of direct causal relationship between IL-17A and ASD.

Even in experimental animals, the following questions remain elusive. First, MIA has been shown to cause abnormalities in fetal brain development, including unexplained cortical dysgenesis. Wong et al. found that adult offspring exposed to MIA on E14.5 had significantly reduced numbers of either $TBR1^+$ or $SATB2^+$ cells in the cortex with cortical patches (26) [see also Shin Yim et al. (31)]. During cortical development, exposure to MIA and IL-17A transiently delays the production of $SATB2^+$ cells on E14.5 and alters cortical neurogenesis or radial migration only at the medial area with cortical patch formation without changing cortical thickness. Furthermore, IL-17RA is only expressed in cortical plates, and the cell type is unknown (26). These phenotypes cannot

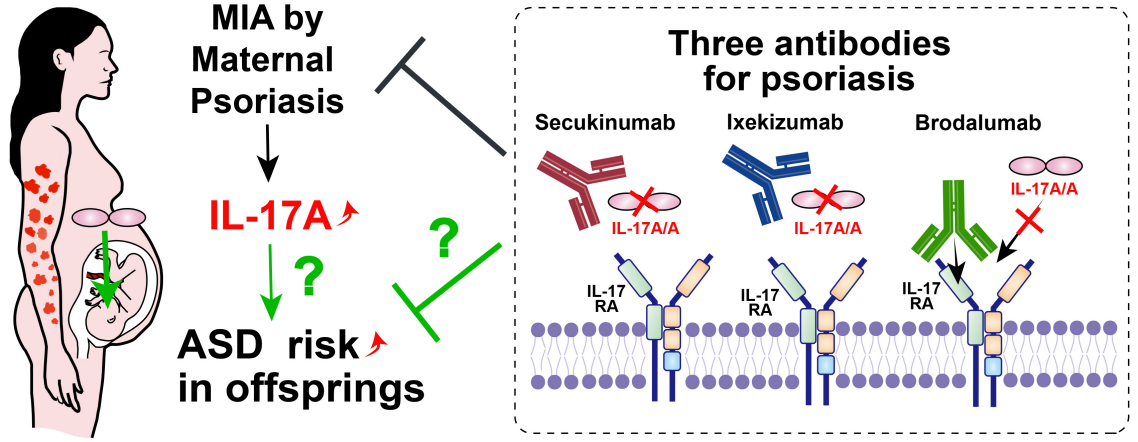

FIGURE 3 | Possible ASD prevention method by modulating MIA with IL-17 related antibodies. Maternal psoriasis is one of the risk factors for offspring ASD. Three available antibodies against IL-17A or IL-17RA for psoriasis, psoriatic arthritis and ankylosing spondylitis could potentially be used for maternal patients. If the risk of ASD was reduced by clinical studies with these antibodies, the direct relationship between MIA and ASD through IL-17 signaling will clearly be revealed.

be explained by abnormalities in neurogenesis or radial migration of the entire radial glia. Another study suggested that microglia may alter the neurogenesis of radial glia or neural migration, as IL-17A injection induces microglia to migrate closer to the lateral ventricles (35). In support of this idea, microglia, but not neurons or other glial types, express the highest amount of *Il-17ra* in the adult stages as examined by databases. Second, it has been suggested that MIA can affect brain development into adulthood with altered systemic immunological responses (30, 72). It has long been unclear why these effects persist, but recent evidence might answer this question. Lim et al. infected pregnant mice with *Yersinia pseudotuberculosis*. Although the infection was restricted to the dam, the offspring surprisingly harbored more intestinal Th17 cells into adulthood via IL-6 signaling (73). Maternal IL-6 induced immediate and long-term effects based on changes in the epigenetic memory of fetal intestinal epithelial stem cells. Therefore, an enhanced response to the microbiota is trained during pregnancy, and the immune response system is already altered at birth (73).

In this review, we summarize how IL-17A affects brain development and adult brain function mostly based on the animal experiments. In the near future, it may be possible to use IL-17A related antibodies to prevent ASD. However, the involvement of IL-17A signaling has not been elucidated yet. Future clinical studies will help to answer this question.

AUTHOR CONTRIBUTIONS

MF: conceptualization and writing—original draft preparation. HM, YO, and XL: writing—review, editing and visualization. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by the Japan Society for the Promotion of Science, JSPS, through 21K16589 and 20K22952 for HM. This research was funded by the Osaka Medical Research Foundation for Intractable Diseases and the Ichiro Kanehara Foundation for the Promotion of Medical Sciences and Medical Care for YO. This research was funded by Shimane Prefecture Technology seeds supporting project: 2021 for MF.

ACKNOWLEDGMENTS

We gratefully acknowledge the work of the present members of our laboratory and Dr. Kuwako's laboratory.

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

frontiers in Psychiatry

REVIEW
published: 09 February 2022
doi: 10.3389/fpsyt.2022.821455

Influence of Prenatal Drug Exposure, Maternal Inflammation, and Parental Aging on the Development of Autism Spectrum Disorder

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Specialty section:
This article was submitted to Autism, a section of the journal Frontiers in Psychiatry

Received: 24 November 2021
Accepted: 12 January 2022
Published: 09 February 2022

Citation:
Sato A, Kotajima-Murakami H, Tanaka M, Katoh Y and Ikeda K (2022) Influence of Prenatal Drug Exposure, Maternal Inflammation, and Parental Aging on the Development of Autism Spectrum Disorder. Front. Psychiatry 13:821455. doi: 10.3389/fpsyt.2022.821455

Autism spectrum disorder (ASD) affects reciprocal social interaction and produces abnormal repetitive, restrictive behaviors and interests. The diverse causes of ASD are divided into genetic alterations and environmental risks. The prevalence of ASD has been rising for several decades, which might be related to environmental risks as it is difficult to consider that the prevalence of genetic disorders related to ASD would increase suddenly. The latter includes (1) exposure to medications, such as valproic acid (VPA) and selective serotonin reuptake inhibitors (SSRIs) (2), maternal complications during pregnancy, including infection and hypertensive disorders of pregnancy, and (3) high parental age. Epidemiological studies have indicated a pathogenetic role of prenatal exposure to VPA and maternal inflammation in the development of ASD. VPA is considered to exert its deleterious effects on the fetal brain through several distinct mechanisms, such as alterations of γ-aminobutyric acid signaling, the inhibition of histone deacetylase, the disruption of folic acid metabolism, and the activation of mammalian target of rapamycin. Maternal inflammation that is caused by different stimuli converges on a higher load of proinflammatory cytokines in the fetal brain. Rodent models of maternal exposure to SSRIs generate ASD-like behavior in offspring, but clinical correlations with these preclinical findings are inconclusive. Hypertensive disorders of pregnancy and advanced parental age increase the risk of ASD in humans, but the mechanisms have been poorly investigated in animal models. Evidence of the mechanisms by which environmental factors are related to ASD is discussed, which may contribute to the development of preventive and therapeutic interventions for ASD.

Keywords: autism spectrum disorder, prenatal drug exposure, valproic acid, selective serotonin reuptake inhibitor, maternal immune activation, hypertensive disorders of pregnancy, advanced parental age

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder with two symptomatic domains: impairments in reciprocal social interaction and repetitive and restrictive behaviors and interests (1). The prevalence of ASD is estimated to be 0.76% worldwide (2), varying across geographic regions, from 0.5% in Asia (3) to 2.5% in the United States (4). Compared with other psychiatric disorders, such as attention-deficit/hyperactivity disorder, ASD has many unanswered questions. There is no evidence of ASD remission in adulthood (2), and there are no effective pharmacotherapies for core symptoms of ASD. Understanding the pathomechanisms of ASD is necessary to develop preventive and therapeutic interventions.

The causes of ASD are divided into genetic alterations and environmental risk factors. Early studies of monozygotic and dizygotic twins estimated that the heritability of ASD is as high as 90% (5). However, subsequent research with improved methodologies showed heritability of 50–60%. Most of this heritability was attributed to common genetic variations, whereas the contribution of rare inherited mutations is lower (6). At the time of this writing, more than 1,200 genes and 2,200 copy number variations are listed in the Autism Database (7) as implicated in ASD (8).

Some of these genes cause congenital disorders, such as Fragile X syndrome and tuberous sclerosis complex. Both of these disorders have been extensively studied in both humans and animal models (9). Genetic analyses of familial ASD cases with no other specific manifestation have also unveiled many copy number variations and genetic mutations (10–12). The introduction of these genetic alterations in animals has contributed to our understanding of common neuronal mechanisms of ASD. For example, brains from mouse models of tuberous sclerosis complex (13, 14) and PTEN tumor hamartoma syndrome (15) exhibited the hyperactivity of mammalian target of rapamycin (mTOR) complex 1 (mTORC1). The inhibition of mTORC1 by rapamycin reversed ASD-related behavioral abnormalities (13–15). Mutations of *SHANK2*, *SHANK3*, *NLGN4*, and *NRXN1α* are considered to cause ASD by altering synaptic connectivity and neuronal excitability, resulting in excitatory/inhibitory (E/I) imbalance (16). A recent large-scale exome sequencing study found significant mutations of 102 genes, most of which were enriched in the excitatory and inhibitory neuronal lineage (17). Investigation of these genetic models has revealed the neuropathological findings in ASD (16). The brain of neuron-specific *Pten* knockout mice exhibited hypertrophy of hippocampal neurons (15). Synaptic density was increased in the cerebellum of *Tsc1*-deficient Purkinje mice (13) and stem cells carrying ASD-related *NLGN4* mutations (16). Lower neuronal excitability was found in *Tsc1*-deficient Purkinje cells (13) and *SHANK3*-deficient neurons (16), whereas neuronal excitability was enhanced in $CNTN5^{+/-}$ and $EHMT2^{+/-}$ cells (16).

The aforementioned evidence, however, does not necessarily explain the increasing prevalence of ASD over recent decades (i.e., from 0.4/1,000 to 2/1,000 in the 1970s to 1.5% or higher in recent studies) (18–20). According to recent studies, environmental risk factors account for the remaining 40–50% of ASD cases (6, 21), which might partially explain the increase in ASD prevalence. Meta-analyses have indicated several significant risk factors, such as advanced parental age, prenatal exposure to antidepressants, and complications during pregnancy (21–23). In contrast to epidemiological evidence, some of these risks are difficult to be reproduced without causing deleterious alterations in rodents, such as premature birth (24, 25) and low birth weight (26, 27), which hampers our understanding of whether and how these perinatal problems might contribute to ASD. The environment-induced ASD models discussed in this review also exhibit neuropathological features similar to those found in genetic models of ASD. However, the relation between each environmental risk and specific feature appears to be complicated. For example, prenatal exposure to valproic acid (VPA) results in ASD through different mechanisms, including disrupted γ-aminobutyric acid (GABA) signaling and mTORC1 hyperactivity. Shorter dendrites and reduced synapse pruning are observed in different ASD models, such as prenatal exposure to selective serotonin transporter inhibitors (SSRIs) and maternal inflammation.

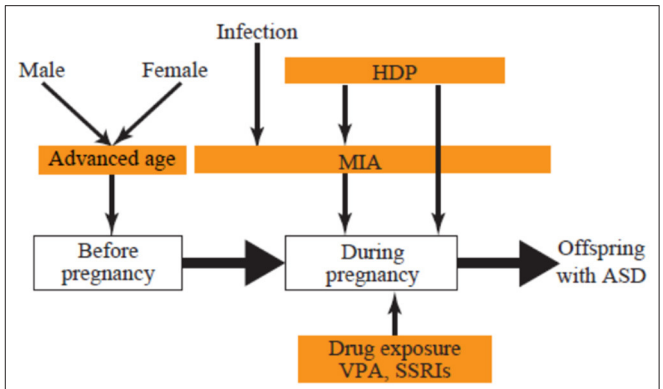

FIGURE 1 | Developmental stage and environmental ASD risks. ASD, autism spectrum disorder; HDP, hypertensive disorders of pregnancy; MIA, maternal immune activation; SSRI, selective serotonin reuptake inhibitor; VPA, valproic acid.

The present review focuses on three groups of environmental risks of ASD: prenatal exposure to drugs, maternal complications during pregnancy, and advanced parental age. We discuss how each of these leads to ASD to better understand the mechanisms, with the goal of mitigating risk and developing potential therapeutics (**Figure 1**).

PRENATAL EXPOSURE TO DRUGS

Exposure to medications during pregnancy is a well-known risk factor for ASD (21–23). Considering the abundance of research on mechanisms, VPA and SSRIs are discussed in this review.

VPA

VPA has been clinically used as an antiepileptic drug for more than 50 years, and it is listed as a first-line drug for

generalized epilepsy (28). Other indications of VPA include the prevention of migraine (29) and treatment of acute mania (30). It has a simple molecular structure (2-propylepntanoic acid) and appears to exert its clinical effects through various mechanisms, including the inhibition of GABA transaminase, voltage-gated Na^+ channels, and T-type Ca^{2+} channels (29).

Despite its excellent clinical efficacy, congenital malformations and neurodevelopmental problems have been observed in children of mothers who took VPA during pregnancy (31). Congenital malformations that are related to VPA include neural tube defects, congenital heart disease, and cleft palate (32). Prenatal exposure to VPA increases the risk of ASD, but other antiepileptic drugs, such as carbamazepine and lamotrigine, do not (33). Children of mothers who used high-dose VPA (>800 mg/day) during pregnancy had a lower IQ at 6 years of age. Low-dose VPA (800 mg/day or less) did not affect IQ in children but was associated with impairments in verbal ability compared with other antiepileptic drugs (34). Prenatal exposure to carbamazepine or clonazepam did not affect the prevalence of ASD in offspring (33), implying that the inhibition of Na^+ channels or potentiation of GABAergic signaling (i.e., effects of VPA) is insufficient to cause ASD. As discussed below, other pharmacological effects of VPA, either alone or combined, may be required. Based on these data, women who are considering becoming pregnant are now recommended to avoid using high-dose VPA by switching to other antiepileptic drugs or reducing VPA to dose to 600 mg/day or less before pregnancy (35). By contrast, those who are found to be pregnant while taking VPA are not advised to switch from VPA to other medications in order to avoid the risk of worsening epilepsy.

In accordance with the human data, rodents that were exposed to VPA *in utero* exhibited similar congenital anomalies and cognitive deficits (36). Rodent models of VPA-induced ASD are generated by administering higher doses of VPA after conception (see **Table 1**), whereas human mothers have an increased risk of having offspring with ASD when they become pregnant while taking VPA (>800 mg/day ≈15 mg/kg/day for increasing the risk of ASD). The methodology by which VPA is used to induce ASD-like behavior is well established, which sheds light on several pathways that lead to ASD.

Alterations of GABAergic Signaling

Although it is one of the main mechanisms of action by which clinical effects manifest, remaining unclear is whether the inhibition of GABA transaminase by VPA is critical for disturbing the development of fetal brains. GABA is synthesized by glutamic acid decarboxylase (GAD), which converts glutamate to GABA, and degraded by GABA transaminase. GABA exerts its action by binding to specific receptors, $GABA_A$ or $GABA_B$ receptors, which results in the opening of Cl^- channels. GABA stimulates the influx of Cl^- ions in the mature brain, resulting in a decrease in intracellular transmembrane potential (90). However, neurons in the immature brain have higher concentrations of intracellular Cl^- because of higher activity of Na-K-Cl cotransporter 1 (NKCC1) that passes Cl^- into neurons. The binding of GABA to GABA receptors in immature neurons causes the efflux of Cl^- and depolarization of neurons (91). This excitatory signaling via GABA is important for normal development of the immature brain, including neurogenesis and synapse formation (92, 93).

The mechanism by which VPA potentiates GABAergic signaling in the fetal brain may be related to the development of ASD. Prenatal treatment with a single dose of VPA, mainly on embryonic day (E) 12.5 in mice (44) and rats (94, 95) but as late as E17 in mice (96) reduced GAD expression in different brain regions that suggests a lower number of GABAergic neurons (94–96), and reduced GABA concentrations and the number of GABA receptors (44). These changes have been associated with impairments in fear conditioning, object recognition (94), and ASD-like social deficits (94, 95). Interestingly, the prenatal inhibition of GABAergic signaling with GABA receptor antagonists, such as picrotoxin administration on E10-12, suppressed neurogenesis in the fetal brain (97) and resulted in ASD-like social deficits in offspring (97, 98). These findings suggest that GABA signaling should be controlled within a limited range to maintain normal development of the fetal brain.

Another mechanism may involve the excitatory-to-inhibitory shift of GABAergic signaling during delivery. This shift may be triggered by oxytocin. Oxytocin blockade on the day before delivery resulted in persistent excitatory GABAergic signaling in the postnatal brain (99). Pups that were born to rats that received VPA on E12 also exhibited diminished excitatory-to-inhibitory shift of GABAergic signaling on postnatal day (P) 15. Maternal pretreatment with bumetanide, an inhibitor of NKCC1, restored the inhibitory GABAergic signaling, and pups of bumetanide-treated mothers exhibited the normalization of isolation-induced ultrasound vocalizations (USVs) (99). In an immature neuron model that utilized cultured cortical neurons that were prepared from P1 rats, 3-day VPA treatment immediately after preparation of the neurons reduced vesicular GABA transporter (VGAT) expression and the number of GABAergic synapses, which persisted for 10 days after cessation of VPA exposure (100). However, VPA did not reduce VGAT expression when treatment began after cultivating the neurons for 8 days. Considering that P1 in rats corresponds to approximately 23 weeks gestation in humans, and P8 around delivery (101), these findings suggest that mid-gestational exposure to VPA produces excitatory-dominant E/I imbalance by blocking the excitatory-to-inhibitory shift of GABAergic signaling or reducing VGAT expression and decreasing GABA synthesis in the postnatal brain. This is further supported by the therapeutic effect of the postnatal restoration of E/I balance for ASD. Postnatal intracerebral administration of the $GABA_A$ receptor agonist clonazepam rescued deficient social novelty in mice that were exposed to VPA on E12.5, whereas the $GABA_B$ receptor agonist baclofen did not (44). The postnatal suppression of glutamatergic signaling using the *N*-methyl-D-aspartate receptor antagonist MK-801 or metabotropic glutamate receptor 5 antagonist MPEP was similarly effective in restoring GABAergic signaling in rodents that were exposed to VPA on E12-13 (41, 81, 86, 102). Prenatal exposure to VPA may cause persistent excitatory-dominant E/I imbalance and ASD, which might be reversed by the postnatal correction of E/I balance.

TABLE 1 | Postnatal treatment in ASD rodent models of prenatal exposure to VPA.

Species/strain	Dose/route/time of exposure	Outcome	Treatment/route/time of exposure	References
ICR mice	300 mg/kg, s.c., E10	↓ Sociability ↓ Social preference, social interaction	0.5–1 mg/kg CP465022, i.p., 30 min before testing 0.15–0.3 mg/kg PF4778574, i.p., 30 min before testing	(37)
ICR mice	300 mg/kg, s.c., E10 400 mg/kg, s.c., E12	↓ Sociability, social preference ↑ Marble burying, self-grooming	0.3 mg/kg donepezil, i.p., P14–40	(38)
C57BL/6 mice	500 mg/kg, i.p., E11	↓ Sociability ↑ Marble burying	30 mg/kg BrBzGCp2, i.p., 10 h before testing	(39)
C57BL/6J mice	600 mg/kg, s.c., E12	↓ Sociability	5 mg/kg rapamycin, i.p., 5 days	(40)
Sprague-Dawley rats	400 mg/kg, s.c., E12	↓ Sociability, social preference ↑ Self-grooming	0.3 mg/kg MK-801, i.p., 30 min before testing 30 mg/kg memantine, i.p., 30 min before testing	(41)
Sprague-Dawley rats	400 mg/kg, s.c., E12	↓ Sociability, social preference ↑ Self-grooming	25–100 mg/kg agmatine, i.p., 30 min before testing	(42)
Sprague-Dawley rats	500 mg/kg, i.p., E12 or E13	↓ Sociability	DBS in bilateral CTN, applied for 3 days	(43)
C57BL/6J mice	500 mg/kg, i.p., E12.5	↓ Sociability, social preference ↑ Marble burying, self-grooming	10 nM clonazepam, i.c., in mPFC, 30 min before testing	(44)
C57BL/6J mice	500 mg/kg, i.p., E12.5	↓ Sociability ↑ Marble burying	10–15 mg/kg E100, i.p., P44–65 1 mg/kg donepezil, i.p., P44–65	(45)
ICR mice	500 mg/kg, i.p., E12.5	↓ Social interaction	50–200 μg/kg oxytocin, i.n., 14 days	(46)
ICR mice	500 mg/kg, i.p., E12.5	↓ Social interaction	0.2 mg/kg risperidone, i.p.,14 days 3 mg/kg aripiprazole, i.p., 14 days	(47)
ICR mice	500 mg/kg, i.p., E12.5	↓ Sociability, social interaction ↑ Marble burying	80–160 μmol/kg betaine, s.c., 20 h before testing	(48)
Tuck-Ordinary mice	500 mg/kg, i.p., E12.5	↓ Sociability, social preference ↑ Marble burying, stereotypy, sociability, social interaction, USVs, marble burying, head-dipping	10–15 mg/kg DL77, i.p., 21 days 1 mg/kg donepezil, i.p., 21 days	(49)
129×C57BL/6J mice	600 mg/kg, i.p., E12.5	↓ Sociability, social preference, USVs ↑ Marble burying	Knock-in of *Oxtr* in bilateral LS	(50)
C57BL/6 mice	600 mg/kg, i.p., E12.5	↓ Sociability, spontaneous alternation	7.5 mg/kg KU55933, i.n., P40	(51)
C57BL/6 mice	600 mg/kg, s.c., E12.5	↓ Sociability ↑ Marble burying, self-grooming	10 mg/kg TC-2153, i.p., 3 h before testing	(52)
C57BL/6J mice	600 mg/kg, s.c., E12.5	↓ Social interaction	10 mg/kg rapamycin, i.p., 2 days	(53)
Sprague-Dawley rats	400 mg/kg, i.p., E12.5	↓ Sociability, social preference ↑ Self-grooming	1 mg/kg rapamycin, i.p., P23–33	(54)
Wistar rats	450 mg/kg, i.p., E12.5	↓ Sociability, social preference, USVs ↑ Self-grooming	3 μg arginine vasopressin, s.c., P1–7	(55)
Sprague-Dawley rats	500 mg/kg, i.p., E12.5	↓ Sociability, social interaction ↑ Marble burying	2.5 μg wortmannin, i.c., in bilateral LA, 30 min before testing	(56)
Sprague-Dawley rats	500 mg/kg, i.p., E12.5	↓ Sociability	DBS in right mPFC, applied for 7 days	(57)
Sprague-Dawley rats	500 mg/kg, i.p., E12.5	↓ Sociability, social preference, spontaneous alternation ↑ Self-grooming	50–500 mg/kg metformin, p.o., P21–50	(58)
Sprague-Dawley rats	500 mg/kg, i.p., E12.5	↓ Sociability ↑ Marble burying, self-grooming	Diet enriched with fenofibrate (∼200 mg/kg), P21–120	(59)
Sprague-Dawley rats	500 mg/kg, i.p., E12.5	↓ Sociability, social preference ↑ Marble burying	0.05 mg/kg URB597, i.p., 2 h before testing	(60)
Wistar rats	500 mg/kg (route not specified), E12.5	↓ Sociability, social interaction, USVs ↑ Head-dipping	1–2.5 ml/kg URB597, i.p., 30 min or 2 h before testing	(61)
Wistar rats	500 mg/kg, i.p., E12.5	↓ Sociability, social interaction, USVs ↑ Marble burying, head-dipping	0.05 mg/kg URB597, i.p., 2 h before testing	(62)
Wistar rats	500 mg/kg, i.p., E12.5	↓ Sociability ↑ Marble burying	10 μg D-cycloserine, i.c., in bilateral LA, 30 min before testing	(63)
Wistar rats	500 mg/kg, i.p., E12.5	↓ Sociability, social interaction, spontaneous alternation	10–20 mg/kg pioglitazone, p.o., P21–48	(64)
Wistar rats	500 mg/kg, i.p., E12.5	↓ Sociability, social interaction, spontaneous alternation	100–200 mg/kg fenofibrate, p.o., P21–48	(65)

(Continued)

TABLE 1 | Continued

Species/strain	Dose/route/time of exposure	Outcome	Treatment/route/time of exposure	References
Wistar rats	500 mg/kg, i.p., E12.5	↓ Sociability, social preference ↑ Self-grooming	20–100 mg/kg cannabidivarin, i.p., P34–58	(66)
Wistar rats	500 mg/kg, i.p., E12.5	↓ Sociability, social preference	1 mg/kg LP-211, i.p., P21–27	(67)
Wistar rats	500 mg/kg, i.p., E12.5	↓ Sociability, social preference, spontaneous alternation	10–20 mg/kg vinpocetine, p.o., P21–48	(68)
Wistar rats	500 mg/kg, i.p., E12.5	↓ Sociability, social preference, spontaneous alternation	3–30 mg/kg papaverine, i.p., P21–48	(69)
Wistar rats	500 mg/kg, i.p., E12.5	↓ Sociability, social preference, spontaneous alternation	30–60 mg/kg cilostazol, p.o., P21–48	(70)
Sprague-Dawley rats	600 mg/kg, s.c., E12.5	↓ Sociability	10 mg/kg PF3845, i.p., 2 h before testing	(71)
Sprague-Dawley rats	600 mg/kg, i.p., E12.5	↓ Social interaction	2.5 ml/kg cerebrolysin (route not specified),14 days	(72)
Sprague-Dawley rats	600 mg/kg, p.o., E12.5	↓ Sociability, social preference, social interaction, spontaneous alternation ↑ Self-grooming	1–30 mg/kg 5-ALA, p.o., P21–56 12 μg/kg oxytocin, i.n., P21–56	(73)
Sprague-Dawley rats	600 mg/kg, i.p., E12.5	↓ Sociability	3.5 mg/kg MS-275, i.p., P35–42 6 mg/kg retinoic acid, p.o., P35–42	(74)
Wistar rats	600 mg/kg, i.p., E12.5	↓ Sociability, social interaction ↑ Stereotypy	Environmental enrichment, P22–35	(75)
Wistar rats	600 mg/kg, i.p., E12.5	↓ Olfactory habituation/dishabituation, social interaction ↑ Self-grooming	80,000 IU/kg vitamin D3, i.m., P12	(76)
Wistar rats	600 mg/kg, i.p., E12.5	↓ Sociability, social preference	1 mg/kg fingolimod, p.o., P15–35	(77)
Wistar rats	600 mg/kg, i.p., E12.5	↓ Social interaction ↑Self-grooming	4 mg/kg rapamycin, p.o., P24–34	(78)
Wistar rats	600 mg/kg, i.p., E12.5	↓ Sociability ↑ Self-grooming	20 μg oxytocin, i.n., P40 3 μg oxytocin, s.c., P0–6	(79)
Wistar rats	600 mg/kg, i.p., E12.5	↓ Sociability, social interaction ↑ Self-grooming	10 mg/kg Dapt, i.p., 10 days	(80)
Wistar rats	600 mg/kg, s.c., E12.5	↓ Sociability, social preference ↑ Self-grooming	0.03 mg/kg MK-801, i.p., P6–10	(81)
Wistar rats	600 mg/kg, i.p., E12.5	↓ Olfactory habituation/dishabituation, social interaction	Diet enriched with n-6 polyunsaturated fatty acid, P21–**77**	(82)
Wistar rats	600 mg/kg, i.p., E12.5	↓ Sociability, social preference, social recognition memory	0.03–0.1 mg/kg cariprazine, p.o., 7 days 0.1 mg/kg risperidone, p.o., 7 days 1 mg/kg aripiprazole, p.o., 7 days	(83)
Wistar rats	600 mg/kg, i.p., E12.5	↓ Sociability, social preference ↑ Marble burying	30 mg/kg dextromethorphan, i.p., P23–43	(84)
Wistar rats	600 mg/kg, i.p., E12.5	↓ Sociability, social preference ↑ Marble burying, self-grooming	1–10 mg/kg JZL184, i.p., PND21–34 40 mg/kg JZL184, i.p., 2 h before testing	(85)
C57BL/6Hsd mice	600 mg/kg, s.c., E13	↓ USVs, sociability ↑ Self-grooming	10 mg/kg MPEP, i.p., 5 min before testing	(81)
C57BL/6Hsd mice	600 mg/kg, s.c., E13	↑ Marble burying, self-grooming	20 mg/kg MPEP, i.p., 10 min before testing	(86)
C57BL/6J mice	400 mg/kg, i.p., E13.5	↓ Social interaction, spontaneous alternation ↑ Rearing, self-grooming	200 mg/kg sodium phenylbutyrate, i.p., P21–63 200 mg/kg sodium phenylbutyrate, i.p., 30 min before testing 320 mg/kg D-cycloserine, i.p., P21–63	(87)
C57BL/6J mice DBA/2 mice	600 mg/kg, i.p., E13.5	↓ Social preference	30 mg/kg resveratrol, i.p., 24 h before testing	(88)
Sprague-Dawley rats	(Not found in text)	↓ Social interaction	0.63–10 mg/kg F17464, i.p., 30 min before testing 0.16–5 mg/kg fenobam, i.p., 30 min before testing 0.16–2.5 mg/kg memantine, i.p., 30 min before testing	(89)

BrBzGCp2, S-p-bromobenzylglutathione cyclopentyl diester; CTN, central thalamic nuclei; Dapt, (3,5-difluorophenacetyl)-L-alanyl-S-phenylglycine-2-butyl Ester; DBS, deep brain stimulation; E, embryonic day; i.c., intracerebral; i.m., intramuscular; i.n., intranasal; i.p., intraperitoneal; LA, lateral amygdala; LS, lateral septum; mPFC, medial prefrontal cortex; P, postnatal day; p.o., per os; s.c., subcutaneous; USVs, ultrasound vocalizations.

Disturbances in Folic Acid Metabolism

The second mechanism by which VPA is implicated in ASD is the association between VPA and folic acid deficiency. Neural tube defects (i.e., a congenital malformation of the spinal cord in which the neural tube fails to close during embryogenesis and results in exposure of the "unclosed" spinal cord, also called spina bifida) were shown to be related to vitamin deficiencies, including folic acid deficiency (103). A randomized control study found that daily folic acid supplementation reduced the risk of neural tube defects, but other vitamins did not (104). Unexpectedly, children who were exposed to VPA *in utero* had a higher risk of neural tube defects (32) and ASD (33), similar to children who were born to women with folic acid deficiency during pregnancy. These observations raised the possibility that folic acid supplementation may be useful for the prevention of VPA-related conditions, such as neural tube defects and ASD. Folic acid supplementation before and during pregnancy significantly reduced the risk of ASD in children who were exposed to antiepileptic drugs, including VPA (105). Interestingly, folic acid supplementation lowered the risk of ASD in offspring to less than half, regardless of the concomitant use of antiepileptic drugs (106).

Despite these clinical findings that strongly suggest a similarity between prenatal exposure to VPA and folic acid deficiency in the development of ASD, little is known about the underlying mechanisms by which these two conditions result in ASD or the mechanisms by which folic acid prevents ASD. In a study of human placentas from women without epilepsy, VPA perfusion for 3 h reduced placental concentration of folic acid by ∼30% and suppressed mRNA levels of the *FOLR1* gene, which encodes folate receptor α (107). VPA is also a non-competitive inhibitor of high-affinity folate receptors, such as folate receptor α (108), and may disturb folic acid metabolism (109). The preventive effect of maternal folic acid supplementation was recapitulated in rodents with regard to neural tube defects. VPA administration in pregnant ICR mice on E8 caused neural tube defects, which were prevented by oral folic acid administration prior to the VPA injection (110). A recent study investigated the effect of folic acid supplementation on VPA-induced ASD. Neuropathological changes in offspring that were prenatally exposed to VPA on E12.5 included an increase in dendritic spine density, an increase in the expression of vesicular glutamate transporter 1 (VGLUT1) and postsynaptic density 95 (PSD95; i.e., markers of excitatory neurons), and a decrease in the expression of GAD65 and gephyrin (i.e., markers of inhibitory neurons) (111), suggesting a net result of excitatory-dominant E/I imbalance. The offspring also exhibited ASD-like behavioral deficits that were dose-dependently prevented by maternal folic acid supplementation from E1 to E12.5 (111), which was consistent with the lower prevalence of ASD that correlates with maternal folic acid supplementation (33). Further studies should investigate how folic acid normalizes VPA-induced alterations in the fetal brain.

Inhibition of Histone Deacetylase

Two other mechanisms may also link *in utero* VPA exposure to ASD in offspring, although they seem less relevant to clinical effects: inhibition of histone deacetylase (HDAC) and activation of the mTORC1 signaling pathway. Trichostatin A, an established HDAC inhibitor, was teratogenic in *Xenopus* embryos similarly to VPA (112). VPA and trichostatin A also reduced VGAT expression in cortical neurons obtained from P1 rats (100). This change is considered to result in lower extracellular GABA levels and the disruption of E/I balance toward predominately excitatory transmission, which was observed in another study that employed a rat model of prenatal VPA exposure (111). Moreover, maternal exposure to VPA and trichostatin A on E12.5 decreased USVs and sociability (113). These alterations that are associated with VPA and trichostatin A appear to be caused by HDAC inhibition. Valpromide, an analog of VPA that lacks HDAC inhibitor activity, failed to recapitulate phenotypic changes that were induced by VPA. At the histological level, valpromide administration did not reduce VGAT expression in cortical neurons (100) or global gene expression in the embryonic telencephalon (114). The number of Nissl-positive cells was comparable in the prefrontal and somatosensory cortices when valpromide was administered (115). No congenital malformation (112), deficits in social interaction, anxiety-related behavior, or learning and memory deficits (115) were observed in rodent offspring that were exposed to valpromide *in utero*. These findings emphasize that the HDAC inhibition by VPA affects normal embryogenesis and neuronal development that leads to ASD.

Activation of mTORC1

Another mechanism of VPA-induced ASD is activation of the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mTORC1 pathway (116). Briefly, the mTORC1 signaling pathway is stimulated by growth factors and altered energy levels, and activates mTORC1 that results in stimulation of cell growth and proliferation (117). These effects are mediated by the phosphorylation of downstream signaling molecules, including ribosomal protein S6 kinases (S6Ks) that regulate global protein synthesis, ULK-51-like kinase 1 (ULK1) that is involved in initiating macroautophagy, and eukaryotic translation initiation factor-4E (eIF4E)-binding proteins (4E-BPs) that control cap-dependent translation (117). mTORC1 hyperactivity underlies ASD in human diseases that are caused by mutations of genes that are upstream of mTORC1, such as tuberous sclerosis complex (13, 14) and PTEN tumor hamartoma syndrome (15). Rodent models in which genes that are downstream of mTORC1 were deleted also exhibited ASD-like behavioral changes that were associated with excessive protein synthesis, impairments in autophagy, and alterations of the gene translation profile (118). In summary, alterations of cell growth and proliferation that are under the control of mTORC1 result in impairments in social behaviors that are relevant to ASD.

Both mice (40, 53) and rats (54) that were exposed to VPA on E12 exhibited an increase in mTORC1 activity in the brain and ASD-like behaviors, such as a decrease in social interaction and increase in self-grooming (40, 53, 54). With regard to developmental delays in children who are prenatally exposed to VPA (34), VPA-exposed mice exhibited deficits in early postnatal development, including eye opening, the righting reflex, and performance in the hanging wire test (53). Related to these

behavioral changes, postnatal brains of these animals exhibited signs of the suppression of autophagy (40, 54), one of the main consequences of mTORC1 hyperactivity (117, 118). The expression of a set of genes was also changed, including Fyb, which functions downstream of the mTORC1 signaling pathway (53). Deficits in behavior, autophagy, and gene expression were all reversed by postnatal treatment with the mTORC1 inhibitor rapamycin (40, 53, 54). The constitutive activation of mTORC1 is likely a treatable mechanism by which social behavior is disrupted in VPA-induced ASD.

SSRIs

SSRIs, Serotonin Metabolism, and ASD in Humans

Serotonin (5-hydroxytryptamine [5-HT]) is a neurotransmitter that is involved in diverse brain functions, including motor activity and emotion. When released from presynaptic vesicles, 5-HT binds to post- and presynaptic receptors. Serotonin is then either degraded or collected by the serotonin transporter (SERT). Serotonin plays a critical role in neurodevelopment, including the development of serotonergic fibers (119). SSRIs are a group of drugs that inhibit the SERT and elevate extracellular 5-HT levels in the brain (120). SSRIs are used as a first-line treatment of major depressive disorder (121). The prevalence of depression during pregnancy is estimated to be as high as 10%, and its treatment is often indicated during pregnancy (122, 123).

The role of alterations of serotoninergic signaling in ASD was suggested by the observation that approximately 30% of individuals with ASD have high mean blood levels of 5-HT (124). The maternal use of SSRIs is speculated to elevate maternal 5-HT levels, which might affect fetal brain development and result in a higher risk of ASD in offspring. Case-control studies found a 2-fold increase in the risk of ASD in children of mothers who reported antidepressant use during pregnancy (125, 126). This effect was particularly strong when antidepressants were used during the first trimester of pregnancy, regardless of whether or not the mothers had depression (125). In another cohort study, the use of SSRIs during the second and third trimesters of pregnancy doubled the risk of ASD in offspring, regardless of a maternal history of depression (127). However, later studies reported inconsistent findings with regard to the association between antidepressant use during pregnancy and ASD in offspring (128). A meta-analysis reported an association between the pre-pregnancy maternal use of antidepressants and ASD in offspring and not during pregnancy (23). Another case-control study found that the risk of neurodevelopmental disorders, including ASD, was associated with maternal psychiatric conditions but not the maternal use of SSRIs during pregnancy (129). A nationwide cohort study in Finland reported the influence of prenatal SSRI use on a higher incidence of depression but not ASD (130). In summary, the relationship between ASD and maternal conditions, such as psychiatric comorbidity and antidepressant use, remains elusive and requires further epidemiological studies.

SSRIs, Serotonin Metabolism, and ASD in Animal Models

Investigations of pregnant rodents have improved our understanding of 5-HT dynamics and its alterations by SSRIs during pregnancy. Analyses of *Pet1* knockout mice, in which most dorsal raphe neurons lacked 5-HT, revealed that 5-HT in the fetal forebrain was of placental and not maternal or fetal origin (131). The blockade of SERTs using the serotonin-norepinephrine transporter inhibitor venlafaxine by gavage from E8 to E20 decreased placental weight and SERT expression in the placenta in rats (132). The inhibition of 5-HT signaling with the 5-HT_2 receptor antagonist ketanserin by gavage from E15 to E20 reduced placental weight and placental blood flow in rats (133). SSRI use during pregnancy could result in lifelong consequences on the brain in offspring (134), but the underlying mechanism is complex, including fetal exposure to SSRIs and alterations of 5-HT supply from the placenta. Decreases in placental weight and blood flow that are associated with maternal treatment with SSRIs could also lower the supply of oxygen and nutrients to the fetus and result in lower offspring weight, which might affect fetal brain development.

In contrast to VPA, few studies have investigated how prenatal exposure to SSRIs affects social behavior in offspring (**Table 2**). At the behavioral level, fluoxetine administration in mice that began before pregnancy or from early gestation to late-gestation or delivery produced ASD-like behavioral deficits in offspring, decreased USVs in pups, disrupted social interaction, enhanced social dominance, and increased tactile hypersensitivity (135–137). Citalopram, an SSRI with particularly high specificity for blocking SERT compared with dopamine and norepinephrine transporters, also altered behavior in mouse offspring, decreased sociability, decreased social preference, decreased locomotor activity, and increased anxiety-related behavior when given during late gestation (138). In fetal brains that were exposed to fluoxetine, neurons in the prefrontal cortex exhibited a reduction of the frequency of inhibitory synaptic currents, and interneurons exhibited an increase in intrinsic and serotonin-induced excitability (136). Prefrontal cortex tissue from the fluoxetine-exposed brain exhibited high mRNA levels of 5-HT_{2A} receptor (136). Striatal extracts from mice that were prenatally exposed to citalopram expressed higher levels of NMDAR1 and CaMKIIα, which were associated with morphological changes in the striatal neurons and decreases in dendritic length, number, and branch patterns (138). Prenatal exposure to SSRIs is suggested to result in excessive 5-HT signaling and altered E/I balance.

The effects of therapeutic interventions in these models also appear to be consistent with these findings. High 5-HT level in the brain that are caused by reexposure to fluoxetine in adulthood recovered tactile hypersensitivity (135). The 5-HT_{2A} receptor antagonist MDL100907 suppressed abnormal excitability in neurons in the prefrontal cortex and reversed social preference in a model of fluoxetine-induced ASD (136). In a citalopram model, high levels of NMDAR1 and CaMKIIα were normalized by postnatal treatment with memantine, which was associated with the recovery of sociability and social preference (138). More

TABLE 2 | Rodent models of prenatal exposure to SSRIs.

Species/strain	Compound/dose/route/time of exposure	Behavioral outcome	Treatment/route/time of exposure	References
C57BL/6J mice	16 mg/kg fluoxetine, p.o., before mating to E16, delivery, or P14	↓ USVs, sociability	—	(135)
C57BL/6 mice	15 mg/kg fluoxetine, p.o., before mating to P14	↓ Sociability, social preference	—	(137)
C57BL/6J mice	0.6 mg/kg fluoxetine, i.p., E4–19	↓ Spontaneous alteration, social preference	0.01 mg/kg MDL100907, i.p., 30 min before testing	(136)
C57BL/6 mice	20 mg/kg citalopram, i.p., E13 to delivery	↓ Sociability, social preference	10 mg/kg memantine, i.p., 20 min before testing	(138)

E, embryonic day; i.p., intraperitoneal; P, postnatal day; p.o., per os; USVs, ultrasound vocalizations.

research is required to determine whether and how exposure to SSRIs *in utero* affects fetal brain development and causes social deficits.

The aforementioned interventions can be partially replicated by manipulating genes that are involved in 5-HT neurotransmission because 5-HT levels are consistently changed in these models (i.e., either elevated or depleted). The most extensively investigated models are *SERT* knockout mice and rats (139). In these animals, extracellular 5-HT levels are several times higher, and consequently the density of 5-HT_{1A} and 5-HT_{1B} receptors is decreased (140–144). Diverse behavioral phenotypes are observed in *SERT* knockout rodents, such as an increase in anxiety and fear (139), that may affect social behavior in *SERT* knockout animals. Intact sociability was observed in *Sert* $^{-/-}$ and *Sert* $^{+/-}$ mice, reflected by the time spent sniffing a novel mouse or a novel object (145). The heterozygous loss of *Sert* aggravated deficient sociability in *Pten*$^{+/-}$ mice, a genetic model of ASD that is associated with the activation of mTORC1 activity (146). Reciprocal social interaction in the resident-intruder paradigm was unaffected in *Sert* $^{-/-}$ and *Sert* $^{+/-}$ rats (147). A recent study reported deficits in social interaction, sociability, and social novelty in *Sert* $^{-/-}$ and *Sert* $^{+/-}$ mice (148). This was associated with high levels of 5-HT in the brain in *Sert* $^{-/-}$ mice but not in *Sert* $^{+/-}$ mice. Impairments in social interaction were ameliorated by restricting the dietary intake of tryptophan (i.e., the precursor of 5-HT), which lowers 5-HT levels in the brain (148). Constitutively elevated 5-HT levels are thus considered to disrupt social behavior.

Reducing 5-HT concentrations in the brain, opposite to *SERT* deletion, may also give rise to ASD. One method to reduce 5-HT levels is to introduce a gain-of-function mutation of the *SERT* gene. *SERT* Ala56 mice that expressed an ASD-associated variant in humans exhibited elevations of 5-HT clearance in the brain and ASD-related social impairments and repetitive behavior, but forebrain 5-HT levels did not change in the mutants (149). This increase in 5-HT clearance was reversed by MW150, a p38α mitogen-activated protein kinase inhibitor, which also normalized social dominance in the tube test (150). Brain 5-HT levels can also be depleted by deleting the tryptophan hydroxylase 2 (*TPH2*) gene, which is essential for synthesizing 5-HT in the brain. *TPH2* knockout mice exhibited diverse ASD-related behaviors, including impairments in social interaction, an increase in marble burying, and deficient early developmental milestones (151). Female *TPH2* knockout mice exhibited high levels of aggression against co-housed mice and an increase in defensive behavior when paired with a knockout mouse (152). These studies suggest that prenatal increases and decreases in fetal 5-HT levels may result in the subsequent development of ASD.

MATERNAL IMMUNE ACTIVATION

Another possible environmental risk factor for ASD is maternal inflammation that is induced by infection during pregnancy. Associations between ASD and prenatal infection with specific pathogens, such as rubella and cytomegalovirus, have been repeatedly reported (153). Meta-analyses revealed a mild but significant increase in the risk of ASD in children of mothers who experienced infection during pregnancy, regardless of the pathogen (154, 155). One study estimated that maternal infection accounts for 12–17% of ASD cases (155). The risk of ASD appears to correlate with the severity of maternal infection, in which the risk was further elevated when mothers required hospitalization because of the infection (154). In another meta-analysis, maternal fever during pregnancy, regardless of whether it was caused by infection, increased the risk of neurodevelopmental disorders (156). The invasion of pathogens does not appear to be essential for the development of ASD in offspring; instead, the maternal inflammatory response itself seems sufficient.

Maternal immune activation (MIA) and cytokine production following infection are likely central mechanisms that link maternal infection and ASD in offspring (157). Briefly, MIA-induced ASD appears to begin with the selective activation of Th1 cells, consequently resulting in high interleukin-6 (IL-6) levels, but other mechanisms may also mediate the development of ASD. IL-6 activates retinoic acid receptor-related orphan nuclear receptor γt (RORγt) in naive $CD4^+$ T cells, which stimulates its differentiation into Th17 cells, Activated Th17 cells produce cytokines, including IL-17A that may be critically involved in the development of ASD (158–160). Microglia are local macrophages in the brain that mediate MIA and neurodevelopment. Normal functions of microglia include neurogenesis and synapse pruning, playing an important role in synaptic plasticity (161). The production of proinflammatory cytokines by MIA, such as IL-6 and IL-17, results in microglial

activation, which suppresses synapse pruning particularly in the hippocampus and disrupts synapse function (162). The relation between MIA and the development of ASD in offspring has been demonstrated in both humans and in rodents.

Maternal Infection, Immune Activation, and ASD in Humans

Findings are limited about markers that are suggestive that children with ASD were exposed to MIA *in utero*. The Early markers for Autism study found that high levels of IL-4, IL-5 and interferon-γ (IFN-γ) in maternal serum at 15–19 weeks of gestation were associated with a 50% higher risk of ASD, whereas high levels of IL-2, IL-4 and IL-6 was associated with developmental delay in the absence of ASD (163). Using a larger sample set, a subsequent analysis found high levels of many cytokines, including IL-1α and IL-6, in mothers of children with ASD and developmental delay compared with children with ASD without developmental delay and controls (164). The cytokine profiles of neonates that were exposed to MIA may reflect immunological alterations in their mothers, but the presence of neonatal cytokines was determined using dried blood spots from the neonates, and cytokines in these samples might be unstable. A study did not find changes in these cytokines, such as increase in IL-6; instead, Th1 and Th2 cytokine levels decreased (165) or were comparable between ASD children and controls (166). High IL-4 levels were associated with a higher risk of severe ASD (odds ratio o = 1.4), and higher IL-1β were associated with mild or moderate ASD (odds ratio = 3.02) (167). Children with ASD had high levels of IL-6 and IL-8 during neonatal periods compared with controls (168). No differences were found in cytokine levels between children with developmental delay in the absence of ASD and controls (167, 168).

High levels of proinflammatory cytokines may persist beyond the neonatal period. Two-to 5-year-old children with ASD had high plasma levels of the proinflammatory cytokine IL-6 compared with age-matched typically developing controls and those with developmental disabilities other than ASD (169). Brains of ASD patients also had high cytokine levels, including IL-6 and the Th1 cytokine IFN-γ, whereas levels of Th2 cytokines (i.e., IL-4, IL-5, and IL-10) were not elevated in brains in ASD patients (170). An increase in serum IL-17A levels was also detected in 6- to 11-year-old ASD children (171). The phytohemagglutinin-induced stimulation of peripheral blood mononuclear cells resulted in the production of IL-17 but not Th2 cytokines IL-4 and IL-13 in 2- to 5-year-old ASD children, whereas cytokine levels at baseline were comparable between ASD children and controls (172). Plasma levels of IL-17 and IL-1β remained elevated in 7- to 15-year-old ASD individuals (173). Heavy cytokine burden may be related to a more severe ASD phenotype. Children with the regressive form of ASD exhibited a significant increase in IL-1β and IL-6 levels, whereas those without the regressive form did not (169). Most children who had high serum IL-17A levels had severe ASD, based on the Childhood Autism Rating Scale (171). Another study, however, did not find a correlation between the cytokine profile and clinical variables, including the severity of ASD, was not found (173). Altogether, exposure to IL-6 and IL-17, rather than direct effects of pathogens, may be important for MIA-induced ASD.

Modeling Maternal Immune Activation in Animals

To investigate the mechanisms of MIA-related ASD in disease models, two approaches have been utilized. One is to inoculate human pathogens, and the other is to administer proinflammatory compounds (**Table 3**).

Pathogens that are administered in pregnant rodents range from viruses to bacteria and parasites, and their effects on ASD-related behavioral changes have been examined. Human influenza virus infection in pregnant mice on E9 reduced social interaction (174, 175). Offspring that were exposed to high virus titers exhibited lower 5-HT levels through an increase in metabolism and decrease in levels of oxytocin (175). An injection of group B Streptococcus, a major bacterial pathogen in pregnant women and neonates, impaired social behavior, decreased social interaction, and decreased USVs when inoculated in late gestation in rats (179, 180). These deficits were accompanied by white matter damage in the external capsule and corpus callosum (179, 180). Mice that were born to mothers that were infected with *Mycobacterium tuberculosis* on E12.5 exhibited deficits in approach to social novelty and social preference, and these effects were associated with high plasma IL-6 and IL-17A levels (176). A maternal injection of the soluble tachyzoite antigen of *Toxoplasma gondii* on E14.5 also elicited MIA and produced ASD-like behavior, such as impairments in social approach, an increase in self-grooming, and an increase in marble-burying behavior in offspring (178). Thus, maternal infection in mid- to late-gestation is considered to lead to ASD, regardless of the specific pathogen.

Given that different pathogens cause ASD-like behavioral alterations in rodents, common downstream mechanisms have been sought. Substances that induce MIA include the synthetic double-strand RNA polyinosine-polycytidylic acid (poly(I:C)), which mimics viral infection, lipopolysaccharide (LPS), which mimics bacterial infection, and ILs that are induced by inflammation, such as IL-6. Double-strand RNA is produced by most viruses during replication. A poly(I:C) injection causes the production of proinflammatory cytokines through Toll-like receptor 3 (221). A maternal injection of poly(I:C) on E12.5 promoted an ASD-like phenotype in offspring, including an increase in USVs, a decrease in sociability, and disorganized cortical cytoarchitecture (195). Offspring from LPS-injected mothers on E14 exhibited a decrease in USVs, an increase in marble burying behavior, and a decrease in interest in a novel mouse (208). These findings suggest that MIA models that use agents that mimic MIA contribute to revealing the detailed mechanisms of MIA-related ASD.

Infection or poly(I:C) induces the production of IL-6, which can cross the placenta and affect the fetal brain (222, 223). A maternal injection of IL-6 on E12.5 was sufficient to cause behavioral alterations of preference for social novelty in offspring. The co-administration of anti-IL-6 antibody ameliorated these alterations, but anti-IFN-γ antibody did not. Moreover, maternal

TABLE 3 | Maternal immune activation models of ASD in rodents.

Species/strain	Compound/dose/route/time of exposure	Behavioral outcome	Treatment/route/time of exposure	References
BALB/c mice	6,000 pfu influenza A, i.n., E9.5	↓ Social interaction	—	(174)
BALB/c mice	75–600 pfu Influenza A (H3N2), i.n., E9.5	↓ Social interaction	—	(175)
Balb/c mice	10^8 cfu *Mycobacterium tuberculosis*, aerosol infection, E12.5	↓ Sociability, social preference ↑ Self-grooming	—	(176)
C57BL/6J mice	200 μg/kg Staphylococcal enterotoxin A or B, i.p., E12.5	↓ Sociability	—	(177)
C57BL/6 mice	90 μg soluble tachyzoite antigen from *Toxoplasma gondii*, i.p., E14.5	↓ Sociability, social preference, social interaction ↑ Marble burying, self-grooming	Transfer of maternal regulatory T cells	(178)
Lewis rats	10^8-10^9 cfu group B Streptococcus, i.p., E19	↓ Social interaction, USVs	—	(179)
Rats	10^9 cfu group B Streptococcus, i.p., every 12 h from E19 to delivery	↓ Olfactory discrimination, social interaction	—	(180)
C57BL/6N mice	5 mg/kg poly(I:C), i.v., E9	↓ Sociability ↑ Marble burying	Maternal injection of 1,25OHD, s.c., E9	(181)
C57BL/6 mice	5 mg/kg poly(I:C), i.p., E10.5, E12.5, and E14.5	↓ Sociability ↑ Marble burying, USVs	—	(182)
C57BL/6 mice	20 mg/kg poly(I:C), i.p., E11.5–12.5	↓ Sociability, USVs ↑ Marble burying	Maternal injection of anti-IL-17A antibody, i.p., E11.5	(183)
C57BL/6J mice	3 mg/kg poly(I:C), i.p., E12.5	↓ Sociability	Genetic removal of *Nox1*	(184)
BTBR mice	20 mg/kg poly(I:C), i.p., E12.5	↓ Sociability ↑ Marble burying, self-grooming (M), USVs (PND8, 10)	—	(185)
C57BL/6J mice	20 mg/kg poly(I:C), i.p., E12.5	↓ Sociability	Maternal injection of anti-IL-6 antibody, i.p., E12.5	(186)
C57BL/6J mice	20 mg/kg poly(I:C), i.p., E12.5	↓ Sociability, reversal learning ↑ Self-grooming	—	(187)
C57BL/6J mice	20 mg/kg poly(I:C), i.p., E12.5	↓ Sociability	—	(188)
C57BL/6J mice	20 mg/kg poly(I:C), i.p., E12.5	↓ Sociability, USVs ↑ Marble burying	20 mg/kg RS102895, i.p., P10 Genetic removal of CCR2 in monocytes	(189)
C57BL/6J mice	20 mg/kg poly(I:C), i.p., E12.5	↓ Social preference	—	(190)
C57BL/6J mice	20 mg/kg poly(I:C), i.p., E12.5	↓ Sociability, social preference, social interaction ↑ Marble burying	0.0625 mg/kg clonazepam, i.p., single injection	(191)
C57BL/6N mice	20 mg/kg poly(I:C), i.p., E12.5	↑ Marble burying	Maternal diet enriched with choline	(192)
C57BL/6N mice	20 mg/kg poly(I:C), i.p., E12.5	↓ Sociability ↑ Marble burying	Genetic deletion of *Il6ra* in placental trophoblasts	(193)
C57BL/6 mice	20 mg/kg poly(I:C), i.p., E12.5	↓ Sociability ↑ Marble burying (M)	—	(194)
C57BL/6 mice	20 mg/kg poly(I:C), i.p., E12.5	↓ Sociability, USV duration ↑ Marble burying	Maternal injection of anti-IL-17A antibody, i.p., E12.5	(195)
C57BL/6 mice	20 mg/kg poly(I:C), i.p., E12.5	↓ Sociability, social preference ↑ Self-grooming, USVs	Maternal diet enriched with docosahexaenoic acid	(196)
C57BL/6 mice	20 mg/kg poly(I:C), i.p., E12.5	↓ Social preference ↑ Self-grooming	—	(197)
C57 mice	20 mg/kg poly(I:C), i.p., E12.5	↓ Sociability ↑ Marble burying, USVs (PND10)	—	(185)
FVB/N EGFP-Tg mice	20 mg/kg poly(I:C), i.p., E12.5	↓ Social recognition, USVs (PND6, 8) ↑ Marble burying, USVs (PND10)	—	(198)
C57BL/6J mice	50 mg/kg poly(I:C), s.c., E12.5	↓ Sociability, social preference	40 mg/kg resveratrol, s.c., E9.5–14.5	(199)

(Continued)

TABLE 3 | Continued

Species/strain	Compound/dose/route/time of exposure	Behavioral outcome	Treatment/route/time of exposure	References
C57BL/6 mice	2 mg/kg poly(I:C), i.p., E12.5 3 mg/kg poly(I:C), i.p., E12.5 and 1.5 mg/kg poly(I:C), i.p., E17.5	↓ Sociability	10–20 mg/kg suramin, i.p. weekly beginning at 6 weeks	(200)
C57BL/6J mice	3 mg/kg poly(I:C), i.p., E12.5 and 1.5 mg/kg poly(I:C), i.p., E17.5	↓ Sociability, spontaneous alternation	10–20 mg/kg suramin, i.p., 2 days before testing	(201)
C57BL/6J mice	3 mg/kg poly(I:C), i.p., E12.5 1.5 mg/kg poly(I:C), i.p., E17.5	↓ Sociability ↑ Marble burying, self-grooming	30 mg/kg JNJ47965567, i.p., (not specified) Genetic removal of *P2rx7*	(202)
C57BL/6J mice	0.25 U/kg poly(I:C), i.p., E12.5 and 0.125 U/kg poly(I:C), i.p., E17.5	↓ Sociability	500 nM clonazepam, i.c., single injection in bilateral ACC	(203)
CD1 mice	5 mg/kg poly(I:C), i.p., E12.5 or E17.5	↓ Sociability, reversal learning	—	(204)
ddY mice	5 mg/kg poly(I:C), i.p., E12–17	↓ Social preference	TPPU, 15 mg/L in drinking water, E12–P21	(205)
ddY mice	5 mg/kg poly(I:C), i.p., E12–17	↓ Sociability, social preference	Maternal diet enriched with glucoraphan, E5–P21	(206)
Sprague-Dawley rats	4 mg/kg poly(I:C), i.v., E15	↓ Sociability	—	(207)
C57BL/6 mice	75 μg/kg LPS, i.p., E11.5–12.5	↓ Sociability (F) ↑ Marble burying (M)	—	(194)
C57BL/6N mice	50 μg/kg LPS, i.p., E14	↓ Sociability, USVs ↑ Marble burying	Maternal injection of anti-IL-17A antibody, i.p., E14	(208)
C57BL/6 mice	75 μg/kg LPS, i.p., E14.5	↓ Sociability, social preference	Maternal injection of inactivated influenza vaccine, i.m., E2.5	(209)
C57BL/6 mice	100 μg/kg LPS, i.p., E15	↓ Sociability, USV duration ↑ Marble burying, self-grooming	—	(210)
Wistar rats	1 mg/kg LPS, s.c., every other day from E7 to delivery	↓ Social interaction, USVs (M) ↑ USVs (F)	—	(211)
Wistar rats	100 μg/kg LPS, i.p., E9.5	↓ Social interaction ↑ Self-grooming	0.8 mg/kg ω-3 polyunsaturated fatty acid, p.o., P30–51	(212)
Wistar rats	100 μg/kg LPS, i.p., E9.5	↑ Self-grooming	—	(213)
Wistar rats	500 μg/kg LPS, i.p., E9.5	↓ Sociability ↑ Marble burying	—	(214)
Sprague-Dawley rats	1.5 mg/kg LPS, i.p., E12	↓ Sociability, social preference	—	(215)
Wistar rats	500 μg/kg LPS, i.p., E16	↓ Social interaction, USVs ↑ Head-dipping	—	(216)
C57BL/6 mice	5 μg IL-6, i.p., E12.5	↓ Sociability	Maternal administration of S31–201, i.p., or diosmin, p.o., E12.5	(217)
C57BL/6J mice	20 μg/kg IL-6, i.p., E12–16	↓ Sociability	—	(218)
C57BL/6J mice	30 μg/kg IL-6, i.p., E12.5–16.5	↓ Sociability, social preference, USVs ↑ Self-grooming	0.1 mg/kg melanotan-II, i.c.v., 7 days	(219)
C57BL/6J mice	0.1 pM pCpG-Mu*il17a*, i.v.	↓ Social preference, social recognition	—	(220)

1,25OHD, 1α-25 dihydroxycholecalciferol; E, embryonic day; i.c., intracerebral; i.c.v., intracerebroventricular; i.n., intranasal; i.p., intraperitoneal; i.v., intravenous; LA, lateral amygdala; LS, lateral septum; P, postnatal day; p.o., per os; s.c., subcutaneous; USVs, ultrasound vocalizations; M, male; F, female.

poly(I:C) treatment did not disrupt social behavior in IL-6 knockout offspring (186). Parasitic MIA also resulted in deficient social recognition memory that was associated with IL-6 upregulation. IL-6 increased at both the mRNA and protein levels in the brain, and serum IL-6 levels were higher in response to *Toxoplasma gondii* antigen (178). Excessive maternal levels of IL-6 may thus play a role in triggering a cascade of events that lead to ASD in offspring.

Supporting the association between ASD and such cytokines as IL-6 and IL-17, experimental models of MIA-induced ASD have elucidated the role of IL-17 in the development of ASD. A poly(I:C) injection stimulated IL-17A production in dams,

followed by higher IL-17A mRNA expression in the fetal brain, an ASD-like phenotype, and cerebral pathological changes in offspring (195). An injection of LPS elevated IL-6 and IL-17A levels only in pregnant mice but exerted ASD-like behaviors in offspring, including a decrease in USVs and lower interest in a novel mouse (208). Similar increases in IL-6 and IL-17A levels were observed in a mouse model of *Mycobacterium tuberculosis* infection, accompanied by behavioral alterations in offspring and increases in the mRNA expression of *NRXN1* and *NLGN1* (176).

Further studies revealed the critical role of fetal exposure to IL-17A but not IL-6 in MIA-associated ASD. The administration of IL-6 in pregnant mice on E12.5 altered social behavior in offspring (186), but an intraventricular injection of IL-6 into the fetal brain on E14.5 did not produce an ASD-like phenotype (195). Instead, an intraventricular injection of IL-17A in the fetal brain on E14.5 caused social deficits and cortical disorganization in wildtype pups (195). The MIA-associated phenotype was blocked by abolishing IL-17A secretion by deletion of the *RORγt* gene from Th17 cells (195), maternal pretreatment with an anti-IL-17A antibody (208), and deletion of the *IL-17Ra* gene in pups (195). The mechanisms of elevations of IL-17A that lead to ASD may involve alterations of the function of regulatory T (Treg) cells. Mice that were exposed to *Toxoplasma gondii*-induced MIA exhibited higher percentages of Th1 and Th17 cells but a lower percentage of Treg cells. The adoptive cell transfer of Treg cells from MIA mothers at 8 weeks of age largely reversed the abnormal behavioral phenotypes of offspring as early as at 9 weeks (178).

Microglial activation following MIA may be also be involved in ASD development, but few studies have investigated direct effect of alteration of microglial function on ASD-related behavioral deficits in offspring. Morphological changes suggest that microglial activation is not fully consistent in mouse models. Daily injection of IL-6 from E12.5 to delivery resulted in morphological changes of microglia, though not associated with ASD-like behavioral deficits (224). Poly(I:C) administration on E12 and E15 impaired direct social interaction and increased velocity of microglial process motility, but morphological changes was not observed (189). Another study showed that single injection of poly(I:C) effectively produced an ASD-like decrease in sociability and increase in stereotypy, but the superimposition of postnatal hypoxia/ ischemia (HI) insult was needed to alter microglial morphology (225). Microglial infiltration in the brain may be difficult to detect. In two models that produced ASD-like behavioral deficits following MIA [i.e., mice that were exposed to two doses of poly(I:C) on E12.5 and E17.5 (211) and rats that were exposed to daily injections of LPS from E7 to delivery (210)], microglial infiltration in the brain was not detected. In Chen et al., HI superimposition on MIA induced microglial infiltration in the hippocampus (225).

Despite the aforementioned limited histopathological observations of microglia, synapses and neurons that are exposed to MIA exhibit alterations that suggest microglial activation. An increase in spine density that was attributable to a reduction of spine pruning was found in the dentate gyrus in mice given LPS on E15 (226) and in the hippocampus in mice that were given 2 doses of poly(I:C) on E12.5 and E17.5 (211). Subchronic IL-6 exposure from E12.5 to delivery in mice delayed the migration of GABAergic progenitors (224). Parvalbumin-positive neurons in the hippocampus were entrapped in perineuronal nets following MIA alone (225), which may impair synaptic plasticity. A single injection of poly(I:C) on E12 reduced parvalbumin-positive cells and impaired GABAergic signaling in the dentate gyrus, which was associated with social withdrawal and deficient spatial memory (227). In this model, spatial memory and abnormal histology were restored by minocycline, which inhibits microglial activity (227). In the combined MIA/HI mouse model of ASD, the pharmacological inhibition of monocyte infiltration after HI insult prevented ASD-like behaviors (225). Interestingly, MIA-associated behaviors, including ASD-like social impairments, were recovered by exercise in adulthood, which was correlated with the normalization of synapse density and suggested intact microglial function in mice (211). Microglial activation and infiltration in the MIA-exposed brain may link high cytokine levels and the development of ASD, and MIA-induced ASD in offspring could be ameliorated by prenatal or postnatal immunological interventions.

HYPERTENSIVE DISORDERS OF PREGNANCY

Hypertensive disorders of pregnancy (HDP) comprise a collection of hypertensions in pregnant women. They are divided into (a) hypertension that arises *de novo* at or after 20 weeks of gestation and (b) hypertension that is known before pregnancy or present in the first 20 weeks of gestation (228). Hypertensive disorders of pregnancy that are accompanied by proteinuria or other maternal organ dysfunction, such as acute kidney injury, are referred to as pre-eclampsia. HDPs occur in ∼5–8% of all pregnancies (229). Epidemiological studies reported an association between HDP and ASD. Both maternal chronic hypertension before pregnancy and gestational hypertension are associated with an ∼40% increase in the odds of ASD in offspring (23, 230). Pre-eclampsia also increases the risk of ASD (231), but the presence of organ dysfunction in pre-eclampsia does not appear to be an additional risk factor (23, 230). Research on HDP has been performed by generating animal models. The central focus of HDP research has been on maternal pathophysiology and fetal growth failure. In contrast, there are sparse findings on how HDP affects fetal brain development and results in ASD-like social deficits in offspring.

Reduced Uteroplacental Pressure

Animal models of HDP were first established in rabbits, dogs, and monkeys by constricting the terminal aorta. This method is called reduced uteroplacental pressure (RUPP) (232). Later, this method was applied to rats and mice (233). The procedure was also improved so that uterine arteries were occluded instead of the abdominal aorta, resulting in the avoidance of hindlimb paraplegia in classic RUPP models (234). In addition to main manifestations of HDP, such as maternal hypertension, proteinuria, and fetal growth restriction (232–234), fetal brains also exhibited changes in response to HDP exposure, but their

direct relevance to ASD remains unclear. Alteration of the brains of guinea pigs born to RUPP mothers included smaller volumes of the basal ganglia and impairments in prepulse inhibition at 12 weeks (235). Pregnant rats that underwent RUPP also exhibited immunological changes that are similar to MIA, including high levels of IL-6 and IL-17A that are produced by $CD4^+$ T cells (236) and an increase in the number of Th17 cells (237). IL-17 recombinant receptor C was used to suppress Th17 cells and resulted in blunted hypertension and the recovery of pup and placenta weight (237). Placental hypoperfusion in animal models of RUPP-induced HDP may mediate morphological and functional changes that are related to MIA-induced ASD in offspring.

Angiotensin II

Numerous clinical observations have indicated a relationship between HDP and angiotensin (AT). Patients with HDP exhibited greater vascular reactivity to AT II (238), and serum from pregnant patients with HDP but not those with essential hypertension contained AT1 autoantibodies (AAs) that could stimulate AT II receptor 1 (239). AT1-autoantibodies that were isolated from women with HDP reproduce key features of HDP in mice (240), and their pharmacological blockade normalized maternal blood pressure during pregnancy (241). Exposure to LPS during pregnancy gave rise to features that are relevant to HDP in rats offspring, which was prevented by treatment with the AT II receptor inhibitor losartan (242). Infusions of AT II in pregnant mice stimulated the production of soluble fms-like tyrosine kinase-1, a circulating antagonist of vascular endothelial growth factor and placental growth factor (243), and IL-6 in the placenta (244). In summary, AT II infusion is sufficient to induce HDP and IL-6 production, and AT II inhibition is sufficient to prevent LPS-induced HDP, suggesting that the stimulation of AT II signaling underlies MIA and HDP. Thus, excessive AT II activity in HDP might give rise to ASD through an increase in cytokine production as observed in MIA. Further research is required to elucidate the neurological phenotype that is induced by AT II in these models.

Vasopressin

In contrast to the AT-related HDP model, pregnant women with preeclampsia are reported to exhibit lower activity of the renin-angiotensin system (245). Women with HDP exhibited higher levels of copeptin, a pro-segment of arginine vasopressin (AVP) (246), raising the hypothesis that high AVP levels during pregnancy play a role in the pathogenesis of HDP. Infusions of AVP during pregnancy resulted in an HDP phenotype in mice (247). The simultaneous inhibition of AVP receptors 1 and 2 abolished the AVP-induced elevation of blood pressure, whereas blocking each receptor alone resulted in the insufficient normalization of blood pressure in mid- and late-pregnancy (247). Infusions of AVP during pregnancy increased IL-17 levels in maternal plasma and the placenta (248), suggesting that IL-17 may also affect fetal brain development following AVP loading. To date, however, only one study has reported neurological and behavioral consequence of AVP-infused HDP in offspring (249). Both male and female offspring that were exposed to AVP on P7 had a smaller neocortex, but caudate-putamen volume decreased in males only and increased relative to the neocortex in females. At the behavioral level, male offspring that were exposed to AVP exhibited an increase in anxiety-like behavior in the elevated plus maze test, but females did not. Social behavior was analyzed in males only, in which an increase in sociability was observed in AVP-exposed males compared with controls (249). Further research on ASD-like phenotypes in the AVP infusion model of HDP is necessary to determine whether AVP-induced HDP recapitulates the higher risk of ASD in humans.

Serotonin Metabolism in the Placenta

Women with HDP have high 5-HT levels in blood (250) and the placenta (251). Such a hyperserotonemic state is attributable to a reduction of activity of monoamine oxidase A and not a defect in 5-HT transport in the placenta (251, 252). Serotonin levels correlated with the severity of maternal hypertension (251), suggesting a causal role for hyperserotonemia in HDP. Serotonin exerts a contraction effect on placental vascular smooth muscle, which may be a primary way 5-HT plays a role in HDP (253, 254). Chorionic arteries from HDP women exhibited a lower contraction response to the *in vitro* perfusion of 5-HT (255), presumably because of chronic exposure to excessive 5-HT during pregnancy. Ketanserin, a 5-HT_{2A} receptor antagonist, attenuated arterial contraction that was stimulated by 5-HT in both normal and HDP samples (255). Moreover, 5-HT administration in normal pregnant rats produced HDP-like alterations, such as a reduction of growth of the placenta and fetus (256). Continuous hyperserotonemia in $Sert^{-/-}$ mice, which was 2-fold higher than controls, produced smaller placentas, marked cell death, and necrotic lesions that were similar to placentas from HDP women (257). Undetectable 5-HT levels in $Tph1^{-/-}$ mice also resulted in smaller placentas and cell death, although these effects were much milder than in $Sert^{-/-}$ mice (257). These similar and contrasting findings from hyperserotonemic and hyposerotonemic models suggest a more deleterious effect of hyperserotonemia on the placenta and possibly on the fetus during pregnancy. As discussed in Section SSRIs above, the relationship between high 5-HT levels and ASD has been demonstrated in different animal models. Investigations of these models in view of HDP may improve our understanding of how HDP causes ASD in offspring.

ADVANCED PARENTAL AGE

Advanced parental age does not appear to accompany the visible effects that are discussed above, such as the effects of medication use and the inflammatory response during pregnancy. A detailed population study sought to identify prenatal and perinatal risks of ASD and found higher parental ages of ASD children compared with non-ASD children (258), prompting further studies of the association between parental age and the risk of ASD.

Effects of Paternal and Maternal Age and Their Interaction on ASD

The risk of higher paternal age for ASD have been consistently reported. In a study in Israel, the odds ratio (OR) of ASD in offspring marginally increased for paternal ages of 30–39 years and significantly increased (OR = 5.75) for paternal ages of 40 years and older when adjusted for maternal age and compared with paternal ages of <30 years (259). A cohort study in Sweden also reported an association between paternal age and the risk of ASD, which began to increase at the paternal age of 30 years (260). The authors then confirmed this association in a meta-analysis of Western and non-Western cohorts, which revealed a dosage effect on the OR of ASD in offspring (1.22 for paternal age 30–39 years, 1.58 for paternal age 40–49 years, and 2.66 for paternal age 50 years and older) (260). A similar linear increase in the risk of ASD according to paternal age was also found in a large-scale cohort study (261). A recent meta-analysis showed that high paternal age was associated with a 55% higher risk of ASD (262).

Maternal age also appears to affect the risk of ASD in offspring, though in a different manner compared with paternal age. In a population study, logistic regression analysis was conducted that included maternal but not paternal age as regression coefficients. This study found that having children with ASD was related to higher maternal age (258). However, the effect of advanced maternal age was marginal when adjusted for parental age (259). Later studies found that the risk of ASD in offspring increased in mothers who were 30 of age or older (261) and 35 years of age or older (263). When the ages of both mothers and fathers were adjusted, the risk of ASD in offspring began to increase in mothers who were 30–39 years old and evident in mothers who were 40 years old (264).

In contrast to consistent findings that advanced parental age elevates the risk of ASD in offspring, remaining to be clarified is whether lower parental age heightens the risk of ASD in children. A cohort study in Sweden found that the risk of ASD for younger mothers was comparable to a reference group (29 years old), whereas children of younger fathers had a lower risk of ASD (261). Another cohort study of European and non-European countries reported a higher risk of ASD in children of mothers who were younger than 20 years old, whereas no association was observed for younger fathers (264). According to a meta-analysis from North America, Europe, Asia, and Oceania, the lowest paternal and maternal age categories were associated with a lower risk of ASD (262). Lower parental age might have a protective effect against the risk of ASD in offspring, which should be investigated further.

Remaining to be determined are which maternal and paternal ages have the greatest impact on the risk of ASD. A Swedish population study reported a higher OR of ASD, particularly ASD with intellectual disability, that was associated with advanced maternal age (2.04 for mothers aged 40–45 years and 1.18 for fathers aged 40–44 years) (261). Another study reported a higher risk of ASD for fathers aged 40–49 years (OR = 1.52) compared with mothers of the same age group (OR = 1.15) (264). A recent meta-analysis found a comparable risk of ASD for the highest age group (OR = 1.41 for mothers and 1.55 for fathers) (262). Further research is needed to distinguish the effects of maternal and paternal ages.

In summary, both advanced maternal and paternal age are likely related to a higher risk of ASD. Data have been insufficient or inconsistent with regard to differences in the impact of maternal and paternal ages and their interaction on ASD.

Modeling Advanced Parental Age in Rodents

The influence of advanced parental age has been investigated in rodent models, mainly for older fathers. Paternal age did not affect the general health of pups, such as the number of pups, body weight, and early mortality (265). Mice from older fathers exhibited alterations of behavioral phenotypes. These offspring took longer to attain the righting reflex, exhibited a decrease in spontaneous motor activity, deficient memory retention in the passive avoidance test (265), and decreases in ambulatory distance and prepulse inhibition (266). ASD-related behavior was also found in advanced paternal age models. Male mice from older fathers exhibited impairments in social preference (267), the deficient discrimination of social novelty (267, 268), an increase in self-grooming (268), and increases in or alterations of USVs that are suggestive of ASD (268, 269). The magnitude of social deficits correlated with paternal age. Lower social interaction was observed in offspring of 40-week-old fathers and further decreased in offspring of 48-week-old fathers (270). Autism spectrum disorder-like social deficits that are caused by advanced paternal age may transmit to the second generation. When both parents were born to fathers that were 12 months of age or older, the offspring exhibited a decrease in sociability, an increase in repetitive behavior, and anxiety-like behavior (267, 268).

Little is understood about what accompanies behavioral changes that are observed in offspring of older fathers. Advanced paternal age may produce morphological changes in the brain in offspring, but the findings are inconsistent. Male mouse offspring of 12- to 18-month-old fathers had a higher rostral cortical volume and lower lateral ventricle volume, but social interaction was unaffected (271). A recent study that used male mice that were older than 12 months of age found that their offspring emitted abnormal USVs. This finding was associated with a smaller cortical thickness of layer 6 of the primary motor cortex, suggesting that alterations in the motor cortex impair vocal communication (269). A possible mechanism of paternal age-induced ASD may be related to alterations of sperm DNA methylation. Older fathers and their offspring shared hypomethylation in regions that flank CpG island promotors, which was not found in younger fathers and their offspring (266). Whole-genome DNA methylome analyses of sperm identified hypomethylated genomic regions that were enriched in RE1-silencing transcription factor/neuron-restrictive silencer factor binding motifs (265). The treatment of young male mice with the DNA-demethylating drug T5-Aza reproduced DNA hypomethylation in sperm, and their offspring exhibited alterations of USV patterns (269). Alterations of DNA

methylation patterns in sperm may contribute to the effect of advanced paternal age on ASD in offspring.

Analyzing advanced maternal age in animal models is challenging because aged females may exhibit changes in nursing behavior. For example, 30-week-old female mice exhibited enhanced nest-building performance during pregnancy and lactation (272). Cesarean section and cross-fostering were applied to avoid confounding effects of alterations of nursing behavior in 15- to 18-month-old female mice, but their pups still exhibited more USVs on P8 and an increase in anxiety-related behavior in the elevated plus maze (273). Associated with the behavioral changes, pups that were born to aged female mice exhibited high hippocampal mRNA expression of genes that are related to ASD, such as *Ada* (which influences ASD development) and *Egr2* (which influences ASD severity). Several other genes were enriched in the Gene Ontology analysis, including "protein folding" and "protein post-translational modification" (273). Younger maternal age in mice (32–35 weeks) also affected cognitive functions in offspring, including impairments in learning in the passive avoidance test, spatial memory in the Morris water maze test, and memory in the novel object recognition test (274). Interestingly, a marked decrease in expression of the vitamin D receptor gene was found in the placenta in aged female mice and their offspring (274). When these aged female mice received vitamin D supplementation before pregnancy, their offspring exhibited intact learning and memory (275). Advanced maternal age likely affects cognitive function in offspring, and possible ASD-like social deficits in these models should also be investigated.

CONCLUDING REMARKS

We discussed the various contributions of environmental risk factors to the development of ASD. Prenatal exposure to VPA and MIA have been extensively investigated in epidemiological and biological studies. The findings indicate the critical role of these risk factors in producing ASD. Hypertensive disorders of pregnancy and advanced maternal age elevate the risk of ASD, but remaining unclear is how these risk factors give rise to ASD-like cognitive dysfunction. The association between maternal SSRI use and ASD in offspring is inconclusive, but rodent models that show alterations of 5-HT metabolism provide a plausible rationale for this association. Future research is expected to develop therapeutic interventions for ASD that target environmental risk factors.

AUTHOR CONTRIBUTIONS

AS wrote the manuscript. HK-M, MT, YK, and KI critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by a grant from the Japan Society for the Promotion of Science (JSPS) KAKENHI (no. 21H03028).

ACKNOWLEDGMENTS

The authors thank Mr. Michael Arends for English proofreading.

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

The reviewer RK declared a shared affiliation with two of the authors, MT and YK, to the handling editor at the time of review.

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frontiers in Psychiatry

HYPOTHESIS AND THEORY
published: 17 February 2022
doi: 10.3389/fpsyt.2022.823096

Maternal Immune Activation and Interleukin 17A in the Pathogenesis of Autistic Spectrum Disorder and Why It Matters in the COVID-19 Era

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OPEN ACCESS

Edited by:
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Reviewed by:
Kohji Fukunaga,
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Toru Takumi,
RIKEN Brain Science Institute (BSI), Japan

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Specialty section:
This article was submitted to Autism, a section of the journal Frontiers in Psychiatry

Received: 26 November 2021
Accepted: 21 January 2022
Published: 17 February 2022

Citation:
Carter M, Casey S, O'Keeffe GW, Gibson L, Gallagher L and Murray DM (2022) Maternal Immune Activation and Interleukin 17A in the Pathogenesis of Autistic Spectrum Disorder and Why It Matters in the COVID-19 Era. Front. Psychiatry 13:823096. doi: 10.3389/fpsyt.2022.823096

Autism spectrum disorder (ASD) is the commonest neurodevelopmental disability. It is a highly complex disorder with an increasing prevalence and an unclear etiology. Consensus indicates that ASD arises as a genetically modulated, and environmentally influenced condition. Although pathogenic rare genetic variants are detected in around 20% of cases of ASD, no single factor is responsible for the vast majority of ASD cases or that explains their characteristic clinical heterogeneity. However, a growing body of evidence suggests that ASD susceptibility involves an interplay between genetic factors and environmental exposures. One such environmental exposure which has received significant attention in this regard is maternal immune activation (MIA) resulting from bacterial or viral infection during pregnancy. Reproducible rodent models of ASD are well-established whereby induction of MIA in pregnant dams, leads to offspring displaying neuroanatomical, functional, and behavioral changes analogous to those seen in ASD. Blockade of specific inflammatory cytokines such as interleukin-17A during gestation remediates many of these observed behavioral effects, suggesting a causative or contributory role. Here, we review the growing body of animal and human-based evidence indicating that interleukin-17A may mediate the observed effects of MIA on neurodevelopmental outcomes in the offspring. This is particularly important given the current corona virus disease-2019 (COVID-19) pandemic as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection during pregnancy is a potent stimulator of the maternal immune response, however the long-term effects of maternal SARS-CoV-2 infection on neurodevelopmental outcomes is unclear. This underscores the importance of monitoring neurodevelopmental outcomes in children exposed to SARS-CoV-2-induced MIA during gestation.

Keywords: ASD, autism, cytokine, maternal immune activation, MIA, interleukin-17A (IL-17A), COVID-19

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by a spectrum of deficits in social interactions and communication combined with stereotypical and repetitive behaviors. Up to 50% of those affected can have intellectual disability (ID) and limited verbal communication (1–3). In recent decades, the prevalence of ASD has consistently increased from approximately 1 in 1,000 in the 1960s (4), to 1 in 44 today in the United States (5). Increasing prevalence may in part, be explained by changes in reporting practices, increased recognition of ASD symptoms, broadening of the ASD diagnosis (1), and improved accessibility to services (6, 7). A significant ratio of 4:1 from male to female still exists with markedly differing prevalence rates between the sexes, 1/38 in males and 1/151 among females (8). Although genetic susceptibilities are recognized, the mechanism of disease development is unknown and does not follow a clear pattern of inheritance (9, 10). This suggests possible mediation by additional unknown biological or environmental factors (11). Both common and rare genetic risk factors have been identified with more than 400 diverse genes now linked to ASD. Singly, these genetic factors each convey only a modest increase in ASD risk (∼1%), however collectively they can contribute to a far greater risk (12, 13). Up to 20% of individuals with ASD may possess copy number variants (CNVs) and *de novo* loss of function single nucleotide variants (SNVs) that are individually rare but in combination, increase an individual's ASD risk (12). While newer methods of genetic analysis (such as whole genome sequencing) are uncovering new candidate genes with regularity (14), the heterogeneity of the clinical and phenotypic groups within ASD strongly suggest that in those with a genetic predisposition, environmental factors may act in concert to bring about a multisystem dysfunction leading to ASD. A well-characterized environmental factor known to impact early fetal brain development and increase ASD risk is maternal inflammation during pregnancy, which is commonly called maternal immune activation (MIA). Numerous epidemiological studies have linked gestational infections with elevated risk of ASD in offspring (15–17), and animal models of MIA have simulated gestational infection resulting in MIA-induced neural and behavioral abnormalities analogous to those seen in ASD (18–20).

Focused early intervention in young children with ASD has been shown to result in normalized patterns of brain activity, and is associated with improved functional outcomes and reduced morbidity (21, 22). Most children affected by ASD can have a reliable and stable ASD diagnosis from as early as 14 months of age (23), yet in spite of this, the average age of ASD diagnosis is closer to 5 years (24, 25). Numerous studies sought to identify blood-based biomarkers of ASD in affected adolescents and adults (26, 27) and have reported alterations of molecules involved in iron transport (28), inflammation (29, 30), brain development (31), and metabolism (32). None to date has identified and validated reliable mechanistic biomarkers with the ability to improve ASD detection in the crucial early developmental period. Multiple descriptive ASD biomarkers such as characteristic MRI brain findings, abnormalities of gaze preference on eye tracking or characteristic EEG findings in infants with ASD; show promise in terms of aiding earlier ASD detection. However, none is directly involved in the pathogenesis of ASD and arises of the condition rather than contributes to it. The infant brain doubles in volume over the first year coinciding with maximal neuroplasticity and synaptogenesis. Recognition of an early mechanistic biomarker gives us the best chance of implementing strategies during this critical early childhood window allowing ASD diagnosis and intervention at the earliest possible stage.

Here, we highlight recent research in this area, both from pre-clinical animal studies and epidemiological human studies, along with a proposed mechanistic pathway, that we can encourage other research groups with access to suitable maternal-child cohorts to examine this question. We encourage researchers to look at the prospective study of children born during the corona virus disease-2019 (COVID-19) era, when their gestations may have been complicated by mild or even asymptomatic severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Otherwise, the long-term effect, if any, of COVID-19 on the fetal brain could remain unknown for years to come.

INFLAMMATION, VIRAL INFECTION, AND ASD: WHAT ARE THE IMPLICATIONS OF THE COVID-19 PANDEMIC?

There is growing scientific evidence that aberrant immune activation occurs in ASD (27, 33) based on studies of autistic children and young adults (34, 35). As early as 1971, Stella Chess reported ASD cases associated with the 1964 Rubella outbreak in the United States (36), and in a 1977 follow up study, Chess et al. quoted ASD prevalence rates of 8–13% in children of mothers who were infected during that outbreak (16). Large epidemiological studies indicate that conditions such as maternal autoimmune disorders and mid-trimester viral infections that trigger gestational pro-inflammatory states (i.e., MIA), are linked with elevated ASD, schizophrenia, and bipolar disorder risk in offspring (16, 17, 37, 38). More recently, a range of conditions associated with proinflammatory states in pregnancy such as obesity, psychosocial stress, and pre-eclampsia were associated with increased ASD risk in children (39, 40). Thus, gestational MIA appears to play a role in the pathogenesis of the ASD phenotype in exposed offspring.

Abbreviations: ACE-2, angiotensin-converting enzyme-2; ADHD, Attention Deficit Hyperactivity Disorder; ARDS, acute respiratory distress syndrome; ASD, autism spectrum disorder; CS, cesarean section; CD8 cell, cluster of differentiation 8, cytotoxic T-lymphocytes; CHD8, chromodomain helicase DNA binding protein 8 gene; CNV, copy number variant; COVID-19, corona virus disease-2019; FMR1, fragile X mental retardation 1 gene; GWAS, genome-wide association study; HLA-G gene, human leukocyte antigen G coding gene; ID, intellectual disability; IL, interleukin; IL17A gene, interleukin 17A gene; LPS, lipopolysaccharide; MERS, Middle Eastern Respiratory Syndrome; MIA, maternal immune activation; mTor, mammalian target of rapamycin; Poly (I:C), polyinosinic:polycytidylic acid; PNS, peripheral nervous system; RORγt, retinoid-related orphan receptor gamma t; SARS-CoV-2, severe acute respiratory syndrome-coronavirus 2; SNV, single nucleotide variant; Th17, T helper 17 cell; TSC1/TSC 2, Tuberous sclerosis complex ½.

MATERNAL IMMUNE ACTIVATION AND NEURODEVELOPMENTAL OUTCOMES

We define MIA as a triggering of the maternal immune system by infectious or infectious-like stimuli resulting in an increase in measurable inflammatory markers during pregnancy (41, 42). Maternal immune activation has been most commonly simulated in preclinical rodent, murine and non-human primate (rhesus macaque) animal models by Poly (I:C) (polyinosinic-polycytidylic acid) or LPS (lipopolysaccharide) injection which, respectively, model viral and bacterial infection (18, 43, 44). Poly (I:C) is a synthetic analog of double stranded RNA, mimics the effects of viral infection (45). The triggered immune response results in offspring with behavioral, immunological, and neurological abnormalities that approximate to autistic symptoms observed in humans, notably, impaired sociability and repetitive behaviors (18, 46, 47). Offspring born to poly (I:C) treated dams have consistently, across all exposure categories [administration of varying doses of poly (I:C) and at varying gestations], shown impairment of social interaction, this is manifest as reduced communication in ultrasonic vocalizations (USV) which are usually triggered by separation from the dam in the first two postnatal weeks. Marble burying, a well-recognized behavioral paradigm to measure repetitive behaviors in rodents, again is consistently increased in murine offspring following poly (I:C) treatment (48). These offspring have proven useful in pre-clinical etiological studies as well as identification of therapeutic targets.

Cytokine dysregulation may play a causative role in observed neuronal dysfunction in pre-clinical models of MIA (20, 46, 49). In a recent study, Choi et al. convincingly demonstrated that simulated MIA in murine models leads to elevation in maternal IL-6, which in turn activates maternal Th17 cells. These maternal Th17 cells produce IL-17, which is thought to cross the placenta triggering increased expression of IL-17AR in the fetal brain and leading to cortical malformations and behavioral abnormalities (18, 50). These malformations parallel abnormalities found in brain development in children, adolescents and adults with ASD (51, 52). Poly (I:C) treatment also leads to raised IL-17A mRNA levels in placental tissue of these mice (18). Through inhibition of IL-6 and IL-17A signaling with antibody blockade of the IL-17A cytokine, Choi at al also determined that a sustained increase in IL-17A expression seemed to be pathogenic in ASD, as IL-17A blockade prevented the development of ASD-like phenotypes (18). Specific behaviors in mice which model core diagnostic features of ASD (including repetitive burying and increased neonatal USV) were normalized in the previously MIA-exposed offspring (53, 54).

Improved fetal resilience is associated with lower intensity of MIA. Autism spectrum disorder risk after prenatal exposure to maternal fever has been found to increase in a dose dependent manner (55, 56) and similar effects were identified in animal models of MIA (57). A balanced maternal diet seems to contribute to improved fetal resilience also (58–60). Exposure to relatively higher grades of immune activation *via* high intensity MIA (40), intrapartum infection (61, 62) and genetic risk factors lead to reduced fetal resilience, and increased likelihood of unfavorable developmental outcomes.

ALTERATIONS IN CYTOKINE EXPRESSION IN HUMAN STUDIES

While many studies have examined the cytokine profiles of individuals with ASD, only a very limited number of studies to date have examined mid-gestation cytokine levels in mothers of children who subsequently develop ASD. Three studies retrospectively analyzed maternal blood sampled during pregnancy. A 2017 study by Jones et al., reported elevated mid-gestation cytokines and chemokines in mothers of children with ASD associated with ID, and particularly early onset ASD (as defined by the authors as early or sustained delays in language or social skills, and excluding those showing clear skill regression) (63). Dysregulation was noted in a number of cytokines including interleukins IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, and IL-17A between 15 and 19 weeks' gestation. An earlier study noted elevations in mid-gestation serum IL-4, IL-5, and IFN-gamma levels in mothers of ASD affected children (15). While, more recently, Irwin et al. demonstrated alterations in IL-4, MCP-1, and IL-10 levels in 28-week gestation serum of mothers who birthed ASD affected children (64). Other authors have examined amniotic fluid at mid-gestation and found elevated levels of IL-4, IL-10, TNF-α, and TNF-β in ASD patients vs. controls (65). Yet, amniotic fluid cytokine concentrations are more indicative of the fetal immune state rather than the maternal state (66, 67). In **Table 1**, we outline a number of the cytokines most frequently found to be dysregulated in the serum or cerebrospinal fluid (CSF) of ASD affected individuals, and gestational serum and amniotic fluid samples from mothers of ASD affected children.

A growing body of evidence supports a role in ASD pathogenesis for Th17 cells and their product cytokine, IL-17A (**Figure 1**) (79, 82). The IL17A gene itself has been identified by a small genome-wide CNV study to have amplified CNVs in ASD affected cohorts (83). Elevated levels of IL-17A have been reported in the blood of ASD affected individuals, and these correlate positively with severity of ASD behavioral symptoms (35, 63, 79). Yet, others have found high concentrations of IL-17A in individuals affected by obesity or high BMI (84), both of which are more likely in ASD groups (85). This is a potential confounder for any retrospective cohort based study designs.

STRING analysis (**Figure 2**) (86) indicates that IL-17A has proven or predicted interactions with IL-2, IL-6, IL-10, IL-13, IL-17F, IL-17RA, IL-17RC, CTLA4, STAT3, and STAT6. Each of these proteins have been previously reported to have altered expression in children with ASD, as outlined below. Of these, the most persistently described, and hence, potential key player is IL-17A, along with its receptor IL17RA and receptor complex, IL17RC.

Network nodes represent proteins—each node represents all the proteins produced by a single, protein-coding gene locus. Edges (lines) represent protein-protein associations that are specific and meaningful, i.e., proteins jointly contribute to a shared function; this does not necessarily mean they are physically binding each other. Blue connecting lines indicate that protein interaction information was derived from curated databases, pink indicates the interaction was experimentally determined, yellow indicates

TABLE 1 | Cytokine dysregulation in ASD affected individuals and in gestational serum and amniotic fluid samples of mothers with ASD affected offspring.

Cytokine	Category	Altered in blood/CSF of ASD individual	Altered in gestational blood	Altered in amniotic fluid	Cytokine characteristics relevance to ASD
TNFα	Pro-inflammatory	(29, 68–70)	(63)	(65)	Apoptosis of infected cells. Elevated in the CSF and blood of ASD affected individuals (29, 68, 69).
IL-1β	Pro-inflammatory	(29, 68, 71, 72)	(63)		A potent pro-inflammatory cytokine involved in both acute and chronic inflammation. Correlated with ASD symptom severity (34).
IL-6	Pro-inflammatory	(29, 68, 70–74)	(63)		Induces production of acute phase proteins and stimulates B-cell antibody production (75). Pleiotropic (affects hematologic, hepatic, endocrine, and metabolic function). Thought to impact synapse formation and neuronal migration (76). Potentially mediates IL-17 linked ASD risk in pregnancy (18, 46).
IFNγ	Pro-inflammatory	(27, 29, 73)	(15, 63)		Interfaces between innate and adaptive immune response. Secreted by NK cells, and promotes NK killing. Activates macrophages, which produce IL-12 and−23, stimulating Th1 and Th17 cell, respectively. Inhibits Th2 cells. Versatile, with a role in defense against intracellular pathogens, tumors surveillance, autoimmunity, allergy, and the protection of the amniotic space during pregnancy (77).
IL-17	Pro-inflammatory, Chemotactic	(29, 35, 70, 74, 78, 79)	(63)		Derived from Th17 cells, a subset of CD4 cells. Potentiates the innate PMN response throughout inflammation. Postulated to trigger alterations in the blood brain barrier and lead to cortical dysplasia (46).
IL-4	Pro-/Anti-inflammatory, Allergy	(72)	(15, 63, 64)	(65)	A Th2 derived cytokine, often linked with asthma and allergic type inflammation (33). Dual role: pro/anti-inflammatory properties. Crucially important in mitigating inflammation during pregnancy (primarily through suppression of Th1 T-cells and associated cytokines (IL-2 and IFNγ).
GM-CSF	Growth factor	(80)	(63)		A colony-stimulating factor. Produced by stromal cells, it targets bone marrow, and precursor cells, mediating hematopoiesis.
IL-8	Chemotactic	(71, 73, 81)	(63)		Produced by fibroblasts, neutrophils, and macrophages. Chemo-attractant for phagocytes at site of inflammation.

The numbers in parentheses indicate the relevant references.

the interaction was determined *via* text mining, black indicates protein co-expression, and lilac indicates protein homology. Analysis was performed on 28 July 2021 *via* the string-db.org domain.

IL-17A ASSOCIATED PRO-INFLAMMATORY MEDIATORS IN ASD

Upregulation of pro-inflammatory pathways has been persistently associated with ASD. IL-6 is a versatile cytokine, with multiple functions throughout the body. It plays roles in immunity, inflammation, hematopoiesis, and oncogenesis. IL-6 works to promote pro-inflammatory Th17 cells (IL-17 producers) and to downregulate anti-inflammatory Treg cells (regulatory T-Helper cells) (87, 88). Th17 cells produce cytokines that cross the placental barrier (20). This transplacental effect has been well-characterized with IL-6, which was shown to alter offspring behavior and brain development (20, 89).

Like IL-17A, IL-17F is also produced by Th17 cells (90). IL-17F is reported to be involved in the regulation of proinflammatory gene expression and responses (91). IL-17RA and IL-17RC are both members of the IL-17 receptor family. In order for IL-17A (or indeed IL-17F) to have biological effects on tissues, IL-17RA must be present (90). IL-17RA is expressed in immune cells, and some children affected by ASD appear to possess higher levels of this receptor compared to neuro-typical controls (92). IL-17RA blockade may reduce monocyte associated oxidative stress which may improve neuro-inflammation associated with ASD (92). IL-17RC is also essential for the formation of the IL-17 receptor complex (46). IL-17RC levels in neutrophils are raised in children with ASD compared to neuro-typical controls. In fact, expression of this receptor (mRNA and protein) was completely absent in a cohort of neuro-typical children. The presence of both IL-17A receptor subunits in ASD patients

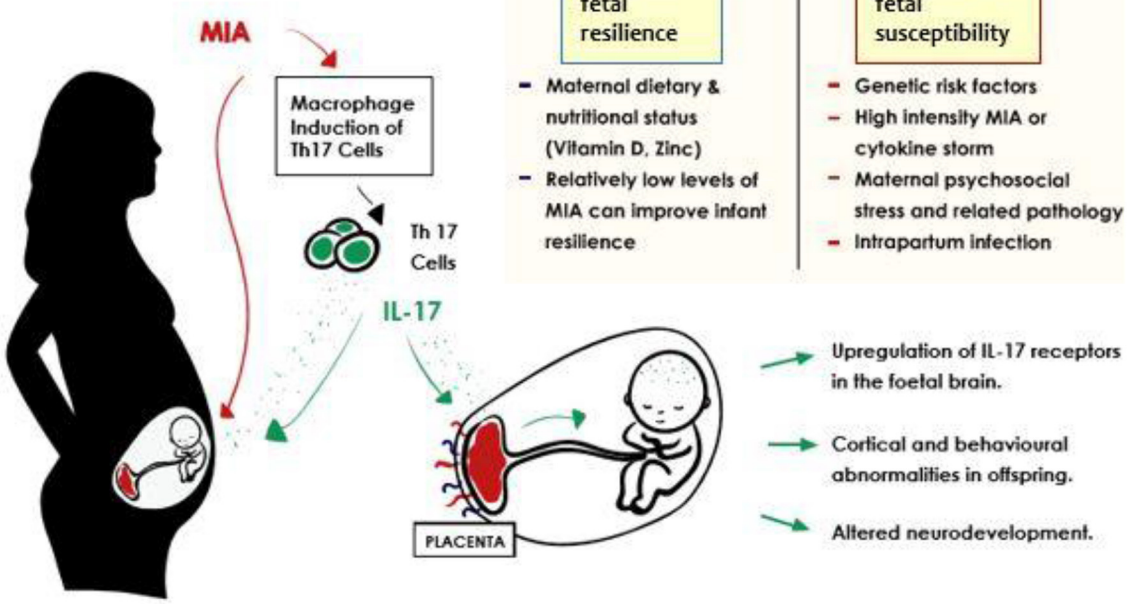

FIGURE 1 | Potential outcomes in the inflammation-exposed fetus in the context of MIA related IL-17 induction. Improved fetal resilience is associated with lower intensity of maternal immune activation. Autism spectrum disorder risk after prenatal exposure to maternal fever has been found to increase in a dose dependent manner (55, 56) and similar effects were identified in animal models of MIA (57). A balanced maternal diet seems to contribute to improved fetal resilience also (58–60). Exposure to relatively higher grades of immune activation *via* high intensity MIA (40), intrapartum infection (61, 62), and genetic risk factors lead to reduced fetal resilience, and increased likelihood of unfavorable developmental outcomes.

may magnify the effects of IL-17A resulting in an autistic phenotype (93).

The transcription factor STAT3 (signal transducer and activator of transcription 3) is a key player in the development of T helper cells and regulates the expression of the T helper cell specific transcriptional regulator—retinoic acid receptor related orphan receptor γ-t (RORγt) *via* IL-6 (94, 95). IL-6 is a potent driver of RORγt activity. RORγt is exclusively found in lymphoid cells such as Th17 cells (CD 4 helper cells), and is required for differentiation of Tregs to Th17 cells (95). STAT3 proteins occur at elevated levels in the peripheral blood mononuclear cells (PBMCs) of children affected by ASD (96). Inhibition of STAT3 mitigates MIA associated behavioral and immunological abnormalities seen in animal models (49), while RORγt KO models reverse outcomes in MIA exposed mouse pups (18).

Lastly, IL-13 is a cytokine derived from T cells, which has both inflammatory and anti-inflammatory properties. IL-13 inhibits the production of other inflammatory cytokines (IL-1α, IL-1β, IL-6) through its effects on inflammatory macrophages (97). IL-13 is recognized as a key driver in allergic and inflammatory airway disease, where its effects are potentiated by IL-17 (98). Raised IL-13 has been noted in the plasma and PMBCs of children affected by ASD (29, 99), particularly those with comorbid asthma (although IL-13 is known to be skewed in those with co-morbid atopic conditions) (35).

IL-17A ASSOCIATED ANTI-INFLAMMATORY MEDIATORS IN ASD

Another member of the STAT family, STAT6, suppresses the IL-17A inflammatory response. In certain conditions, STAT6 signaling attenuates IL-17A producing T-cells, reducing their production of IL-17A (100). IL-4 mediated inhibition of Th17 cells and IL-17A production is STAT6 dependent (101). In human studies, children with ASD reportedly have reduced levels of STAT6-expressing CD45 cells ($CD45^{+}STAT6^{+}$) in their PBMC profile compared to neuro-typical controls (80). STAT6, as part of the IL-4 signaling cascade can enhance the expression of anti-inflammatory mediators. This pathway is critical for acceptance of the fetal graft, through reduction of Th17 cells and increase of both IL-4 and Tregs in the fetal environment (102, 103).

In addition to downregulation of the STAT6 mediated pathways, downregulation of other anti-inflammatory cytokines is also reported in autism. Anti-inflammatory cytokine IL-10 acts as a "master" immuno-regulator (104) and IL-10 concentrations are significantly lower in ASD children compared with neuro-typical controls (79, 105). Cytotoxic T-lymphocyte antigen 4 (CTLA4) is a glycoprotein located on T cells (106) and is induced following T cell activation. This anti-inflammatory molecule is expressed at lower levels in the PBMCs of children with ASD (107). Reductions in the levels of these anti-inflammatory and

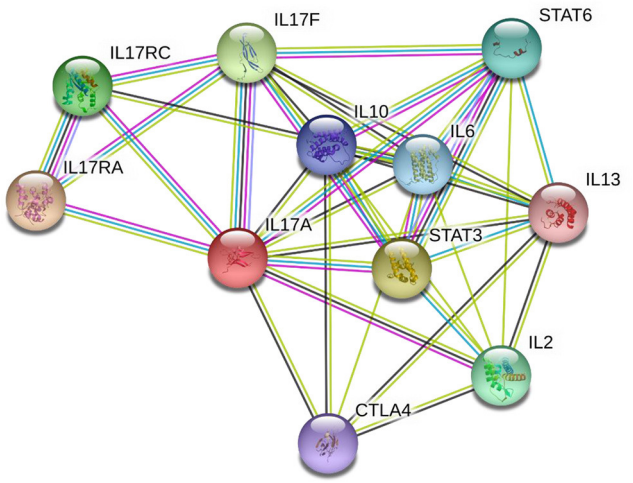

FIGURE 2 | STRING diagram illustrating the known and predicted protein interactions for IL-17A. Network nodes represent proteins—each node represents all the proteins produced by a single, protein-coding gene locus. Edges (lines) represent protein-protein associations that are specific and meaningful, i.e., proteins jointly contribute to a shared function; this does not necessarily mean they are physically binding each other. Blue connecting lines indicate that protein interaction information was derived from curated databases, pink indicates the interaction was experimentally determined, yellow indicates the interaction was determined *via* text mining, black indicates protein co-expression, and lilac indicates protein homology. Analysis was performed on 28 July 2021 *via* the string-db.org domain.

regulatory proteins may lead those with ASD to acquire a more pro-inflammatory state.

LINKING IMMUNITY AND GENETICS IN ASD

Bioinformatics analysis of large CNV studies suggest strongly that innate immune processes are implicated in ASD risk (108), this may indicate that immune dysfunction in ASD may be genetically driven or influenced. Maternal immune activation downregulates expression of susceptibility genes known to be highly penetrant in ASD and heavily involved in neurogenesis, cell signaling, synaptogenesis, and axonal guidance in the early stages of fetal development (108, 109). When compared with curated ASD associated gene sets [e.g., *via* the SFARI Gene database (http://gene.sfari.org/)], MIA downregulated genes were substantially enriched. The strongest enrichment of MIA downregulated genes was observed in the ASD gene categories with the highest likelihood of a link to ASD i.e., SFARI "High Confidence" or "Syndromic" ASD gene sets. This suggests that MIA may bestow increased ASD risk through downregulating the expression of the same genes that are highly penetrant in ASD during the early stages of fetal development.

Loss of function mutations in TSC1 and TSC2 genes are linked to syndromic ASD, and these genes are critical upstream regulators of the mammalian target of rapamycin (mTor) pathway. mTor has important functions in innate immunity and metabolism in particular (52, 110, 111).

Maternal immune activation also has downstream effects, in some cases influencing the transcriptome rather the genes themselves. Fragile X mental retardation 1 gene (FMR1) and CHD8 are both highly penetrant genes for ASD, yet MIA does not seem to influence expression of these genes directly. Rather, it wields an influence on downstream gene targets such as FMRP (fragile X syndrome protein complex). This raises the possibility that MIA may act as an environmental factor disrupting crucial early developmental genomic pathways through influence on downstream gene targets (108). This might suggest that MIA could act both in a direct (genetic) and indirect fashion (epigenetic/regulatory) with the end effects converging on similar pathways.

As previously, mentioned, normal pregnancy is associated with suppression of immunity, allowing the fetus to develop inside the mother's innate immune system. Human leukocyte antigen G coding gene antigen recognition controls the placental immune response and allows acceptance of the fetal graft. Human leukocyte antigen G coding gene interacts with the CD8 cell surface antigen found on most cytotoxic T-lymphocytes that mediate efficient cell–cell interactions within the immune system (112). Higher rates of HLA-G mutations have been found in mothers of children with ASD (113). The Th17 pathway in particular has been identified as a likely effector of inflammatory changes on the developing fetal brain, with downstream effects on behavior and cognitive development (46, 114). We hypothesize that the physiological changes in maternal immunity during pregnancy are dysregulated in some mothers of children with ASD.

In summary, many of the inflammatory proteins reported to have altered expression in ASD are linked to pro-inflammatory Th-17 cells, their product IL-17A, and the IL-17 receptors and receptor complexes. It appears that IL-6 activation (regulated by STAT3 and STAT6 *via* RORγt activity) of IL-17 expression, and subsequent upregulation of IL-17 receptors and receptor complexes may have a key role in the pathogenesis of ASD. The majority of linked molecules identified above are pro-inflammatory and found in higher quantities in those with ASD, with a corresponding downregulation of anti-inflammatory proteins. Whether this dysregulation of IL-17 is an inherent or acquired state is unclear.

Circulating T cell and IL-17A levels are altered in a subset of children with ASD. Maternal immune activation (including IL-17A) seems to play a role in altering important developmental pathways through direct interaction with ASD susceptibility genes, and indirectly, through interaction with their gene products. Circulating levels of IL-17A are dysregulated during pregnancy in mothers of children who develop ASD and ID (63, 79, 83). Murine models support a causative role for IL-17A in the pathogenesis of ASD. We conclude from the existing evidence that IL-17A dysregulation in the mother or developing infant could play a causal role in the development of at least some subsets of ASD and may be the link between environmental exposure and genetic susceptibility. Understanding the role of IL-17A and its associated targets on neurodevelopmental outcomes is now becomin increasingly important.

WHAT IS THE RELEVANCE OF THE ONGOING COVID-19 PANDEMIC TO MIA-INDUCED ASD RISK?

Coronavirus disease 2019 (COVID-19), a disease caused by the novel coronavirus, SARS-CoV-2, has become a pandemic, affecting every corner of the globe. Although, the disease (COVID-19) affects primarily the respiratory systems of those affected, it has been found to affect and damage other organs, including the kidneys (115), liver (116), brain (117, 118), and heart (119, 120). Worldwide reported cases and COVID-19 related mortality are most likely an underestimate due to variability of public health capacities between countries, but as of August 2021, there have been almost 200 million confirmed cases of COVID-19, and over 4.2 million deaths reported to the WHO (121).

Our current knowledge of COVID-19 is based only on our limited experience with SARS-CoV-2 since December 2019 and analogously, through our experience of other coronaviruses (SARS CoV and MERS, Middle East Respiratory Syndrome). The long-term consequences of *in-utero* SARS-CoV-2 exposure and/or congenital infection are almost entirely unknown. There is clear evidence that prenatal exposure to viral infections increases the risk of adverse developmental, neurological, and psychiatric outcomes in later childhood and adult life (38, 44, 122). In this next section, we discuss the implications of the COVID-19 pandemic in the context of MIA-induced alterations in neurodevelopmental outcomes.

COVID-19 AND CYTOKINE STORM

Preclinical work shows that MIA, which stimulates interleukin-17A release from Th17 cells, can establish sustained fetal-placental inflammatory responses. This inflammatory milieu can persist into childhood and affect the development of the young "primed" brain. Remarkably, in murine models, social difficulties in MIA-exposed offspring are remediable through a variety of mechanisms including IL-17 blockade (18, 46). Cytokine storm is a general term applied to maladaptive cytokine release in responses to infection and other stimuli (123). In the context of sepsis, cytokine storm is considered one of the major causes of acute respiratory distress syndrome (ARDS), systemic inflammatory response syndrome (SIRS), and multi-organ failure (124, 125). In COVID-19, cytokine storm seems to play a role in disease aggravation and correlates positively with severity of disease (126). IL-17A target IL-6 and C-reactive protein (CRP) specifically, have been shown to correlate positively with increased mortality (127). Elevated numbers of Th17 cells have been isolated in the blood of individuals with fatal COVID-19 infection (128), while many authors have demonstrated significantly elevated levels of IL-17A in those with both mild and severe COVID-19 (129–131). Coronavirus infection results in macrophage, and dendritic cell activation and IL-6 release (132). This instigates an amplification cascade (JAK–STAT1/3 pathway) that results in cis signaling (binding of cell membrane bound IL-6 receptors) in lymphocytes with downregulation of Tregs and increased differentiation of TH17 cells; as well as trans-signaling (binding of soluble IL-6 receptor) effects on many other cell types (endothelial cells). This widespread immune activation and cytokine production contributes to the pathophysiology of severe COVID-19 (133). Indeed, some authors have specifically suggested therapies intended to target both Th17 cells and IL-17A in COVID-19 disease (134, 135). We have already outlined how Th17 specific (T-helper 17 cell) pathways are initiated *via* activated macrophages that produce IL-6 and IL-1β. As outlined, IL-6 in particular, is a potent potentiator and trigger for IL-17A release (123, 134, 136). IL-17A therefore, may be a key player in the COVID-19 cytokine storm.

CORONAVIRUS (SARS-CoV-2) NEUROTROPISM AND NEUROLOGICAL EFFECTS

Coronaviruses have a demonstrated specific neuro-tropism that allows them access to, and to proliferate in, the host's CNS (137, 138). Cell entry seems to occur through the angiotensin-converting enzyme-2 (ACE-2) and transmembrane protease serine 2 (TMP S2) receptors, both of which are widely expressed in the placenta and at the feto-maternal interface. While trans-placental infection of the fetus is, yet to be proven conclusively, vertical transmission is certainly plausible and may lead directly to inflammatory processes in the fetal brain, in addition to indirect effects *via* the host/maternal immune response. The neurological sequelae of COVID-19 are wide-ranging and relatively common. The majority of neurological presentations so far have fallen into five categories, (i) Encephalopathy (including delirium and impaired consciousness), (ii) Inflammatory CNS disorders [including encephalitis and Acute Disseminating Encephalomyelitis (ADEM)], (iii) Cerebrovascular accident (CVA)/stroke, (iv) PNS disorders [including Guillain-Barré Syndrome (GBS) and cranial nerve palsies], (v) "Miscellaneous" central neurological disorders (such as raised intracranial pressure, seizures, and myelitis) (139). Hyposmia/Anosmia and hypogeusia (140) are recognized as two important hallmarks of acute SARS-CoV-2 infection, while more severe neurological complications have included CVAs, encephalitis, encephalopathy, and neuropsychiatric disorders (118, 141). Protein-protein network analysis for GBS and COVID-19 revealed that the combined gene set showed an increased connectivity as compared to COVID-19 or GBS alone, this was particularly true of genes related to Th17 cell differentiation. Transcriptome analysis of PBMC from patients with COVID-19 and GBS demonstrated the activation of interleukin-17 signaling in both conditions (142). Viral RNA has been isolated in clinical CSF samples in those with COVID-19 and neurological symptoms (143), and post-mortem examination of brain tissue has identified both viral RNA and neutrophilic infiltrates suggestive of aberrant immune response (144).

Recent pluripotent stem cell derived organoid models have been used to model SARS-CoV-2 infection in a wide range of tissues including gut, lung, liver, kidney, and brain (117, 145). These models demonstrate the virus' ability to infiltrate and

proliferate in a variety of different cell/tissue types. Within the brain, the areas with the highest avidity for SARS-CoV-2 are the choroid plexus and the hippocampus (117). This is an interesting finding, as the choroid plexuses themselves represent the interface between CSFand blood compartments (in a similar fashion to the blood-brain barrier). They are located in each of the four ventricles, and are intimately related with immediately adjacent CSF, capillary blood supply, and neural tissue. Angiotensin-converting enzyme-2 receptors also appear to be highly expressed in the choroid plexus (146). In this sense, they provide a comprehensive roadmap upon which SARS-CoV-2 can potentially travel. The neurological features on COVID-19 infection are diverse and wide-ranging. Most studies to date have focused on symptomology in adult patients, but novel models of SARS-CoV-2 infection in a variety of human and animal tissues is casting new light on the mechanisms underlying COVID's infectivity and its ill-effects. There appears to be a variety of mechanisms underlying COVID's pathogenicity, not limited to direct viral effects on tissue, but also collateral effects *via* immune and thrombotic processes (147). Although there is little research on the effects of COVID on fetuses in early pregnancy, the same processes of direct viral effects and secondary immune and inflammatory effects are likely to be at play.

MATERNAL COVID-19 INFECTION AND PERINATAL EXPOSURE

Pregnant women are not thought to be more susceptible to contracting coronavirus than the general population (148), but given alterations in the pregnant immune state (103), they may be more susceptible to more severe infection (149, 150). Studies from previous pandemics, H1N1 influenza (2009), SARS (2003), and MERS (2012), suggest the possibility of significant maternal and neonatal morbidity and mortality (151, 152). Indeed, both MERS and SARS resulted in maternal death in a significant number of cases, but the specific risk factors for a fatal outcome during pregnancy are not clear. Our experience with these previous coronaviruses indicates higher risk of adverse outcomes for the fetus and infant including fetal growth restriction (FGR), and preterm delivery, both of which have previously been linked to increased ASD incidence (153) as well as NICU admission, spontaneous abortion, and perinatal death. As with other Coronaviruses, maternal SARS-CoV-2 infection has been associated with negative perinatal outcomes. Preterm delivery, fetal distress, stillbirth, and perinatal death have been widely reported (150, 154–156). Figures from China show that while up to 3% of pregnant women infected with COVID-19 required admission to intensive care (157, 158), a UK study showed 1% of pregnant women admitted with SARS-CoV-2 required ECMO (Extra-corporeal membrane oxygenation) and 10% Intensive Care Unit (ICU) management (159).

Cesarean section (CS) has been implicated as a risk factor for the development of ASD in offspring. The mechanisms underlying this are unclear, yet the risk of ASD is increased by approximately 33% in both elective and emergency CS procedures (160). In a systematic review of perinatal and maternal outcomes during the pandemic, CS rates were reported at extremely high levels, up to 90% in some centers (range from approximately 50–90%) (161). For comparison in work published in 2020, Turner at al noted an all-cause national CS rate in Ireland of approximately 26% (162). These higher rates were observed in most centers in spite of recommendations from the Royal College of Obstetrics and Gynecology (RCOG) and the International Federation of Gynecology and Obstetrics (IFGO) against decisions for CS being influenced by maternal SARS-CoV-2 status.

More specifically to neonatal outcomes, the WHO quotes worldwide preterm delivery rates of approximately 10% (163). Two large review studies reported preterm delivery rates of 20–25% in SARS-CoV-2 affected pregnancies (164, 165). Women with SARS-CoV-2 seemed to be more likely to endure a preterm delivery (165). The majority of these deliveries were iatrogenic, but in some reviews, up to half were attributable to either fetal or maternal compromise (166).

Maternal and neonatal ICU admission rates were also higher in the SARS-CoV-2 affected cohorts. Maternal ICU admission and mechanical ventilation rates were high vs. age matched non-pregnant women (165). While rates of stillbirth and neonatal death appear similar to uninfected fetuses, NICU admission rates were notably higher in COVID affected pregnancies (159), commonly as a precautionary step in the care of the neonate. Neonatal morbidity was higher in the SARS-CoV-2 affected groups and was associated with preterm delivery in mothers with more severe COVID-19 primary infection. Hypoxemia and respiratory difficulties in mothers had knock on effects of reduced placenta perfusion, pre-placental hypoxemia, fetal distress, and preterm delivery (167).

Given our knowledge of the potential developmental effects of Th17 activation in pregnancy, children *in-utero* during this pandemic may have significant inflammatory exposures if maternal infection occurs. There remain unanswered questions about the impact that asymptomatic and mild maternal infection has on the fetal brain in early pregnancy. Prospective follow up studies will need to follow infants whose mothers were infected as well as health unaffected controls. There is enormous potential to leverage archived serological samples from pregnancy and neonatal cohorts to study the relationships (or associations) between markers of maternal inflammation and later neurodevelopmental outcomes in offspring born during the pandemic. While in general, the likelihood of intrauterine maternal-fetal transmission of coronaviruses is low—there have been no documented cases of vertical transmission occurring with either SARS or MERS. There are current reports of possible vertical transmission of SARS-CoV-2 in several cases of third trimester maternal infection (168–170). Little to no information exists about children exposed in the first and second trimesters yet. While generally placental seeding does not seem common, some cases have reported strong evidence of placental infection with the demonstration of high viral load and immuno-histological evidence of SARS-CoV-2 in placental tissue (168). Currently, we can only surmise what the true effect (if any) of gestational COVID-19 on the incidence of ASD will be, but already some have concerns that the incidence

may increase (171, 172). No studies have yet been reported on neurodevelopmental outcomes, as the oldest offspring are still in early childhood. Still, the evidence we have outlined within this review from MIA studies examining IL-17A and its pathway members provides a strong basis to build upon our current hypothesis and ask the question; could COVID-19 induced MIA act *via* IL-17A signaling to increase the risk of ASD-like phenotypes in vulnerable offspring?

DISCUSSION: IMPROVING OUTCOMES FOR ASD AFFECTED INDIVIDUALS AND FAMILIES

We believe that in spite of the tragedy of the COVID-19 emergency, we are presented with a serendipitous opportunity to progress scientific knowledge regarding prenatal exposures and ASD risk. During the COVID-19 pandemic, we have witnessed a novel infection, affect an immunologically naïve population within an extremely well-defined period of exposure. COVID-19 is now a notifiable illness, and has been characterized and monitored more than any illness in history. Many countries have developed stringent mandatory testing protocols, and track and trace programmes. Within all this, exists an opportunity to study the longitudinal effects of this infection on offspring of those affected by gestational COVID. Further investigation of mid-gestational cytokine profiles (IL-17A in particular) and their potential for genetic interplay could be a crucial cog in the development of actionable and cost-effective improvements in the current models of ASD care. Identification of pathways of immune dysregulation during pregnancy could lead to the identification of a risk marker of ASD that could be characterized in broader ASD cohorts. This would facilitate the identification of a predictive marker of ASD allowing earlier dedicated ASD screening in at risk children. Coupled with these potential biochemical markers, known early clinical signs of ASD exist. Crystallization of the ASD diagnosis can be as early as 14 months old according to some authors, and there are clinically detectable signs of ASD from a younger age still (23, 173, 174). The first children born of this pandemic are now reaching their toddler years, and they may represent a group with increased risk of ASD or other developmental conditions. Taken together, a postulated early biochemical marker and established early clinical markers could allow targeted early ASD screening, which would lead to earlier intervention, and improved outcomes. Therapies instituted in this age group have the potential to significantly improve clinical outcomes in ASD affected children. The timing of therapy is important with the most dramatic symptomatic and developmental improvements in those detected at an earlier age of diagnosis (175, 176).

We believe that it is the obligation of the scientific community to glean what benefit we can from this pandemic. In spite of social distancing measures, systematic national "lockdowns," and working from home, there has been unprecedented scientific collaboration to try to counter the scourge of COVID. This has led to some outstanding success, not least in the development of two highly effective mRNA vaccines. In order to facilitate international research, the development of an international gestational COVID-19 consortium and registry would be an important step in coordinating research activities and aims. Isolation of relevant clinical bio-samples and prospective identification of patients will have already begun in some centers, and should be facilitated by the public health infrastructures that have been built up around the pandemic. Multidisciplinary collaborative follow up programmes should be established to identify, assess, and treat children with potential negative post-COVID outcomes.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

MC wrote the manuscript, reviewed the literature, and synthesized the hypothesis. SC, LGi, and LGa commented on the manuscript at all stages. GO'K commented on the manuscript and aided with literature review. DM commented on the manuscript, helped to synthesize the hypothesis, review the literature, and was the key supervisor. All authors have read and approved the final manuscript.

FUNDING

Funding was provided to MC by the National Children's Research Center (NCRC) (grant D/19/1), Our Lady's Children's Hospital, Crumlin, Dublin 12, Ireland.

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frontiers in Psychiatry

ORIGINAL RESEARCH
published: 30 March 2022
doi: 10.3389/fpsyt.2022.859198

Transcriptome Analysis in Hippocampus of Rats Prenatally Exposed to Valproic Acid and Effects of Intranasal Treatment of Oxytocin

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Autism spectrum disorder (ASD) is a heterogeneous disorder characterized by repetitive behaviors and social impairments, often accompanied by learning disabilities. It has been documented that the neuropeptide oxytocin (OXT) ameliorates core symptoms in patients with ASD. We recently reported that chronic administration of intranasal OXT reversed social and learning impairments in prenatally valproic acid (VPA)-exposed rats. However, the underlying molecular mechanisms remain unclear. Here, we explored molecular alterations in the hippocampus of rats and the effects of chronic administration of intranasal OXT (12 μg/kg/d). Microarray analyses revealed that prenatal VPA exposure altered gene expression, a part of which is suggested as a candidate in ASD and is involved in key features including memory, developmental processes, and epilepsy. OXT partly improved the expression of these genes, which were predicted to interact with those involved in social behaviors and hippocampal-dependent memory. Collectively, the present study documented molecular profiling in the hippocampus related to ASD and improvement by chronic treatment with OXT.

Keywords: autism spectrum disorders, hippocampus, oxytocin, transcriptome analysis, valproic acid

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Autism, a section of the journal Frontiers in Psychiatry

Received: *21 January 2022*
Accepted: *04 March 2022*
Published: *30 March 2022*

Citation:
Matsuo K, Shinoda Y, Abolhassani N, Nakabeppu Y and Fukunaga K (2022) Transcriptome Analysis in Hippocampus of Rats Prenatally Exposed to Valproic Acid and Effects of Intranasal Treatment of Oxytocin. Front. Psychiatry 13:859198. doi: 10.3389/fpsyt.2022.859198

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by social deficits and repetitive behaviors (1). ASD involves heterogeneous and complex causal factors, both genetically and environmentally. While it has been documented that various copy numbers or single nucleotide variations are associated with ASD, multiple environmental factors such as maternal infection, exposure to drugs or toxicants, and immune dysregulation have also been implicated (2). Such diverse causes, their interactions, and the resultant complex symptoms make it difficult to focus on particular targets and therapeutic approaches in ASD.

Abbreviations: ASD, autism spectrum disorder; DEG, differentially expressed gene; GO, gene ontology; HDAC, histone deacetylase; LTP, long-term potentiation; MCODE, molecular complex detection; OXT, oxytocin; SHANK, SH3 and multiple ankyrin repeat domains; VPA, valproic acid.

The neuropeptide oxytocin (OXT) facilitates socio-communicative behaviors in mammals (3). OXT or OXT receptor deficiency impairs multiple social behaviors (4, 5). In addition, plasma levels of OXT are significantly lower in children with autism than in their normal counterparts (6, 7). Clinical studies have documented that the intranasal administration of OXT improves socio-emotional impairments in patients with ASD (8, 9). Thus, OXT could be considered a suitable candidate for treating the core symptoms in ASD.

Maternal use of the anti-epileptic drug valproic acid (VPA) during pregnancy is suggested to increase the risk of teratogenicity and ASD onset in offspring (10, 11). These features are reproducible in animals; they represent defects in the limbs and tail and ASD-like social deficits (12, 13). In addition, transcriptome analyses have identified some key molecular pathways for ASD in the amygdala and prefrontal cortex of the model (12, 14, 15); alteration in signaling of protein kinase A and Rho GTPases in the amygdala and calcium signaling in the prefrontal cortex. These pathways are related to synaptic plasticity, a pathological hallmark in ASD (16–19), suggesting that molecular alterations in these emotion-related regions are involved in the pathogenesis of ASD. Especially, GTPases signaling defects are well documented in neurodevelopmental disorders including ASD (20); Rho GTPase Cdc42, a regulator of neurite outgrowth, is reduced in autistic patients (21). Prenatal VPA exposure decreases mRNA levels of Rho GTPase-activating protein p250GAP (22). In addition, p21-activated protein kinase exchange factor, a Rho GTPase regulatory protein, interacts with SH3 and multiple ankyrin repeat domains (SHANK) proteins in spines to regulate postsynaptic structure (23). Since mutations in SHANK gene have been associated with neurodevelopmental and neuropsychiatric disorders, including ASD (24), it is noteworthy that recent findings show that OXT restores neurite abnormalities in hippocampal cultures with SHANK3 deficiency through amelioration of Rho GTPase levels (25). Thus, prenatally VPA-exposed animals are a valid ASD model in terms of behavioral phenotypes and epigenetic modulation of gene expression as an environmental factor.

In our previous studies, we had reported that prenatal VPA exposure impairs learning and long-term potentiation (LTP) in the hippocampus of rats and that chronic administration of intranasal OXT ameliorates learning disabilities (26, 27). In line with our observations, intranasal administration of OXT blocked the learning disability in prenatally VPA-exposed or restraint stress-exposed animal models (28–30). In contrast, Hara et al. demonstrated that a single dose of OXT was effective for social impairment only for a short period and not for memory impairment (31). These reports suggest that the mechanisms of action of chronic OXT would involve molecular alterations in addition to the acute activation of oxytocinergic signaling. However, it remains unclear which molecular pathways are affected by chronic administration of OXT. Furthermore, molecular profiling in the dorsal hippocampus, a critical region for learning and memory, and partly implicated for social behaviors (32, 33) has not been investigated in a prenatally VPA-exposed model.

In the present study, we explored transcriptome profiling in the hippocampus of prenatally VPA-exposed rats. It was seen that prenatal VPA exposure altered the expression levels of genes involved in multiple behaviors and developmental behaviors, some of which were documented as candidate genes in ASD. We also demonstrated that the chronic administration of intranasal OXT partly ameliorated these alterations.

MATERIALS AND METHODS

Animals

Animal studies conformed to the Regulations for Animal Experiments and Related Activities at Tohoku University and were approved by the Committee on Animal Experiments at Tohoku University (approval number: 2020PhA-007). Every effort was made to use minimum the number of rats and minimize their discomfort. Animals were bred in a conventional environment (temperature, 21–23°C; humidity, 50–60%; 12-h light-dark cycle) with free access to normal chow and water. Three pregnant Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) received a single administration of oral VPA (600 mg/kg; Sigma-Aldrich, St. Louis, MO, United States) on day 12.5 as described previously (27). Two control rats received water in the same way. Rats gave birth from 7 to 14 pups per litter, of which 3–8 were males (sex ratio of male to female was about 1:1.1). Sizes and body weights of pups were not affected by VPA treatment, as reported by a previous study (26). Only male pups were included in this study because of the higher incidence of ASD in males than in females and even in prenatally VPA-exposed models (34).

Oxytocin Treatment

On day 21 of birth, the rats were randomly divided and subsequently received vehicle or OXT administration: 3 pups from 2 control rats and 6 pups from 3 VPA-treated rats, with the latter further divided into 3 vehicle- or OXT-treated groups of 3 pups each. Male pups received intranasal OXT (Peptide Institute, Osaka, Japan) dissolved in saline at a dose of 12 μg/kg/d, which is in a range of that promoting social behaviors in rodents (35–37), using a pipette tip on postnatal day 21–55. The liquid volume of OXT solution was changed in the range of 2–10 μL according to the growth of rats. The dose and period of treatment were the same as those in a previous study where OXT attenuated autistic behaviors in prenatally VPA-exposed rats (27).

Identification of Differentially Expressed Genes in Microarray Analysis

Total RNA was extracted from the dorsal hippocampus on postnatal day 56. As with most transcriptome analyses, the number of samples per condition is 2–5 for the analyses using VPA-treated ASD models (12, 14, 15). Therefore, we decided that three samples per condition would be sufficient for this study. Expression profiles were determined using Rat Gene 2.0 ST array systems (Affymetrix, Santa Clara, CA, United States) and analyzed using Transcriptome Analysis Console software (version 4.0; RRID:SCR_018718; Affymetrix).

Differentially expressed genes (DEGs) were evaluated as follows: (1) $P < 0.05$, in a one-way analysis of variance computed by limma (38), (2) more than 1.5 fold-change (both increase and decrease) between control and vehicle-treated VPA groups. (3) Expression levels (in $\log_2$ scale) more than 6.6 at least in one group. Among these, significantly different (improved) values between the vehicle-treated VPA and OXT-treated VPA groups were further investigated. Expression levels were normalized to those of the control group. Microarray data were deposited in the GEO database (accession number: GSE196500).

Gene Ontology Enrichment Analysis

Gene ontology (GO) enrichment analysis was performed using Metascape (RRID:SCR_016620[1]) (39). To obtain the most comprehensive data, gene identifiers were converted from *Rattus norvegicus* to *Homo sapiens* orthologs. GO terms classified in biological processes were collected under the following conditions: a minimum count of 3, $P < 0.01$, and an enrichment factor (the ratio between the observed counts and the counts expected by chance) > 1.5. Cytoscape App (RRID:SCR_003032) was used to create and plot the enrichment network (40).

Protein-Protein Interaction Enrichment Analysis

To investigate protein networks consisting of proteins that form physical interactions each other in DEGs, protein-protein interaction analysis was performed using databases including BioGrid (RRID:SCR_007393) (41), InWeb_IM (42), and OmniPath (43) in Metascape. The molecular complex detection (MCODE) algorithm (RRID:SCR_015828) (44) was applied to identify tightly connected modules in each network. MCODE analysis was performed with default settings; detection when there are at least three genes in a network.

Gene-Disease Association and Regulatory Interaction Analysis

To reveal the involvement of DEGs in diseases and their transcriptional regulation, DisGeNET (RRID:SCR_006178) (45) and TRRUST (46) were performed in Metascape. The algorithm was the same as that of the GO enrichment analysis discussed above.

Overlapping With Autism Spectrum Disorder Risk Genes and Predictive Analysis of Interaction

Two databases were used to explore the overlap between DEGs in the VPA model and candidate genes in ASD patients: SFARI (RRID:SCR_004261[2]) and Krishnan's datasets Genome-wide predictions of autism-associated genes[3] (47). Gene lists in SFARI were scored as follows: S (syndromic), 1 (high confidence), 2 (strong candidate), and 3 (suggestive evidence). In Krishnan's dataset, gene list associated with ASD are separated between brain regions and developmental periods, and the hippocampus in middle-late childhood (ID: HIP. 11) was selected to be appropriate for comparison with the results of this study. Additionally, to predict the interactive networks between DEGs improved by OXT and ASD candidate genes, Krishnan's dataset was used.

[1]http://metascape.org

[2]https://gene.sfari.org/

[3]http://asd.princeton.edu

RESULTS

Gene Expression Profiles in the Hippocampus of Prenatally Valproic Acid-Exposed Rats

Gene expression profiles of the hippocampus were compared between the control, vehicle-treated VPA, and OXT-treated VPA groups ($n = 3$ per group). Hierarchical clustering tended to separate the vehicle-treated VPA group from the others, except for one sample from the OXT-treated VPA group (**Figure 1A**). Microarray analysis revealed that 377 genes differed significantly by more than 1.5-fold between the groups (**Figure 1A**). Among these, 174 genes were considered DEGs (see section "Materials and Methods"). We then analyzed the biological processes of DEGs between the control and vehicle-treated VPA groups. GO enrichment analysis revealed that DEGs were roughly associated with multiple functions, including signaling, behavior, and developmental processes (**Figure 1B**). An in-depth analysis further indicated that DEGs are involved in chemical synaptic transmission, short-term memory, brain development, as well as nervous system development (**Figure 1B**). These results suggest that prenatal VPA exposure affects multiple genes in the hippocampus that are involved in synaptic function, learning and memory, and neurodevelopment, all of which are the key features of ASD. In particular, the enrichment network analysis also indicated that chemical synaptic transmission (#1) serves as a hub function in these biological processes (**Figure 1C**). Protein-protein interaction analysis further predicted that several interactive networks are formed among the DEGs (**Figure 1D**). Interestingly, histone deacetylase 1 (HDAC1) and specificity protein 1 (SP1), both of which are regulated by VPA (48, 49), were identified as factors for the transcriptional regulation of DEGs (**Figure 1E**).

Autism Spectrum Disorder-Associated Molecular Changes in the Hippocampus by Prenatal Valproic Acid Exposure

We next addressed which human diseases are associated with DEGs. Notably, DEGs are known to be enriched in ASD-associated [mental disorders, Gilles de la Tourette syndrome (50), and various types of epilepsy] and learning disability-associated (cognition disorders and mental deterioration) diseases (**Figure 2A**). To further reveal the relationship with human ASD, the overlap between DEGs and ASD candidate genes was investigated using two databases. Eight DEGs were included in the SFARI database, and *Ahi1* was indicated as a cause of syndromic ASD (**Figure 2B**). In addition, 13 DEGs

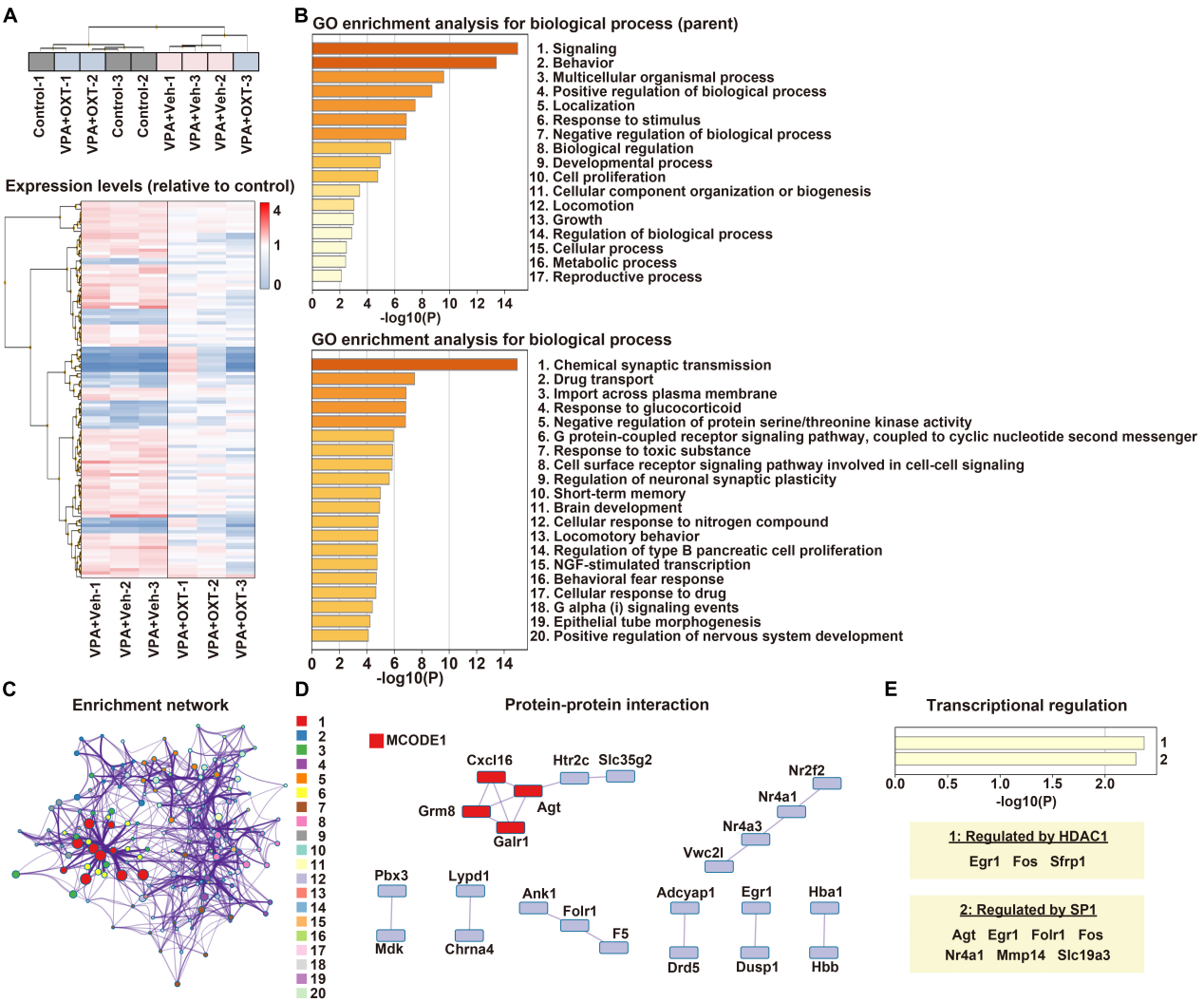

FIGURE 1 | Transcriptome profiling in the hippocampus of prenatally VPA exposed rats. **(A)** Hierarchical clustering of DEGs between groups. Expression levels were normalized to those of the control. **(B)** Biological processes of DEGs in GO enrichment analysis. Top, parent; Down, child annotations, respectively. **(C)** Enrichment networks between clusters of biological processes. Shown numbers are the same with those of child annotations in panel **(B)**. **(D)** Protein-protein interactive networks between DEGs. Shown by MCODE1 represents a strongly connected network. **(E)** Analysis of transcriptional regulation of DEGs. HDAC1 and SP1 are detected as significant regulators. VPA, valproic acid; DEG, differentially expressed gene; GO, gene ontology; MCODE1, molecular complex detector; HDAC, histone deacetylase; SP1, specificity protein 1.

overlapped with gene sets in the hippocampus of ASD in middle-late childhood in Krishnan's database (**Figure 2C**). Collectively, these results suggest that molecular profiles in the hippocampus could underlie autistic behaviors, including learning disabilities seen in prenatally VPA-exposed rats.

Effects of Chronic Administration of Intranasal Oxytocin on Gene Expression in the Hippocampus of Prenatally Valproic Acid-Exposed Rats

To explore the molecular mechanisms by which chronic OXT treatment improves the prenatal VPA exposure induced autistic behaviors, significantly improved populations by OXT among DEGs were extracted (**Figure 3A**). Chronic administration of intranasal OXT (12 μg/kg/d) significantly upregulated 13 genes and downregulated two genes among the DEGs (**Figure 3B**). GO enrichment analysis revealed that these improved genes belonged to partly similar processes with DEGs (**Figure 1B**), including the developmental process, signaling, and import across the plasma membrane, and different genes such as those involved in epithelial tube morphogenesis and response to steroid hormone (**Figure 3C**). These GO genes formed networks with each other except for aging, and epithelial tube morphogenesis, and thus would serve as a hub annotation (**Figure 3D**). Finally, interactive networks were predicted between ASD candidates and DEGs improved by OXT using Krishnan's database (except for *Mt-nd3*, which were not registered in the database). The

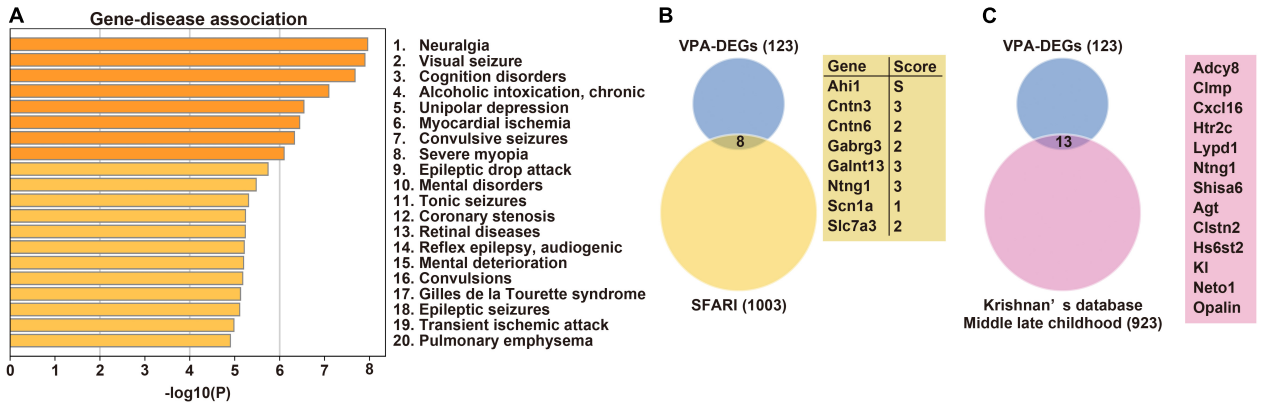

FIGURE 2 | Disease-associated profiling and overlap with ASD candidate genes. **(A)** Association between DEGs and human diseases. Venn's diagrams depict overlap between DEGs and candidate genes for ASD registered in SFARI **(B)** and Krishnan's database **(C)**, respectively. ASD, Autism spectrum disorder; DEG, differentially expressed gene.

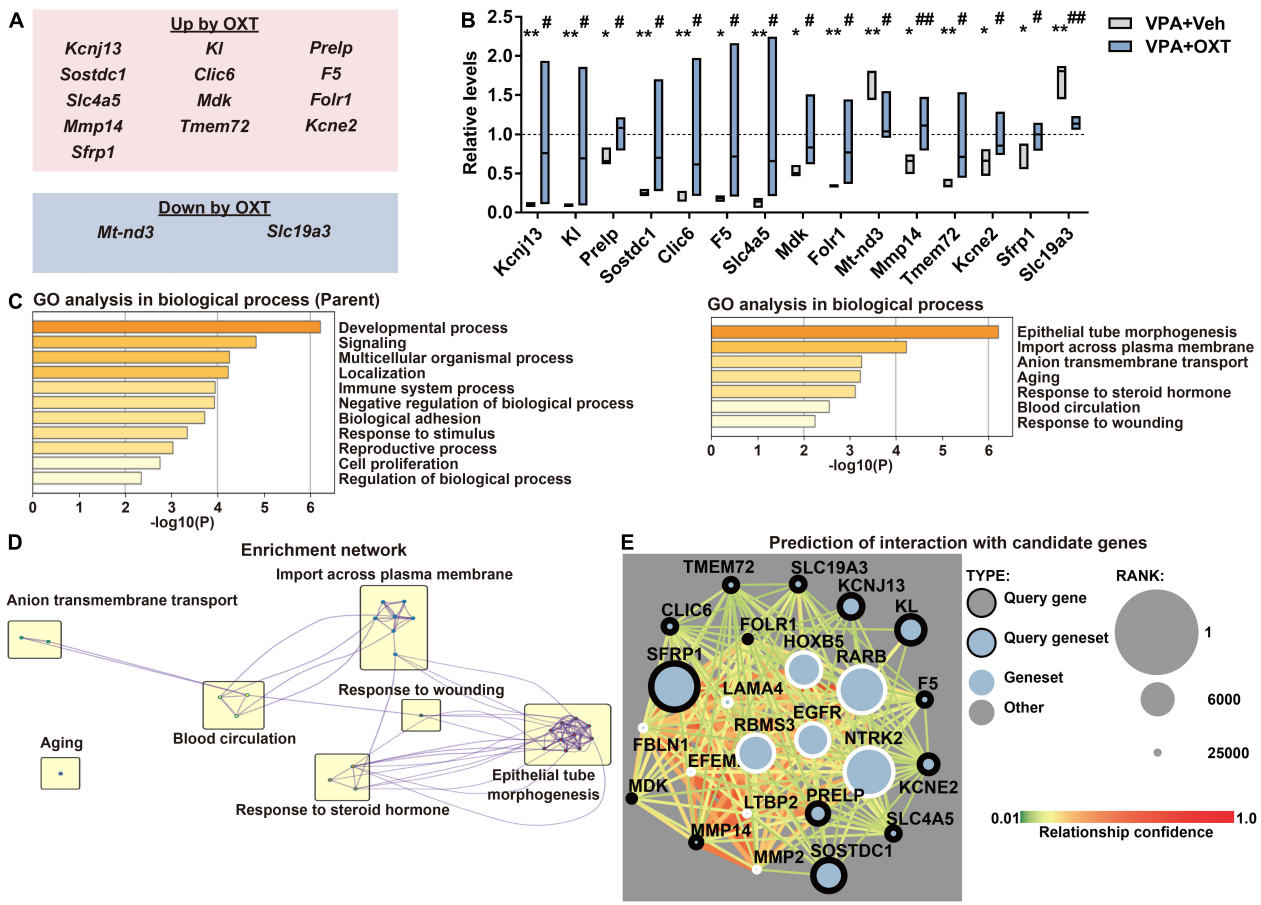

FIGURE 3 | Effect of chronic treatment of intranasal OXT on prenatal VPA exposure-induced molecular changes. **(A)** Summary of (top) upregulated and (bottom) downregulated DEGs by OXT (12 μg/kg/day). **(B)** Quantification of expression levels of genes improved by OXT. The levels were normalized to those of the control. The top and bottom of the bar depict the highest and the lowest of values, respectively. The line within the bar shows the median (n = 3 per group). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. vehicle-treated VPA. **(C)** Biological processes of DEGs in GO enrichment analysis. Left, parent; Right, child annotations, respectively. **(D)** Enrichment networks between clusters of biological processes with child annotations. **(E)** Predictive interaction between DEGs improved by OXT and ASD candidates in Krishnan's database. Shown are top 10 of candidates connecting strongly with our gene sets. DEG, differentially expressed gene; OXT, oxytocin; GO, gene ontology.

predicted top 10 genes that interacted with 14 of the DEGs improved by OXT are shown in **Figure 3E**. Of these, three genes were reported to be involved in social behaviors, learning, and memory (**Table 1**). Chronic administration of intranasal OXT has the potential to partly regulate the molecular pathways in the hippocampus involved in ASD-like behaviors induced by prenatal VPA exposure.

DISCUSSION

Valproic acid is an inhibitor of HDACs that epigenetically modulates gene expression (48). It has been suggested that VPA-induced teratogenicity results from HDAC inhibition. The effects of HDAC inhibitors on fetal teratogenicity are suppressed by their analogs with lower potency against HDACs (51, 52). Interestingly, it has been reported that the prenatal VPA exposure-induced social deficits were ameliorated by chronic administration of HDAC inhibitors at postnatal days (13), supporting the critical role of epigenetic modulation by HDAC inhibition for both onset and amelioration of ASD. In the present study, HDAC1 and specificity protein 1 were implicated as transcriptional regulators of DEGs, suggesting the relevance of the hippocampus in ASD-like molecular profiling and behaviors induced by prenatal VPA exposure.

The dorsal and ventral hippocampus primarily function in cognition and emotion, respectively; inhibition of protein synthesis in the dorsal hippocampus decreases fear memory consolidation (53). The dorsal hippocampus lesion also impairs spatial memory (54). Optogenetics experiments suggested that activity in the ventral hippocampus is required for social memory recall in mice (55). In terms of emotional behaviors, in contrast, the dorsal and ventral parts are reported to oppositely regulate anxiety in rodents; muscimol infusion into the dorsal hippocampus provokes anxiety, while into the ventral part has anxiolytic effects (56). In addition, dorsal-ventral neural circuits in the hippocampus contribute to social memory (57). These reports suggest that the dorsal hippocampus is also involved in emotion, although the degree of contribution is likely to be less than in the ventral part. It is noteworthy that hippocampal volume is altered in ASD patients compared to typically developed individuals, although there is a discrepancy whether the volume increases or decreases (58, 59). Functional magnetic resonance imaging showed that neural connectivity in the anterior hippocampus (ventral part in rodents) was reduced in ASD patients (60). However, there are few reports on the anatomical and functional findings of the dorsal hippocampus in ASD patients.

In this study, 174 genes were identified as DEGs in the hippocampus of prenatally VPA-exposed rats. Among these, only 32 genes were also identified as DEGs in the amygdala or prefrontal cortex of prenatally VPA-exposed models (12, 14, 15), suggesting a distinct pattern of molecular changes between the brain regions in models such as ASD patients (47). According to the gene-disease analysis, prenatal VPA exposure affects a subset of genes in the hippocampus involved in both ASD- and learning disability-associated diseases (**Figure 2A**). Using transcriptome profiling, our study thus demonstrated that the hippocampus is a key region in terms of its contribution to ASD phenotypes, including learning disabilities.

We identified some key molecular changes induced by prenatal VPA exposure and upregulated by OXT. Secreted frizzled-related protein 1 is an endogenous inhibitor of Wnt signaling (61), which gets activated in both the brains of ASD patients and the hippocampus of prenatally VPA-exposed rats (62, 63). Interactive networks also revealed a relatively higher connection between *Sfrp1* and *Rarb* (**Figure 3E**). Transcription of cluster of differentiation 38, which is critical for OXT release (64), is regulated by RARs (65). The severity of ASD has been reported to negatively correlate with serum levels of vitamin A (66). Notably, Lai et al. reported that maternal deficiency of vitamin A in rats induced ASD-like behaviors and decreased the expression levels of cluster of differentiation 38 and retinoic acid receptor β in the hypothalamus and OXT in the serum of the offspring, all of which were rescued by maternal supplementation of vitamin A (67). These reports suggest that the molecular pathways involved in Wnt signaling, including secreted frizzled-related protein and retinoic acid receptor β, play a critical role in ASD pathology and improve following chronic OXT treatment. Epidermal growth factor receptor and tropomyosin receptor kinase B were also predicted to interact with DEGs improved by OXT (**Figure 3E** and **Table 1**), and these genes are reported to be involved in social behaviors, learning, and hippocampal LTP (68–70). Oxytocinergic signaling activates epidermal growth factor receptor to promote LTP maintenance (71). Both *Ntrk* deletion, specifically in oxytocinergic neurons and *Bdnf* deletion, impair maternal behaviors against offspring in mice (72). These reports support that OXT is involved in the molecular pathways underlying not only social behaviors but also hippocampus-dependent learning and memory. To the best of our knowledge, this is the first report to evaluate effects of OXT on molecular alterations in the dorsal hippocampus of an animal model of

TABLE 1 | Relationship of the predicted genes to hippocampal function and ASD-like behaviors.

Genes	Function in the hippocampus and ASD phenotypes	Sources
Egfr	Enhancement of LTP through recruitment of NMDA receptor GluN2B subunit by EGFR activation	(68)
Rarb	Impairments of LTP, AMPA receptor-mediated synaptic transmission, spatial memory, and social recognition by reduction in RARβ levels	(74)
Ntrk2	Impairments of hippocampal LTP and learning in TrkB knockout mice; Reduction of TrkB levels and LTP in the hippocampus and impaired learning and sociability in an inbred ASD model	(69, 70)

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; EGFR, epidermal growth factor receptor; NMDA, N-methyl-D-aspartate; RARβ, retinoic acid receptor β; TrkB, tropomyosin receptor kinase B.

ASD. However, a limitation of this study is that it did not evaluate the effects of OXT on molecular alterations in the amygdala and prefrontal cortex, regions related to emotion and well-investigated in ASD. As mentioned above, it is likely that the pattern of molecular alterations is distinct between brain regions in ASD. In order to further confirm the efficacy of OXT for ASD, the effects on molecular alterations in these regions should be investigated in the future.

In this study, chronic administration of OXT was conducted at adolescence just after weaning in order to avoid the risk of parental abandonment. Interestingly, maternal administration of OXT is implicated to suppress postnatal pathogenesis in animal models of ASD through enhancing excitatory/inhibitory switching of γ-aminobutyric acid during development (73). This suggests the possibility of maternal administration of OXT may reduce the risk of postnatal development of ASD, even when epilepsy patients are medicated with anticonvulsants including VPA during pregnancy. Further study is needed in the future to investigate the possibility of maternal OXT medication for restoration of postnatal ASD development and molecular alterations.

In summary, the present study demonstrated that prenatal VPA exposure affects the molecular pathways involved in ASD-like phenotypes in the hippocampus. Chronic administration of intranasal OXT partly ameliorated these alterations, which would underlie the improvement of social and learning disabilities seen in the prenatally VPA-exposed ASD model.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE196500.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee on Animal Experiments at Tohoku University.

AUTHOR CONTRIBUTIONS

KM and YS performed the experiments. NA and YN verified the methodology and provided the materials and apparatus. KM and KF wrote the manuscript. KF designed the study. All authors contributed to the article and approved the submitted version.

FUNDING

This work was partly supported by grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (Kakenhi 19H03406 to KF), and grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (18J20651 to KM).

ACKNOWLEDGMENTS

This work was partly performed in the Cooperative Research Project Program of the Medical Institute of Bioregulation, Kyushu University. We thank E. Koba and M. Oda (Laboratory for Technical Supports Medical Institute of Bioregulation, Kyushu University) for performing the microarray analysis.

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REVIEW
published: 15 April 2022
doi: 10.3389/fpsyt.2022.842755

Neuronal Cell Adhesion Molecules May Mediate Neuroinflammation in Autism Spectrum Disorder

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Autism spectrum disorder (ASD) is a complex neurodevelopmental condition characterized by restrictive and repetitive behaviors, alongside deficits in social interaction and communication. The etiology of ASD is largely unknown but is strongly linked to genetic variants in neuronal cell adhesion molecules (CAMs), cell-surface proteins that have important roles in neurodevelopment. A combination of environmental and genetic factors are believed to contribute to ASD pathogenesis. Inflammation in ASD has been identified as one of these factors, demonstrated through the presence of proinflammatory cytokines, maternal immune activation, and activation of glial cells in ASD brains. Glial cells are the main source of cytokines within the brain and, therefore, their activity is vital in mediating inflammation in the central nervous system. However, it is unclear whether the aforementioned neuronal CAMs are involved in modulating neuroimmune signaling or glial behavior. This review aims to address the largely unexplored role that neuronal CAMs may play in mediating inflammatory cascades that underpin neuroinflammation in ASD, primarily focusing on the Notch, nuclear factor-κB (NF-κB), and mitogen-activated protein kinase (MAPK) cascades. We will also evaluate the available evidence on how neuronal CAMs may influence glial activity associated with inflammation. This is important when considering the impact of environmental factors and inflammatory responses on ASD development. In particular, neural CAM1 (NCAM1) can regulate NF-κB transcription in neurons, directly altering proinflammatory signaling. Additionally, NCAM1 and contactin-1 appear to mediate astrocyte and oligodendrocyte precursor proliferation which can alter the neuroimmune response. Importantly, although this review highlights the limited information available, there is evidence of a neuronal CAM regulatory role in inflammatory signaling. This warrants further investigation into the role other neuronal CAM family members may have in mediating inflammatory cascades and would advance our understanding of how neuroinflammation can contribute to ASD pathology.

Keywords: autism spectrum disorder, cell adhesion molecules, neuroinflammation, inflammatory cascade, neuroinflammatory signaling, glial cells

OPEN ACCESS

Edited by:
Hideo Matsuzaki,
University of Fukui, Japan

Reviewed by:
Katsuhiko Tabuchi,
Shinshu University, Japan
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Specialty section:
This article was submitted to Autism, a section of the journal Frontiers in Psychiatry

Received: *24 December 2021*
Accepted: *15 February 2022*
Published: *15 April 2022*

Citation:
Eve M, Gandawijaya J, Yang L and Oguro-Ando A (2022) Neuronal Cell Adhesion Molecules May Mediate Neuroinflammation in Autism Spectrum Disorder. Front. Psychiatry 13:842755. doi: 10.3389/fpsyt.2022.842755

INTRODUCTION

Autism spectrum disorder (ASD) is a complex neurodevelopmental condition characterized by restrictive and repetitive behaviors, combined with deficits in social interaction and communication (1). ASD is estimated to currently affect between 0.6 and 2% of the global population, with data suggesting prevalence is increasing over time (2–5). The etiology of ASD remains largely unknown, although it is hypothesized that a combination of environmental and genetic factors contributes to its pathogenesis (6). Monozygotic twin studies show ASD has high heritability, with estimates as great as 83%, but these figures also demonstrate the incomplete genetic concordance (7). ASD affects predominantly males compared to females for reasons yet unidentified (8).

Extensive, current research collated by the Simons Foundation Autism Research Initiative (SFARI) highlights numerous candidate genes linked to ASD development, with many of these encoding for neuronal cell adhesion molecules (CAMs) (9). Neuronal CAMs are together a diverse collection of cell-surface proteins that play roles in neurite formation, neuronal outgrowth, and axon guidance within the nervous system (10, 11). These molecules are classified into families founded on their structure, including the neurexin (NRXN) family, neuroligin (NLGN) family, and immunoglobulin superfamily CAMs (IgCAMs) (12). Amongst them, the IgCAMs represent the largest family of neuronal CAMs, encompassing neurofascins (NFASCs), neural CAMs (NCAMs), and the six-member subfamily, contactins (CNTNs) (12). Of note, variation in *CNTN* gene expression is linked to ASD, and various studies have demonstrated their importance in nervous system development (13). CNTNs have been reported to modulate neuronal communication and synaptic transmission, essential in the assembly of neural circuits and the formation of behavioral pathways (10, 12–15).

One key neuronal CAM, scored by the SFARI Gene database as a strong, syndromic ASD candidate gene is *CNTN-associated protein-2* (*CNTNAP2)* (16). CNTNAP2 is localized to myelinated axons within the juxtaparanode of the nodes of Ranvier. Here, it interacts with CNTN2 and organizes axonal voltage-gated K^+ channels (17). Consistent evidence reports rare variants within *CNTNAP2* as a susceptibility factor for ASD and suggests that CNTNAP2 has a function in the early stages of neurodevelopment and later language development (18–21). Animal models deficient for Cntnap2 show symptoms synonymous with ASD in humans, including epileptic seizures, behavioral abnormalities, and cognitive dysfunction (22). Functional magnetic resonance imaging has illustrated atypical frontal cortex circuitry in children with ASD carrying common *CNTNAP2* variants, compared to neurotypical controls (21). A case report presented by Al-Murrani and colleagues depicted a 3-year-old boy with a deletion in *CNTNAP2* who displayed language delay, communication difficulties, and consequently, behavioral problems (23). However, it is important to note that two of his family members also carried the same deletion and yet exhibited no ASD phenotype. This demonstrates the presence of substantial phenotypic heterogeneity that makes it difficult to identify a solely genetic origin for many cases of ASD and, therefore, suggests an additional environmental trigger contributes to ASD symptom presentation.

Environmental factors can be categorized as prenatal, natal, and postnatal. Common prenatal risk factors include older paternal age, maternal mental and physical health, as well as familial socioeconomic status (24). A particularly promising avenue of research into the natal and postnatal environmental triggers for ASD is inflammation. The relationship between inflammatory disease, proinflammatory cytokines, and impaired immunity has been well-documented in those with ASD. Specifically, immune disorders such as asthma, gastrointestinal (GI) disorders, allergy, and maternal immune activation (MIA) have been regularly identified in studies investigating ASD comorbidities (25–30). MIA occurs when the pregnant mother acquires an infection or is exposed to immunogenic materials and has been identified to increase susceptibility to a child developing ASD (31). Consequences on the developing fetal brain can include modified expression of neuronal migration genes, increased number of microglia, aberrant dendritic morphology within the prefrontal cortex, and excessive neurogenesis (32–35). It is thought that the mother's immune response poses a risk to fetal neurodevelopment, rather than the specific pathogen or immunogenic material (35). In murine models, ASD behaviors are observed in offspring when, throughout pregnancy, dams are exposed to immunostimulants such as lipopolysaccharide (LPS), interleukin-17 (IL-17), and polyinosinic-polycytidylic acid [poly (I:C)] (29, 36, 37). In one study, blocking IL-17A in LPS-immune activated dams resulted in the reversal of ASD behaviors in their offspring (36). These studies demonstrate that altered neuroimmune signaling adversely affects fetal neurodevelopment. Notably, MIA has also been shown to facilitate the transfer of maternal antibodies targeting fetal neural proteins, which could further impair neurodevelopment (38).

Although inflammation has been identified as an environmental risk factor for ASD, it is not entirely clear whether the neuronal CAMs discussed above may play roles in modulating neuroimmune signaling. Microglia are the main source of cytokines within the brain and, therefore, are vital in mediating inflammation in the central nervous system (CNS) (39). There is suggestion of neuronal CAMs influencing signaling cascades that then cause a proinflammatory phenotype in microglia (40).

Inflammatory processes from subsequent microglial activation indicate that neuronal CAMs may play a role in inflammatory cascades, a concept relatively unexplored in current literature. Studies of several CAMs, including CNTNAP2, have gradually revealed their functional involvement in inflammatory signaling. However, there is limited information that discusses the relation between CAMs and inflammatory systems that are risk factors in ASD. This review aims to address the association between neuronal CAMs and inflammatory systems within the scope of ASD (**Figure 1**). This association has been largely unexplored but is important when considering the impact of environmental factors and the inflammatory response on ASD. The following sections will cover recent research reports exploring the role of neuronal CAMs in inflammatory signaling cascades and proinflammatory cytokine production

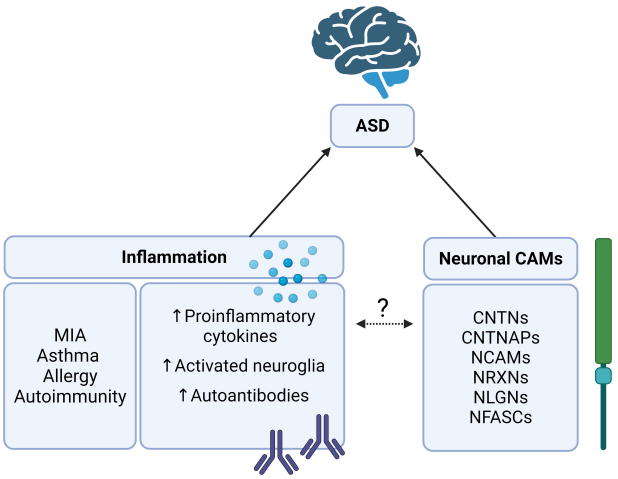

FIGURE 1 | The relationship between autism spectrum disorder (ASD), inflammation, and cell adhesion molecules (CAMs). Neuronal CAMs such as the CNTN, CNTNAP, NCAM, NRXN, NLGN, and NFASC families of proteins are consistently implicated as risk genes for ASD (9, 16). Likewise, inflammation, particularly in MIA, has been identified as an environmental risk factor for ASD (24, 31). What is yet unclear is whether these neuronal CAMs may be involved in regulating inflammatory responses or cascades in ASD. Contactin (CNTN), CNTN-associated protein (CNTNAP), neural cell adhesion molecule (NCAM), neurexin (NRXN), neuroligin (NLGN), neurofascin (NFASC), and maternal immune activation (MIA). Created with BioRender.com.

associated with neuroinflammation found in ASD. Additionally, we will focus on the dysfunction of inflammatory cascades underpinning inflammatory diseases that may link to ASD pathology. Finally, we will assess how neuronal CAMs are involved in the activation, differentiation, and proliferation of glial cells, leading to an inflammatory response and how this may impact neurodevelopmental pathways implicated in ASD.

AUTISM SPECTRUM DISORDER AND INFLAMMATION

Signaling Pathways Involved in Neuroinflammation

Several signaling pathways are involved in the neuronal inflammatory response. This review will primarily focus on three of these; Notch, nuclear factor-κB (NF-κB), and mitogen-activated protein kinase (MAPK) signaling cascades, which all play key roles in inflammatory responses. Notch is a transmembrane protein that, once activated, transduces signals *via* direct cell-cell communication (41). There are four Notch receptors in mammals that, upon binding with a corresponding ligand, causes the intracellular domain of the Notch receptor to be cleaved and translocated to the nucleus (42). Importantly, neuronal CAMs, such as CNTN1 and CNTN6, act as ligands for Notch1 in the CNS (43, 44). In the immune system, Notch1 signaling is important for the differentiation of T cells into T-helper (Th) and regulatory T cells, whilst inhibiting the differentiation of other lymphoid lineages (45–47). Chronic inflammatory diseases associated with ASD, such as inflammatory bowel disease (IBD) and asthma, may be influenced by Notch signaling (41). Experimental alterations of the Notch signaling pathways, such as the removal of constituents responsible for Notch signal transduction, have exposed a potential role of Notch in the proinflammatory response (48, 49). Moreover, proinflammatory cytokine IL-6 is secreted upon activation of Notch2 by tumor necrosis factor-α (TNF-α) in rheumatoid arthritis models (50). Hence, inhibition of Notch signaling may be beneficial in the treatment of inflammatory diseases. However, whether neuronal CAMs acting as Notch ligands modulate the secretion of proinflammatory cytokines is unclear.

NF-κB is another signaling pathway involved in inflammation and is strongly implicated in inflammatory disease. Present in all cell types, NF-κB regulates the expression of genes encoding for proteins vital in the immune response (51). Activation of NF-κB is inducible by inflammatory cytokines, markers of infection, or stress-activated protein kinases (52). Under normal cell conditions, NF-κB is inhibited from nuclear translocation by IκB (inhibitor of κB) until activated (53). NF-κB signaling influences differentiation states of cells and regulates the production of anti-apoptotic factors (54). However, the primary role of NF-κB is to increase the production of cytokines, chemokines, and adhesion molecules involved in the immune response (55). Proinflammatory cytokines such as IL-6, IL-12, and TNF-α form common target genes for activated NF-κB (56). Although intentional activation of NF-κB is protective against pathogens and cancer, uncontrolled or dysfunctional NF-κB signaling can be causative of acute or chronic inflammatory disease (52). Within the CNS, inappropriate activation of the NF-κB signaling cascade leads to aberrant expression of proinflammatory cytokines, which can be particularly damaging. NF-κB activation in microglia, astrocytes, and oligodendrocytes has been implicated in neurodegenerative disease (57). Of note, NF-κB signaling can also be initiated downstream of the MAPK cascade (58).

The MAPK signaling cascade is activated by cytokines, mediators of stress, and inflammatory markers, such as ligands of Toll-like receptors (59). Through a series of phosphorylation cascades, MAPK proteins transduce, and propagate extracellular signals within the cell (60). During inflammation, MAPK signaling is mainly induced by Toll-like receptor activation, resulting in the phosphorylation of transcription factors (61). Once phosphorylated, these transcription factors translocate to the nucleus and transcribe genes encoding proinflammatory cytokines, such as IL-6 and TNF-α (62). Within the CNS, IL-6 signaling forms a positive feedback loop through activation of MAPK signaling, which is reported to promote neurogenesis (63). Microglial activation can also induce the MAPK signaling cascade, promoting a neuroinflammatory environment that contributes to neurodegeneration (64). Although it is uncertain, neuronal CAMs may also play roles in MAPK signaling. For example, NCAM1 was shown to activate MAPK signaling in mesenchymal stromal cells, regulating their migration to the site of inflammation where they contribute to tissue repair (65).

During an inflammatory response, Notch, NF-κB, and MAPK signaling pathways can all be activated by proinflammatory cytokines (52, 59, 66).

Cytokine and Chemokine Profiles in Autism Spectrum Disorder

In the context of the immune system, cytokines are proteins secreted by cells to coordinate, signal and recruit, normally in response to an immune insult. Generally, these signaling proteins are proinflammatory in nature and are important in orchestrating the immune response (67). Most cell types can produce cytokines, but the majority of proinflammatory cytokines are released by macrophages and lymphocytes (68). Chemokines, for example, IL-8, manage leukocyte adhesion and chemotaxis (69). Interleukins play a role in directing cell differentiation, growth, and activation (68, 70). IL-1 and IL-6 are, for the most part, produced by macrophages to stimulate the generation of proinflammatory cytokines and evoke elevation of body temperature (70). The growth and proliferation of eosinophils, T cells, natural killer cells, and B cells are mediated by a vast range of interleukins (70). Cytokines can also be anti-inflammatory and regulate or suppress an escalating immune response. For instance, IL-10 suppresses proinflammatory cytokines and modulates macrophages to dampen the immune response (71, 72).

Irregular cytokine and chemokine profiles have been recorded in the cerebrospinal fluid (CSF), blood, and brain of those with ASD (73–76). Plasma samples from male ASD subjects revealed elevated levels of IL-1β, IL-5, IL-8, IL-12, IL-13, and IL-17 compared to controls (76). In another multiplex cytokine screen, similar differences were found in IL-1β, IL-12, and IL-17, but also IL-6, in individuals with ASD compared with age-matched typically developing children (77). A recent meta-analysis conducted with 1,393 patients with ASD found greater concentrations of IL-6 in the blood, as well as increased concentrations of proinflammatory cytokines IL-1β, TNF-α, and interferon-γ (IFN-γ) within the periphery (78). Within the CNS, localized cytokine and chemokine variances have been reported. Higher levels of IL-6, IL-8, TNF-α, and IFN-γ are observed in the frontal cerebral cortex of ASD patients compared to age-matched control cortices (79). Moreover, evidence of elevated TNF receptor I levels in the CSF of ASD children further indicates inflammation not just in the periphery, but also within the CNS (80). Although inflammatory markers may differ depending on genetic predisposition, when screening panel results are normalized to parental cytokine expression, IL-8 expression was still seen to be elevated in children with ASD (81). These cytokine profiles have been found to differ in males and females, indicating that testosterone may impinge on the inflammatory processes underlying ASD (82). Cytokine profiles may also be useful as biomarkers to predict comorbidities. Children with ASD that also suffered from epilepsy had lower peripheral IL-6 levels compared to ASD children without epilepsy (77). Distinct cytokine profiles have also been shown in ASD children who have attention deficit hyperactivity disorder, with differences in blood IL-8 levels compared to children with only ASD (74). Specific cytokine profiles have the potential to act as an ASD biomarker and aid the diagnosis of comorbidities.

For a more detailed overview of which cytokines and chemokines are most frequently found to vary in concentrations within individuals with ASD compared to typically developing controls, see **Table 1**. In summary, IL-1β, IL-6, IL-8, and IL-17 are the most consistently observed cytokines to be upregulated in cases of ASD, which indicates a proinflammatory response underlying neuroinflammation in ASD.

Interleukin-1β

Interleukin-1β is a proinflammatory cytokine found upregulated in autoinflammatory disease, chronic inflammation, and acute inflammation (83). Expression of IL-1β by monocytes and macrophages, stimulated by pathogen-associated molecular patterns and cytokines, triggers phagocytic cell activation (84). IL-1β has a role in neuropathogenesis but also neuroprotection within the CNS (85). During the development of the nervous system, IL-1β expression regulates the proliferation of neural progenitor cells (86). Hence, abnormal IL-1β levels may contribute to neurological deficits observed in ASD brains. Increased concentrations of IL-1β were shown in several studies, including a meta-analysis, in individuals with ASD compared to healthy controls (76, 78, 87, 88). Microglia can generate vast quantities of cytokines, particularly IL-1β, within the CNS (89). Extracellular vesicles isolated from the serum of children with ASD were shown, *in vitro,* to activate microglia to produce increased levels of IL-1β compared to neurotypical controls (40). Interestingly, IL-1β is found to be elevated in the serum and blood of both males and females with ASD, although mainly males have been included in ASD cytokine

TABLE 1 | Summary of cytokines and chemokines reported to have altered expression in autism spectrum disorder.

Cytokine or chemokine associated with ASD	References
IL-1	(76, 78, 88, 90, 184)
IL-2	(88)
IL-4	(88, 109)
IL-5	(76)
IL-6	(73, 75, 78, 79, 88, 90, 100, 116, 118, 184, 256)
IL-7	(81, 88)
IL-8	(76, 79, 81, 88, 90)
IL-10	(114)
IL-12	(76, 88, 90)
IL-13	(76)
IL-17	(26, 29, 73, 75, 76, 88, 114, 116, 117, 184)
IL-23	(257)
TNF-α	(73, 78, 79, 88, 184)
IFN-α	(81)
IFN-γ	(78, 79, 81, 109)

ASD, autism spectrum disorder; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon.

profile studies (76, 82). This increase is not as high of an increase as would be expected to be found in a person with autoimmune or inflammatory disease, suggesting that the effect of ASD on the inflammatory system is unique (76). One study showed that increased IL-1β was predominantly found in children with regressive ASD, supporting that IL-1β is influential during post-natal neurodevelopment (90). The same study also found a correlation between IL-1β concentration and aberrant behaviors (90).

Interleukin-8

Interleukin-8 production in neutrophils and mast cells is stimulated by IL-1β (91). IL-8, also known as CXCL8, is a potent chemoattractant produced by T cells and macrophages in order to recruit neutrophils and other leukocytes to a site of inflammation (92). Neutrophils can produce IL-8 to self-recruit, stimulated by IL-1 and TNF-α (92). Besides neutrophil chemotaxis, IL-8 has a role in neutrophil morphology, upregulation of adhesion molecules, migration, and exocytosis of proteolytic enzymes (92, 93). Although crucial in peripheral immunity, IL-8 has also been found to be increased within the frontal cerebral cortex of ASD brains (79). The frontal cortex is essential for cognition, emotion, and social behavior, therefore, is a brain region associated with the pathology of ASD (94). Localized inflammation due to IL-8 could affect frontal cortex processing in those with ASD. IL-8 is produced by macrophages to recruit neutrophils, eosinophils, and leukocytes (95). Increased peripheral levels of IL-8 have been found in ASD subjects compared to matched controls (76, 87, 88, 96). With IL-8 having such a key role in innate immunity, it suggests that immune dysfunction in ASD is linked to the innate immune system. Activated microglia may be able to recruit cells of the innate immune system *via* IL-8 secretion to exacerbate the neuroinflammatory response. One study showed that not only was serum IL-8 concentration increased in ASD children compared to healthy controls, but IL-8 was yet higher in concentration in patients with childhood ASD compared to those with Asperger syndrome (96). This data may allude to different pathophysiological mechanisms for different levels on the ASD spectrum. Once more, an increase in severity of ASD phenotype is correlated with proinflammatory cytokine concentration (90). IL-6 plasma concentrations are also demonstrated to be positively correlated with the severity of ASD traits (90).

Interleukin-6

Interleukin-6 is a pleiotropic cytokine that has both inflammatory and anti-inflammatory purposes throughout the human body (97). Functions of IL-6 during inflammation include B-cell differentiation, induction of acute-phase protein release, inhibition of regulatory T-cell differentiation, and maintenance of Th17 cell differentiation (70, 97). IL-6 activity is not limited to the immune system. It is well-documented to play roles in neurodevelopment, such as promoting neurite outgrowth, neurogenesis, and gliogenesis pathways that are implicated in ASD pathology (6, 94, 98–100). Many studies have shown increased plasma and blood IL-6 levels in individuals with ASD (73, 75, 76, 78, 88). Elevated IL-6 levels within the cerebellum are associated with impairment of neuronal cell adhesion and migration, as well as influencing synapse formation (101). CAMs are essential for the formation of behavioral pathways, synapse development, and neuronal plasticity, therefore, CAM dysfunction due to IL-6 may contribute to some ASD pathophysiologies (12, 13, 101). What is more, this data suggests IL-6 may regulate the function of neuronal CAMs (101).

There are distinguished neurological changes in the brains of those with ASD such as neuronal overgrowth in the frontal cortex and microglial activation (94, 102). In the GFAP-IL-6 mouse model of chronic neuroinflammation, where IL-6 is overexpressed in astrocytes, neurological variations like astrocytic gliosis and neurodegeneration were observed (103, 104). Increases in IL-6 serum, blood, and CSF levels have also been observed in adults with ASD, indicating IL-6 can be upregulated over a prolonged period of time, potentially contributing to neurological changes in ASD brains, similar to those observed in chronic neuroinflammation models (78, 102).

An increase of IL-6 was found in the anterior cingulate gyrus of ASD patients compared to controls (102). The anterior cingulate gyrus is responsible for emotional expression, attention allocation, and mood, the dysregulation of which are all core ASD deficits (105). Localized inflammation within this brain region *via* IL-6 could contribute to deficits in communication observed in those with ASD.

IL-6 appears to have a key role in MIA, one of the strongest examples of inflammation associated with ASD development. The immune response of the mother, directed against a pathogen or immunogenic molecule, adversely affects fetal neurodevelopment (31). In animal models, MIA is commonly induced in pregnant mice *via* administration of LPS or poly(I:C), which mimics bacterial or viral infection, respectively (106). Behavioral changes in the offspring of poly(I:C)- and LPS-induced MIA mice were observed in multiple studies (29, 36, 37, 107–109). These behaviors included enhanced marble-burying or self-grooming (represents repetitive and restricted behavior), sociability impairments, and reduced ultrasonic vocalization (which may suggest a change in social communication) (36, 108). Administration of IL-6 to pregnant dams gave rise to ASD-like behavioral traits in their offspring and further experiments utilizing poly(I:C) MIA models found offspring ASD behavior was prevented by co-administering anti-IL-6 antibodies with poly(I:C) (110). In this same study, MIA IL-6 knockout mice sired offspring without any of the behavioral deficits that MIA wild-type offspring possessed (110). Together with the upregulated IL-6 levels observed in human studies, there is substantial evidence implicating altered IL-6 signaling in the neuroimmune dysfunction underlying ASD.

Interleukin-17

Interleukin-17 is an important cytokine in the protection and clearance of bacterial and fungal infections (111). There are six members of the IL-17 family, the most studied of which is IL-17A (112). Able to act on myeloid and mesenchymal cells, IL-17 upregulates proinflammatory genes through NF-κB and MAPK signaling (113).

Th17 cells are characterized by their production of IL-17 and it has been suggested that Th17 cells are implicated in the

development of inappropriate inflammatory responses in ASD (114, 115). A study conducted by Moaaz *et al.* found children with ASD had significantly increased Th17 cell production, alongside fewer regulatory T cells and decreased concentrations of both IL-10 and transforming growth factor-β (TGF-β), which dampen the inflammatory responses (114). This could indicate that regulation of the immune system may be malfunctioning in some cases of ASD.

Increased plasma levels of IL-17 in those with ASD have been outlined in numerous studies (73, 77, 114, 116). Additionally, increased expression of the receptor for IL-17A was found in phagocytes isolated from individuals with ASD, and IL-17 messenger RNA expression was also nearly four times higher in ASD children compared to typically developing children (114, 117, 118). One Turkish study detected reduced IL-17 expression in ASD individuals' peripheral blood mononuclear cells (PBMC), though this could be due to demographic differences within the sample (75). The proinflammatory IL-17 signaling pathway is associated with chronic inflammatory neurological diseases (119). Activation of IL-17 receptors (IL-17R) *in vitro* in monocytes and neutrophils isolated from individuals with ASD led to upregulated NF-κB expression, resulting in increased expression of proinflammatory genes (117, 118). Application of anti-IL-17R antibody to ASD monocytes reversed this enhanced NF-κB expression and could be viewed as a beneficial treatment for managing the inflammation found in some cases of ASD (117).

The above data suggests that upregulation of IL-17 is associated with ASD, but the origin of IL-17 dysregulation may be maternal. In pregnant mothers, IL-17 is able to transfer from the placenta to the fetus, increasing IL-17R levels within the fetal brain (120). Additional IL-17R further increases IL-17 signaling within the fetal brain, likely initiating a neuroinflammatory response (121). This may cause a predisposed sensitivity to IL-17 and explain increased IL-17 concentrations in individuals with ASD. Abnormal IL-17 levels have featured in MIA models. IL-6 and TGF-β together facilitate Th17 cell differentiation from their $CD4^+$ progenitors (115). Th17 cells are characterized by their production of IL-17. A rise in Th17 cell numbers is associated with autoimmune disorders and chronic inflammatory disease (122). It has been found that an increase in serum IL-6, ensuing from an immune assault, yields an elevated production of Th17 cells, increasing IL-17 levels (115). Interestingly, in two studies, the enhanced marble-burying behavior was ameliorated by IL-17A blocking, indicating that IL-17A has a role in repetitive ASD behavioral traits (36, 107). Blocking IL-17A in LPS-administered MIA dams also reversed the reduction in ultrasonic vocalization and social interaction deficits in their offspring (36). Increased levels of maternal IL-17A have been strongly associated with ASD-like behavior in rodent MIA offspring. This amplification of IL-17 has additionally been noticed in both murine MIA offspring and human ASD individuals.

In summary, specific cytokine profiles seem to highlight the presence of a proinflammatory response in individuals with ASD during early neurodevelopment, up to adulthood. Attention is drawn to abnormally elevated IL-1β, IL-6, IL-8, and IL-17 levels in children and adults with ASD (73, 74, 96). Increased IL-6 and IL-17 levels in MIA models suggest that dysregulation of inflammatory cascades begin during prenatal neurodevelopment (100). There is also some evidence that the degree of severity in ASD phenotype may be positively correlated with the concentrations of proinflammatory cytokines (90). Activation of inflammatory signaling in glial and neuronal cells by proinflammatory cytokines may alter neuronal cellular function. Significantly, IL-6 overexpression can promote astrocytic gliosis, causing neuroinflammation similar to that observed in ASD brains (104). In turn, the neuroinflammatory environment activates MAPK and NFκB signaling to enhance the neuroimmune response (52, 59). These inflammatory cascades can be regulated by NCAM1, implicating neuronal CAMs in ASD-related inflammation (65, 123). Additionally, extracellular vesicles isolated from ASD serum may activate microglia to produce increased IL-1β, further suggesting dysfunction of the neuroinflammatory response (40). IL-1β regulates the proliferation of neural progenitor cells, therefore, an increase in IL-1β in the brains of those with ASD may alter neuronal development (86). This may have implications in ASD behavioral pathways that contribute to the core deficits seen in ASD.

INVOLVEMENT OF NEURONAL CELL ADHESION MOLECULES IN INFLAMMATORY AND IMMUNE DISEASE ASSOCIATED WITH AUTISM SPECTRUM DISORDER

Inflammatory and Immune Disease Associated With Autism Spectrum Disorder

Dysregulation of inflammatory signaling cascades and abnormal proinflammatory cytokine signaling is often present in those with ASD (77). Chronic inflammatory diseases such as asthma, IBD, and persistent neuroinflammation have frequently been reported as comorbidities to ASD, alongside immune-mediated disease (124).

Asthma is a chronic inflammatory disease of the respiratory system (125). Current literature is divided over support of a correlation between asthma and ASD. A meta-analysis of 175,406 participants found no proof of an association between asthma and ASD (126). Another study also showed no association between ASD and asthma or allergy, although allergy was linked with increased repetitive behavior (127). The same study did, however, highlight that food allergies and sensitivities were associated with ASD. Other studies report that asthma is 35% more frequently diagnosed and is more prevalent in children with ASD than in typically developing controls (26, 128). Interestingly, PBMCs isolated from children with both ASD and asthma are reported to produce higher levels of IL-17 following stimulation compared to PBMCs from children with ASD but without asthma (26). It is worth noting that upregulated IL-17, as previously

discussed (previous Interleukin-17 section), can contribute to more severe ASD phenotypes.

Gastrointestinal sensitivities and general GI issues are further comorbidities to ASD (124). Children with ASD are frequently reported to have intolerances to food, abdominal pain, bloating, diarrhea, constipation, ulcerative colitis, and Crohn's disease (129). There has been a lot of interest in the "gut-brain axis," where gut microbiota and immune responses have a bidirectional relationship with the CNS (130). Children aged 2–18 with ASD had 67% higher odds of having Crohn's disease and ulcerative colitis compared to a typically developing control group (131). Across four different study populations, the rates of IBD among individuals with ASD were higher than their age-matched controls (132). Impaired gut barriers from localized inflammatory cytokine production leads to increased gut permeability, allowing cytokines to access the CNS (133). These cytokines, originating from the gut, could instigate inflammatory responses within the brain and, therefore, impact cognitive function (130). Reciprocally, inflammation originating from the CNS or plasma could cross the intestinal mucosal barrier and trigger inflammatory signaling in gut-associated lymphoid tissue (133, 134).

The gut-brain axis may indirectly play a role in the neuroimmune system. Increased gut permeability from GI inflammation allows molecules that would otherwise be restricted from entering the bloodstream (30). Microglia are primarily responsible for neuroinflammation within the CNS and, once activated, produce vast quantities of proinflammatory cytokines (89). Activation of microglia and astrocytes were shown in brain tissue of patients with ASD (102). The vagus nerve interacts with the peripheral immune system, constantly surveying gut health (135). GI inflammatory markers can be sensed by the vagus nerve which transmits this information to the CNS, affecting microglial activation (136). Therefore, inflammation from the gut can trigger proinflammatory cytokine secretion *via* microglia activation, causing neuroinflammation (135).

Similar to ASD, autoimmune disease is thought to develop through a genetic predisposition with an environmental trigger that activates the immune system (137). The connection between autoinflammatory disease and ASD is not well-defined amongst current literature. However, several previous studies have revealed an interesting relationship between ASD and autoimmune disease. Zerbo *et al.* discovered autoimmune disease and psoriasis were diagnosed more frequently in males and children over the age of 12 with ASD compared to controls (138). It has also been established that a familial history of autoimmune disease may increase the chance of offspring having ASD, especially when the disease is targeting the CNS, and skin or mucosal membranes (139). These results suggest that there are overlaps in the genetic predisposition to ASD and autoimmune disorders (particularly those affecting the CNS). Interestingly, an autoimmune disease in the pregnant mother of ASD children may predispose the child to an IL-17 sensitivity *via* MIA and as described previously, an increase in IL-17 and the Th17 cells that produce it, is linked to the development of autoimmune disorders (119, 120).

Autoantibodies against proteins found within the CNS of subjects with ASD, including myelin basic protein, have been reported in several studies (140–142). Myelin is important in nerve function and protects the axon from damage. Without myelin, nerves cannot effectively conduct electrical signals in the CNS, resulting in neuronal network dysfunction (143). Demyelination, resulting from inflammation and cytokine infiltration, is a leading cause of neurological disease, impacting sensory, motor, and cognitive function (144, 145). White matter denotes brain regions consisting mainly of myelinated axons (145). Alterations in white matter volume of ASD brains, detected through magnetic resonance imaging, suggests dysregulation of myelination in those with ASD (146). It must be acknowledged that although demyelination is not commonly reported in those with ASD, the presence of autoantibodies against myelin basic protein in those with ASD suggests the potential for demyelinating disease to affect ASD brains (140). Additionally, one case study did present a 6-year-old with ASD and demyelinating neuropathy (147).

Evidence reports that neurodegenerative disease is more prevalent in adults with ASD (148). Alzheimer's disease (AD) is a neurodegenerative disease that has similar mechanisms supporting the pathogenesis of ASD (149). A key protein implicated in the pathology of AD is amyloid precursor protein (APP). Aberrant cleavage of APP into toxic amyloid beta (Aβ) plaques are one of the hallmarks of AD, although several studies reveal similar alterations of APP processing in those with ASD (150). A significant increase in secreted β-amyloid, a product of the pathogenic APP processing pathway, have been found in the plasma of children with severe ASD (151). Additionally, a greater intraneuronal Aβ load and increased Aβ accumulation were observed in astrocytes and some microglia in subjects with ASD and 15q11.2-13q duplication syndrome (152). Activated microglia and astrocytes clear Aβ plaques, reducing their accumulation at the synapse (153, 154). In AD models, microglia respond more readily to Aβ, producing increased proinflammatory cytokines that degrade neuronal synapses, leading to cognitive decline (155). Moreover, transgenic AD mice models showed that increased expression of *APP* led to elevated levels of proinflammatory cytokines, such as IL-1β and IFN-γ, in the brain (154). An increased Aβ load, as demonstrated in those with ASD, would suggest enhanced astrocyte and microglial activation, similarly, increasing proinflammatory cytokine production (152). Furthermore, in the ASD brain, astrocytes and microglia may respond more readily to Aβ, damaging synapses that could contribute to altered cognition (155).

To review, there is conflicting data for the association of asthma with ASD, but there is a strong correlation between allergy and ASD (126). The GI system is particularly susceptible to environmental triggers through ingested materials. Individuals with ASD may have a predisposition to GI disorders that cause an inflammatory immune response once activated by environmental factors. Inflammation originating from the gut may be influencing inflammatory signaling in the CNS attributable to the gut-brain axis (135). Alternatively, GI inflammation may be instigated by inflammatory signaling deriving from the CNS.

There is some evidence to link autoimmunity and dysregulation of immune function to ASD, although there is a lack of support for the presence of autoantibodies in those with ASD (140). More so, there is a suggestion that myelin dysregulation may render axons vulnerable to proinflammatory cytokines that cause demyelination and cognitive impairment (144). Finally, ASD has a complex pathogenesis involving a neuroinflammatory environment comparable to some aspects found in AD.

Contactins and Contactin-Associated Proteins in Inflammatory and Immune Disease

Alongside a documented link between ASD and inflammatory disease, current literature (**Table 2**) also supports an association between CNTNs and CNTNAPs with inflammatory and immune-mediated disease.

Recent exosome research has identified a link between CNTN1 and asthma. CNTN1 was found to induce Notch2 signaling in asthma to activate Th17 and Th2 cells (156). CNTN1 is present on the surface of exosomes containing allergens and acts as a Notch2 ligand for monocyte-derived dendritic cells (156). These dendritic cells secrete IL-4, IL-5, IL-6, IL-13, and IL-17A to drive an enhanced inflammatory response in the airways, suggesting that CNTN1 may act as an inflammatory mediator in the pathology of asthma (156).

Likewise, chronic inflammatory demyelinating polyneuropathy (CIDP) has been strongly correlated with CNTNs alongside additional associations to CNTNAPs. CIDP is an immune-mediated neuropathy, caused by damage to the myelin sheath, with large heterogeneity (157). Antibodies against neuronal CAMs such as CNTN1, CNTNAP1, CNTNAP2, and NFASC have been found in the periphery of patients with CIDP (158–175). All these CAMs are localized to the nodes and paranodes of myelinated axons within the CNS (159). The most common autoantibody shown in seropositive CIDP patients were anti-CNTN1 IgG4 subclass antibodies (169, 176). Cytotoxic effects on cerebellar neurons were identified with chronic administration of IgG4 anti-CNTN1 serum of a patient with CIDP (177). Proteins in the CSF of seropositive CIDP patients show probable blood-brain barrier breakdown, which could increase the likelihood of anti-CNTN1 antibodies entering the brain parenchyma (163, 178). It was demonstrated that CNTN1 expression was reduced in dorsal root ganglion neurons and cerebellar granule neurons after long-term exposure to anti-CNTN1 autoantibodies (177). Notably, Vargas *et al.*, showed that microglia and astrocytes within the cerebellum of ASD patients were activated upon the degeneration of granule cells within their vicinity (102). This raises the possibility that anti-CNTN1 antibodies targeting the cerebellar granule cells cause degeneration resulting in the activation of microglia and astrocytes. These then go on to produce proinflammatory cytokines. Like ASD, within the CSF of CIDP patients, high concentrations of proinflammatory cytokines have been observed (179). Interestingly, anti-CNTNAP2 IgG4 and anti-CNTN1 IgG1 antibodies were unable to cross the paranodal barrier, indicating these autoantibody subtypes may be less pathogenic than anti-CNTN1 IgG4 (169). However, once anti-CNTN1 IgG4 antibodies progressively deteriorate the paranode, other CAM autoantibodies may then be able to pass the paranodal barrier and accelerate demyelination.

Similar antibodies against CNTNs have been found in some cases of multiple sclerosis (MS) (180). Anti-CNTN2 antibodies were reported in a patient with MS, alongside CNTN2-specific T cells. These CNTN2-specific T cells were able to cause permeations in the blood-brain barrier and were revealed to form cortical lesions in animal models (181). Cortical lesions are the result of the inflammatory response against the myelinated sheath and can cause cognitive impairment (182). Although this data may not be directly applicable to ASD, MS demyelination pathology may provide insight into pathogenic mechanisms in ASD cases where CNS antibodies are present (140).

It has been established that the occurrence of neurodegenerative diseases is greater in adults with ASD (148). Evidence of chronic inflammation, such as activated microglia and proinflammatory cytokines, have been shown in the brains of patients with ASD, similar to that of AD (183, 184). The Notch signaling pathway regulates neurogenesis, axon guidance, and synaptic plasticity but in most cell types, can also initiate proinflammatory signaling cascades (41, 185). Therefore, dysfunction of the Notch signaling pathway has been thought to have implications in AD pathophysiology (185). By interacting with Notch1, CNTN1 may influence downstream inflammatory effects such as the expression of proinflammatory cytokines (e.g., IL-6 and IL-17) (41, 43, 186, 187). Notch signaling is found ubiquitously within the human body and is responsible for the homeostasis of many functions and for that reason, is hard to target pharmaceutically (41). However, anti-inflammatory therapies aimed at blocking Notch signaling may be able to be CNS-targeted through specifically impeding CNTN1-driven Notch signaling (188). This could be a useful approach to target neuroinflammation in ASD brains arising from aberrant Notch signaling.

TABLE 2 | Overview of inflammatory diseases and immune disorders that are associated with neuronal cell adhesion molecules.

Inflammatory disease	Associated gene	References
Asthma and allergy	*CNTN1*	(156)
	NRXN1	(193, 194)
Gastrointestinal dysfunction	*NLGN3*	(207, 210–212)
Chronic inflammatory demyelinating polyneuropathy	*CNTN1*	(158, 160–165, 167–174, 176–178)
	CNTNAP1	(158, 162, 163, 167, 173, 174)
	CNTNAP2	(169)
	NFASC	(160–166, 168, 170, 175)
Multiple sclerosis	*CNTN2*	(180, 181)
Neurodegenerative disease	*CNTN2*	(190)
	CNTN4	(192)
	NRXN3	(201, 204)

CNTN, contactin; CNTNAP, CNTN-associated protein; NRXN, neurexin; NLGN, neuroligin; NFASC, neurofascin.

As illustrated previously, Aβ has a key role in the neuropathology of AD (189). Interestingly, the CNTN family of proteins may play roles in regulating this process. Lower CNTN2 expression is observed in and around Aβ plaques that were within the hippocampus of patients with AD compared to controls (190). Additionally, CNTN2 concentrations within the CSF of those with AD were significantly reduced (190, 191). Although it is unclear what the relationship between CNTN2 and APP is, this may point toward a role for CNTN2 in the modulation of Aβ production. A similar role has been posited for CNTN4, which is known to interact with APP and promote its processing *via* the non-amyloidogenic (non-pathogenic) pathway (192). Altered expression of CNTN2 and CNTN4 in ASD could result in increased amyloidogenic processing of APP to Aβ, leading to plaque formation and subsequent inflammation. Furthermore, considering that CNTN2 expression in AD is correlated with increased expression of IL-1β and IFN-γ, it could be pertinent to explore how the CNTN proteins may facilitate Aβ production in ASD.

Neurexins in Inflammatory and Immune Disease

Neurexin 1 has also been implicated in asthma and ASD pathology (**Table 2**). In one study, 43% of patients with a 2p16.3 deletion in NRXN1 were reported to have ASD and of these, 33.5% suffered from asthma and/or allergies (193). Similarly, a *de novo* mutation in NRXN1α was found in a child exhibiting ASD-associated behaviors and developmental delay, alongside asthma that required recurrent hospitalization (194). NRXN1 is co-expressed with CNTN1, suggesting they may be under control of similar transcriptional regulatory programs and have similar regulatory roles in asthma to CNTN1 (195–198). Nonetheless, there is currently no evidence to support a direct link between NRXN1 and asthma, likely indicating asthma occurrence in these case studies may be unrelated to NRXN1 mutations.

In addition to the CNTNs, NRXNs may also play a role in Aβ-induced neuroinflammation. NRXNs interact with Aβ oligomers that are located between deteriorated synapses, as found in pathogenic AD brains (200, 199). NRXN3 is expressed within the hippocampus and cerebral cortex, two important regions of the brain for memory and cognition (201, 202). Variants of the *NRXN3* gene have a strong association with ASD, but not much is known about its function within the scope of AD (16, 203). It was discovered that expression of NRXN3 was reduced in the hippocampus of those with AD and that this expression was inversely correlated with NLRP3 (NOD-, LRR-, and pyrin domain-containing protein 3) expression, which is a constituent of the inflammasome (204). NLRP3 inflammasome signaling leads to the production of IL-1β and IL-18, upon activation by a pathogen or cellular damage (205). This dysregulation of NRXN3 may allow deterioration of neural synapses, causing cellular damage (200). Cellular damage could activate NLRP3, resulting in the release of proinflammatory cytokines, instigating AD pathogenesis (205). Similar disruption in the brains of those with ASD could occur, equally triggering the NLRP3 inflammasome secretion of proinflammatory cytokines IL-1β and IL-18, contributing to the neuroinflammatory environment.

Neuroligins in Inflammatory and Immune Disease

Available data indicates that NLGNs play a role in GI inflammation (**Table 2**). The ADAMs (a disintegrin and metalloproteinase) family are enzymes that are able to cleave transmembrane neuronal CAMs including NLGNs and NRXNs, both of which are associated with ASD (16, 206). ADAMs are expressed throughout the human body but notably, ADAM10 and ADAM17 are found both in the CNS and intestines (207). ADAM17 regulates GI and neural inflammation through the cleavage of TNF-α (increased TNF-α cleavage promotes inflammation) (208). Genetic studies have revealed that whilst ADAM17 expression decreases with age in control groups, in individuals with ASD, *ADAM17* expression increases with age (209). Increased ADAM17 expression in those with ASD facilitates increased TNF-α-mediated inflammation in the gut and brain. Moreover, increased cleavage of NLGN3 by ADAM10 may be causative in decreased intestinal transit seen in people with ASD. Nlgn3-deficient mice were shown to have increased colonic motility, suggesting impaired control of gut motility by the enteric nervous system (210). Like ADAM10 and ADAM17, *NLGN3* is expressed in both the GI system and the CNS (16, 207). *Nlgn3* mutant mice, which display ASD-associated behaviors, were found to have GI symptoms affecting the small intestine and colon function (211). Cecal weight was also decreased in Nlgn3-deficient ASD mice models, alongside increased density of enteric macrophages (212). Due to NLGN3's role in both CNS and enteric systems, mutations affecting this gene have apparent consequences to the immune system.

Neural Cell Adhesion Molecules in Inflammatory and Immune Disease

Despite there being little evidence to support the involvement of NCAM in inflammatory and immune disease, there is some indication of NCAM1 mediating inflammatory cascades that underlie inflammatory disease. Distinct gene expression profiles were found within GI mucosal tissue in people with ASD and GI problems. However, these profiles overlapped significantly with transcriptome profiles of those with IBD, proposing a unique ASD-associated IBD variant. Genes that were exclusively differentially upregulated in ileal and colon samples from the ASD-GI group, compared to neurotypical IBD patients, included *IL-2 receptor alpha (IL2RA)* and *IL-4-induced 1 (IL4I1)* (213). IL4I1 promotes CNS remyelination and IL2RA can activate the MAPK signaling pathway (214, 215). Both remyelination processes and MAPK signaling have been associated with NCAM1 (65, 216). Not only does this link ASD with inflammatory GI disorders, but it may also implicate neuronal CAMs in their pathophysiology's. Due to the ambiguous nature of this potential relationship, we have not included reference to NCAM1 in **Table 2**. This relationship needs further investigation before we can conclude its existence.

Collectively, we can conclude that *NLGN3* appears to be a CAM of interest in the pathology of ASD-related GI disease owing to its expression in both the GI system and CNS, on top of its status as an ASD candidate gene (16, 210). Autoantibodies against paranodal CAMs in inflammatory autoimmune disease prove the importance of neuronal CAMs in axon myelination, which is implied to be impaired in ASD brains *via* white matter dysregulation, but the origin of this dysregulation is unclear (146, 164). Seropositive CIDP cases illustrate how dysfunction of neuronal CAMs, including CNTNs and CNTNAPs, leads to a neuroinflammatory response in the CNS (164). NRXN3, CNTN2, and CNTN4 stand out as key CAMs that play a role in AD-derived inflammation within the CNS (190, 192, 204). Regulation of synaptogenesis by CAMs in the development of neural pathways may also have implications in both ASD and AD. A summary of neuronal CAMs that are associated with inflammatory diseases can be found in **Table 2**.

GLIAL CELLS AND NEURONAL CELL ADHESION MOLECULES

Glia in Autism Spectrum Disorder

Glial cells encompass microglia, astrocytes, and oligodendrocytes, all found within the CNS (217). Collectively, glial cells have a major role in neuroinflammation and neurodegeneration, in addition to neuronal repair after insult (218, 219). Glial cells are sensitive to environmental cues within the CNS, such as inflammation or injury (220). Microglia act as resident macrophages of the CNS and once activated, produce proinflammatory cytokines and mediators, proliferate, migrate, and even present antigens to T cells (221). The secretion of proinflammatory cytokines, for instance, IL-1β and TNF-α, by microglia recruit immune cells to escalate the immune response and initiate the activation of astrocytes (39). Astrocytes have a similar role in inflammation as microglia. They are responsible for blood-brain barrier maintenance, immune cell activation, secretion of proinflammatory cytokines, and the induction of inflammatory-associated signaling cascades (222). After acute inflammation has been resolved through glial activation, microglia can regulate their own deactivation by the secretion of anti-inflammatory cytokines such as IL-10 and TGF-β (223). When the homeostasis of the CNS is altered, chronic activation of microglia or astrocytes can occur. This results in a prolonged inflammatory response, consequently causing damage to neuronal cells (224). Often, this is a characteristic of neurological or inflammatory disease (224). Oligodendrocytes myelinate axons within the CNS, which are important for synapse transmission and neuronal communication (225). As explained previously, dysfunction of myelination leaves neuronal cells unprotected against proinflammatory damage that may also impair cognition and sensory processing (144).

Examination of postmortem ASD brains identified microglial dysfunction as a feature of ASD pathophysiology (89, 226). Astrocytes undergo reactive gliosis and change morphology, much like microglia (227). A pivotal manuscript by Vargas *et al.* gave insight into the glial state within the brains of individuals with ASD. Dynamic neuroinflammation was observed within the cortex, cerebellum, and white matter of ASD subjects, as well as obvious activation of astrocytes and microglia (102). In another study, morphological changes were apparent in microglia from the prefrontal cortex of males with ASD, including decreased branching and thickening of the filopodia (226). Collectively, a neuroinflammatory state with involvement of glial cells, denoted by a change of phenotype, appear to characterize the pathophysiology of ASD. Glial reactivity could be, in part, a consequence of localized neuronal dysfunction onset by ASD and, therefore, could exacerbate synaptic and axonal aberrancy already present (226). Alternatively, glia may become activated in response to environmental cues such as LPS (89). In a rat model of LPS-induced MIA, microglia and astrocytes were activated within the fetal cortex soon after LPS administration (228). This poses an environmental source of glial activation resulting in a neuroinflammatory state. Regardless of the cause, prolonged glial activation is detrimental to neuronal health and consequently, cognitive function in ASD (224).

Microglia-derived cytokines, such as TNF-α, have been reported to regulate the pruning of neuronal synapses (229, 230). Efficient synaptic pruning is most active from the age of two and is vital for brain plasticity, which is thought to be lacking in ASD (231, 232). Researchers have found an increase in synapse number and evidence of under-pruning when examining the brains of children with ASD (233). This would agree with the onset of ASD-associated behaviors around the age of three, as well as behaviors in response to over-stimulation (3). However, there is also evidence that overproduction of TNF-α by microglia in ASD brains increases synaptic scaling, theoretically over-pruning synapses (79, 230). This may also have a detrimental effect on the formation of behavior pathways, especially during early neuronal development (234). As of yet, no consensus has been reached over these pruning hypotheses.

Neural Cell Adhesion Molecules in Glial Cell Differentiation, Proliferation, and Phenotype

Glial cell proliferation is imperative in neural development. Astrocytes originate from radial glial cells and oligodendrocytes stem from oligodendrocyte precursor cells (OPCs), whereas microglia are thought to derive from resident macrophages in the yolk sac (225, 235). OPCs, astrocytes, and microglia are incredibly sensitive to sources of deterioration, including inflammation, and respond by proliferating (218, 225, 235). Dysregulation of cell proliferation and differentiation in the prefrontal cortex may be implicated in ASD (99).

It is important to acknowledge that various neuronal CAMs implicated in ASD are also expressed by glial cells. NCAM1, for example, is expressed on the surface of astrocytes in the brain and is vital in axonal regeneration after neuronal insult (236). However, there is further evidence that NCAM1 may be an important molecule for regulating neuroimmune signaling. Studies reveal that astrocyte-derived NCAM1 can alter NF-κB activity in both bulk rat brain tissue and cerebellar granule neurons (123, 237). NF-κB-mediated transcription

in neurons and astrocytes was also increased with NCAM1 homophilic binding, whereby purified NCAM1 was added *in vitro* (123). This indicates that NCAM1 actively moderates NF-κB activity in astrocytes and neurons, hence, altering the levels of inflammatory cytokines produced and subsequently, the neuroimmune response. The Ig domains of NCAM1 are also reported to inhibit astrocyte proliferation *via* inhibition of MAPK signaling (237). As astrocytes are a major source of cytokines in the CNS, their reduced proliferation presents as a mechanism by which NCAM1 can regulate inflammatory responses in the CNS. Importantly, the Ig domain of NCAM1 does share homology with other members of the IgCAM superfamily (238). Therefore, there is also the possibility that other neuronal IgCAMs implicated in ASD could also exert similar effects on astrocytes, initiating NF-κB signaling or regulating astrocyte proliferation. The introduction of purified NCAM1 to rat forebrain astrocytes inhibited astrocyte proliferation, even with the addition of growth factors (239). Likewise, the application of anti-NCAM1 IgG showed the same inhibition of astrocyte proliferation (237, 239). Genetic variation in *NCAM1*, as demonstrated in ASD subjects by Zhang et al., may result in the production of a structurally ineffective form of NCAM1, meaning unrestricted astrocyte proliferation may occur in ASD brains in response to an inflammatory stimulus (240). In turn, this may contribute to the increased brain volume and astrocyte density observed in human ASD brains and ASD mouse models (99, 241). A polysialylated form of NCAM (PSA-NCAM) has also been implicated in postnatal spinal cord myelination in mice (242). PSA-NCAM, a post-translational modification of NCAM, is expressed on the surface of demyelinated axons, reactive astrocytes, and OPCs (216, 242). PSA-NCAM seems to be associated with OPC migration as well as myelination, as its expression is downregulated once the production of myelin begins (243). Alterations in myelination and white matter have been associated with ASD pathology, possibly linking these findings to PSA-NCAM (146, 244). Although, it is still unclear if PSA-NCAM may regulate the way OPCs respond to inflammatory stimuli or influence their production of inflammatory cytokines. Altogether, it appears that NCAM1 may regulate astrocyte proliferation in response to inflammatory stimuli and alter NF-κB signaling in both neurons and astrocytes. Additional research is still needed to better understand this process and to ascertain if other IgCAMs may perform similar roles in modulating neuroinflammation.

Contactins and Contactin-Associated Proteins in Glial Cell Differentiation, Proliferation, and Phenotype

Another family of proteins that may alter neuroimmune responses is the CNTNs and their interacting partners, the CNTNAPs. CNTNAP1-deficient mice were found to have increased Notch signaling, which in turn promoted astrocytogenesis within the cerebral cortex (245). CNTNAP1 is expressed by the radial glial cells that differentiate into astrocytes, however, no changes in radial glial cell number were observed in CNTNAP1-deficient neonatal mice brains (245). Most radial glial cells transform into astrocytes shortly after birth, leaving differentiated astrocytes to generate new astrocytes (217, 246). During neuroinflammation, astrogliosis occurs, whereby Notch signaling promotes the proliferation of local astrocytes (247). Although CNTNAP1 deficiency may not directly alter the number of astrocytes or astrocytic function, it may alter Notch-mediated astrocyte proliferation (245). Similarly, CNTNAP2 does not appear to directly affect microglia or astrocyte number, however, it could influence astrocyte progenitor number. CNTNAP2-deficient mouse models revealed that the number of radial glial cells were decreased in the hippocampus of 5- to 6-month-old mice compared to wild-type mice (248). No differences in the number of mature astrocytes were indicated, suggesting CNTNAP2 may regulate astrocyte progenitor cell number but not the number of differentiated astrocytes. Additionally, CNTNAP2-deficient conditions alter the way astrocytes respond to stimuli. CNTNAP2-deficient mice exhibited a greater number of reactive astrocytes in the hippocampus following an induced seizure compared to wild-type mice (22). Combined, this data proposes that CNTNAP2 may moderate astrocyte activity at two neurodevelopmental stages; *via* astrocyte progenitor cell number at a younger age and also altering how differentiated astrocytes respond to stimuli later in life. Further experimentation on early postnatal transgenic mice would provide an understanding of alterations in the glial population as neurodevelopment progresses.

Altered OPC proliferation has been implicated in ASD pathogenesis alongside abnormalities of the white matter within the brains of those with ASD (146, 249). Analysis of differentially expressed genes in syndromic ASD models supports dysregulation of oligodendrocyte number (244). PTPRZ (receptor protein tyrosine phosphatase zeta), an interacting partner of CNTN1, is expressed in astrocytes, OPCs, and oligodendrocytes in the adult CNS (250). In the CNS, glial PTPRZ interaction with neuronal CNTN1 triggers cell signaling between glia and neuronal cells, promoting neuronal outgrowth important in neurodevelopment (251). CNTN1 is able to bind to PTPRZ at its Ig domain (252). A previous study observed that CNTN1-PTPRZ interaction on the surface of OPCs impairs OPC proliferation and induces oligodendrocyte differentiation (**Figures 2A,B**) (250). This indicates that CNTN1 and PTPRZ act as modulators of oligodendrogenesis. Although unexplored, alterations in CNTN1 (and other CNTN family members) expression may lead to changes in oligodendrocyte number or responses to inflammatory stimuli. Additionally, altered CNTN expression could contribute to dysfunctional myelination that exposes neurons to proinflammatory damage.

To summarize, glial cells are the key source of cytokines within the CNS and, therefore, are vital when considering the impact that neuroinflammation has in ASD (102). Neuronal CAMs seem to play a role, either directly or indirectly, in regulating glial activity. Importantly, neuronal CAMs may influence how glial cells respond to inflammation. One key example of this is CNTNAP2 acting as a potential moderator in astrocyte response after a stimulus (22). Over-proliferation of glial cells may contribute to ASD pathology, although there is some evidence of decreased OPC proliferation (99, 146, 226, 249). NCAM1 appears to directly regulate astrocyte proliferation *via* NF-κB signaling

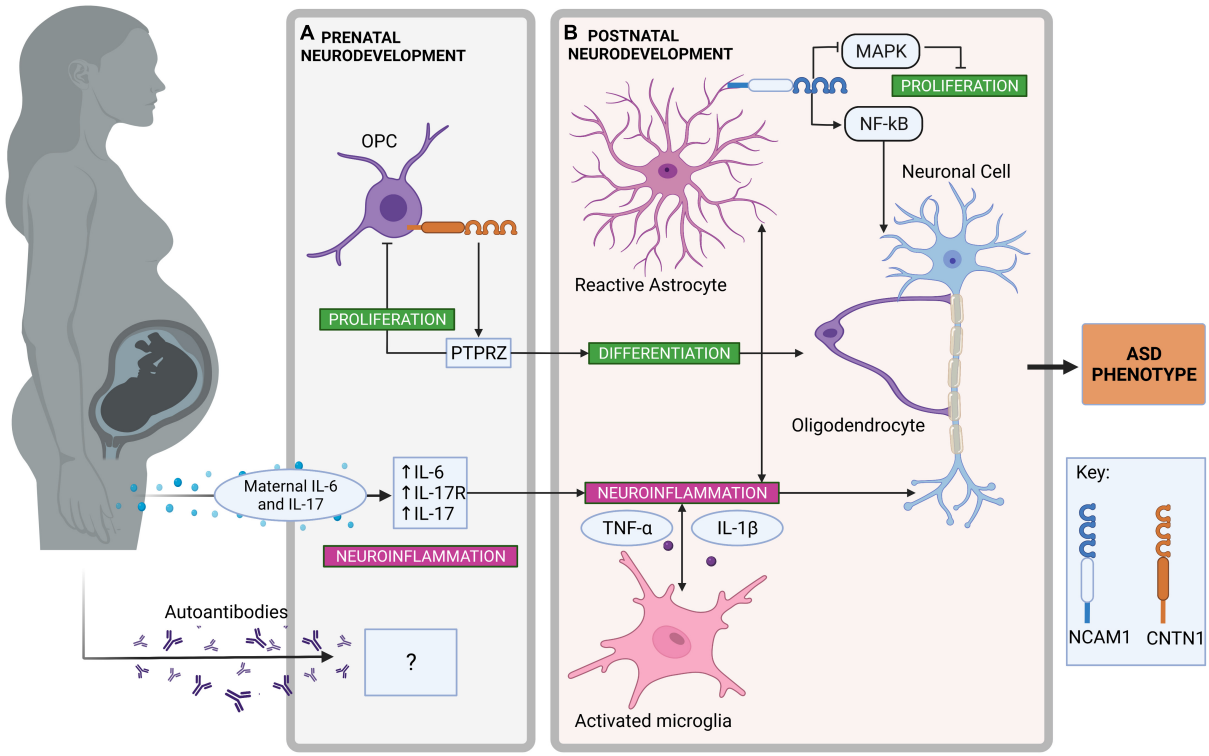

FIGURE 2 | Neuroinflammatory pathways in prenatal and postnatal neurodevelopment contributing to autism spectrum disorder (ASD). **(A)** Neuroinflammatory pathways in prenatal neurodevelopment. Contactin-1 (CNTN1), expressed on the surface of oligodendrocyte precursor cells (OPC) promotes OPC differentiation to oligodendrocytes by interacting with receptor protein tyrosine phosphatase zeta (PTPRZ) (250, 258). Simultaneously, OPC proliferation is inhibited by the same PTPRZ interaction (250). The transfer of maternal proinflammatory cytokines such as interleukin (IL)-6 and IL-17 *via* maternal immune activation can promote neuroinflammation during prenatal neurodevelopment (115, 121). As a result, increased receptor IL-17 (IL-17R) expression is found within the fetal brain, enhancing further neuroinflammatory signaling (107). Although there is some evidence for autoimmune antibodies in association with ASD, their origin and effect on neuroinflammation in the developing ASD brain is unclear (140). **(B)** Neuroinflammatory pathways in postnatal neurodevelopment. Astrocytes are activated by proinflammatory cytokines and neuroinflammation in the central nervous system (227). Neural cell adhesion molecule-1 (NCAM1) expressed on the surface of astrocytes can activate nuclear factor-κB (NF-κB)-mediated transcription of proinflammatory genes (123). NCAM1 can also inhibit astrocyte proliferation through the inhibition of mitogen-activated protein kinase (MAPK) signaling (237). Changes to OPC proliferation have been observed in ASD brains, which can alter the production of myelin and possibly expose neuronal cells to inflammatory insult (146). Microglia are also activated by proinflammatory cytokines and neuroinflammation in the central nervous system (220). Activated microglia to produce proinflammatory cytokines including IL-1β and tumor necrosis factor (TNF)-α (39). Created with BioRender.com.

and may contribute to the increased brain volume observed in individuals with ASD (237, 239). CNTN1 may indirectly regulate OPC proliferation through the interaction of PTPRZ, which subsequently may affect neuronal myelination (250). These ideas are summarized in **Figures 2A,B**, depicting the role of neuronal CAMs in neuroinflammatory pathways during prenatal and postnatal neurodevelopment that may contribute to ASD. PSA-NCAM is expressed during early neurodevelopment making it a good marker to investigate the role of neuronal CAMs during behavioral pathway formation (216). Future investigations may be able to utilize PSA-NCAM to further explore the role of CAMs in inflammatory systems during neurodevelopment.

FUTURE DIRECTIONS

It is believed that both genetic and environmental factors play a role in the pathology of ASD (16, 24). Inflammation has been identified as an environmental risk factor for ASD (220). There is strong evidence highlighting the presence of immune dysfunction in those with ASD, as well as the characterization of ASD-associated behaviors in MIA models (24, 36, 80, 109, 253).

In a healthy model of inflammation, immune cells proliferate and produce proinflammatory cytokines in response to pathogens or immunogenic materials (67). This response is facilitated by signaling pathways that can be activated by cytokines including the Notch, NF-κB, and MAPK signaling cascades (41, 55, 254). Chronic inflammatory signaling can occur if there is dysfunction of the immune system, resulting in unnecessary tissue and cellular damage, instigating the pathogenesis of autoimmune or inflammatory disease (41, 224).

Abnormally elevated proinflammatory cytokines (most commonly IL-1β, IL-6, IL-8, and IL-17) are consistently observed in the CSF and blood of children and adults with ASD (73, 74, 96). Although it is not fully understood, several studies have demonstrated that chronically elevated levels of

these proinflammatory cytokines impair neurodevelopmental processes and neural cell function, including impaired synaptic pruning by microglia, irregular migration of neurons, and altered synaptic plasticity (57, 101, 230). It could be of particular interest for future studies to investigate whether specific cytokine profiles can be associated with inflammation originating from different environmental triggers (e.g., upregulated IL-6 and IL-17 are often associated with MIA) (**Figure 2A**) (110). Additionally, it is not yet certain which cell types, either in the CNS or the periphery, are the main drivers of inflammation in ASD (and the main cell types affected by the chronic inflammation).

Although it is unclear how the neuronal CAMs commonly implicated in ASD may mediate inflammation, investigating other disorders linked to ASD with an inflammatory or immune component can advise potential molecular mechanisms. As discussed in previous sections, the gut-brain axis provides the opportunity for peripheral inflammation to influence neuroimmune signaling and glial activation within the CNS through cytokines and vagal innervation (135). Most likely, GI inflammation is triggered by an exogenous source, such as in the case of food intolerance. In cases of ASD with GI dysfunction, the treatment of GI disorders to reduce inappropriate inflammation could improve the consequences of neuroinflammation. NLGN3 is of distinct interest concerning GI inflammation and ASD, owing to its interactions with ADAM proteins which may influence the production of proinflammatory cytokines in the gut and CNS (16, 210). Further epigenetic studies to explore environmental influences on *NLGN3* expression, and whether its dysregulation alters proinflammatory cytokine production, may be pertinent. There is also some evidence supporting a link to autoimmunity in dysregulation of the immune function in ASD, but literature is unclear on the origin of these autoantibodies (**Figure 2A**) (140, 148, 255). Additionally, there is no proof, as of yet, of the presence of autoantibodies against neuronal CAMs in those with ASD. Autoantibodies against paranodal CAMs in CIDP and MS demonstrate the importance of neuronal CAMs in the protection of neuronal cells against inflammatory damage, however, it remains uncertain how this is relevant to ASD (164). Although ASD is not typically characterized by neurodegeneration, an increase in secreted β-amyloid in the plasma of children with severe ASD identifies an association between the pathology of ASD and AD (151). CNTN4 regulates APP processing, whilst CNTN2 seems to have an association with Aβ (189, 190). The disruption of APP processing pathways may interfere with the balance of Aβ production, the clearance of which is mediated by activated astrocytes and microglia, causing a neuroinflammatory response (155). Therefore, altered expression of these CNTNs may increase Aβ production, contributing to neuroinflammation (153, 189, 192). Future research into the mechanisms by which CNTNs regulate APP processing, and whether dysregulation of this can alter glial cell activity, may reveal more about CNTNs functional role in modulating neuroinflammation.

Glial cells are the key source of cytokines within the CNS and, therefore, are vital when assessing neuroinflammation in ASD brains (102). In response to inflammation, glial cells change their morphology and proliferate (39). There is evidence that neuronal CAMs can play a direct or indirect role in the regulation of glial activity and, therefore, may influence glial responses to inflammation. CNTNAP2 may moderate astrocyte activity by influencing astrocyte progenitor cell numbers and affect how astrocytes respond to external stimuli (22, 248). Compellingly, NCAM1 has emerged as a key player in regulating neuroinflammatory cascades. NCAM1 homophilic binding can initiate NF-κB-mediated transcription in neurons and astrocytes, instigating a proinflammatory response (237). NCAM1 appears to directly regulate astrocyte proliferation (**Figure 2B**) and its dysregulation may, in part, account for the increased brain weight observed in individuals with ASD (237, 239). Additionally, CNTN1 may indirectly regulate OPC proliferation through interacting with PTPRZ (**Figures 2A,B**), which subsequently may affect neuronal myelination (250). Myelin is paramount in protecting axons from damage that may impair cognition and sensory processing (144). Further definition of neuronal CAMs regulatory role in glial activity and response to inflammation is missing from current literature. Experiments investigating CAM expression in glia at different developmental stages, and if neuroinflammation arising from this dysregulated expression can be treated in later life, would be of value for targeted anti-inflammatory therapeutics in ASD. Specifically, pre- and immediately postnatal neurodevelopment would be of interest, owing to ASD phenotypes presenting before the age of three (1).

The concept that neuronal CAMs may mediate or influence inflammatory cascades is largely unexplored in current literature. This review highlights the available evidence on the potential part neuronal CAMs play in neuroinflammation, with a particular focus on ASD. Further investigation into the role of neuronal CAMs within the context of inflammation is clearly warranted and would advance our understanding of neuroinflammation in ASD pathology.

AUTHOR CONTRIBUTIONS

AO-A: concept, research design, editing figures and artwork, and manuscript writing and editing. ME: research, generating figures and artwork, and manuscript writing. JG: research and manuscript writing and editing. LY: research and manuscript editing. All authors read and approved the final manuscript.

FUNDING

This research was supported by the Northcott Devon Medical Foundation Research Grant (AO-A), ARUK Southwest Small Pump Priming Grant (JG and AO-A), and QUEX Ph.D. studentship (ME).

ACKNOWLEDGMENTS

We acknowledge the insightful discussions with Dr. Rosie Bamford, Dr. Charli Harlow, and Miss Emily-Rose Martin (University of Exeter).

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