MOLECULAR AND GENETIC MECHANISMS IN NEURODEVELOPMENTAL DISORDERS: FROM BENCH TO BEDSIDE

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OPINION
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Future Horizons for Neurodevelopmental Disorders: Placental Mechanisms

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INTRODUCTION

To improve care for the estimated 17.1 million children with psychiatric disorders in the United States (1), it is critical to explore all possible connections to better understand these disorders' origins. The onset of neurodevelopmental/psychiatric disorders varies person-to-person. However, even for disorders diagnosed after infancy, there is a growing appreciation that the origins of these disorders are at the earliest stages of brain development—prenatally. Furthermore, not only is it crucial to understand *what* is unusual during development, but also *why* this occurs.

A significant contributor to abnormal prenatal brain development is physiological stress during pregnancy (2–6). Physiological stress induces a significant shift from homeostasis, and may arise from chemical exposures, psychological stress, infections, and illnesses such as preeclampsia or gestational diabetes. Epidemiological studies link maternal stress with offspring neurodevelopmental impairments (2), and animal studies have demonstrated causality of this relationship. For example, preeclampsia—a disorder with disrupted maternal vascular and immune biology—increases risk of neuropsychiatric problems among children (3). Evidence has come from human and non-human preeclampsia studies implicating *what* in the offspring brain has changed: its morphology, white matter, and vasculature. When we ask the further question of *why* these changes occur with preeclampsia or any maternal stress, it is critical to consider the biology of not only mother and offspring but also their link—the placenta. Changes in placenta may be a critical factor for offspring neurodevelopment (**Figure 1**).

The placenta forms after conception and is delivered along with the offspring. During gestation, placenta serves as the mediator between mother and fetus, supplying nutrients and oxygen to the fetus and removing waste and CO_2. The role of the placenta has been emphasized previously, and continues to warrant attention when examining disorders with developmental origins (7). Many cultures bury the placenta after birth, out of respect for its role as a "guide" through pregnancy or its link to the child's future (8). The level of respect for the placenta these cultures offer seems fitting, as growing evidence suggests its importance in long-term neurodevelopmental outcomes.

PLACENTAL BIOLOGY

The nutrient and waste exchange of the placenta that supports fetal development is just part of its critical role. The placenta also produces critical hormones, growth factors, proteins for metabolizing endogenous molecules (e.g., 11βHSD2 breakdown of elevated cortisol in normal pregnancy to limit fetal exposure) or transporting exogenous factors (e.g., efflux transporters for xenobiotic chemicals), and other molecular substrates (e.g., such as serotonin which is directly supplied to the fetal brain (9–11). All of these factors impact fetal development and regulate

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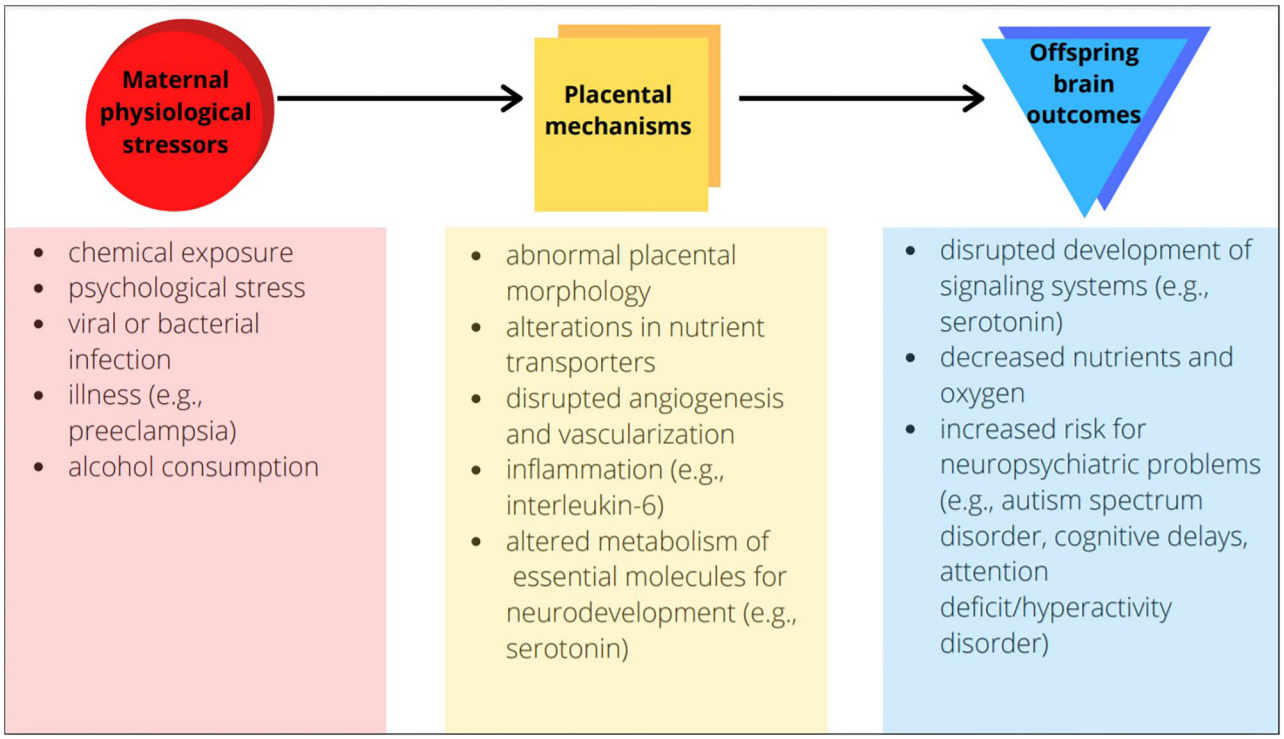

FIGURE 1 | Examples of placental mechanisms as the link between maternal physiological stressors and offspring brain outcomes.

communication between the separate but linked biological domains of the mother and fetus. These processes of normal pregnancy are dependent on proper placental structural formation and its function throughout gestation (12). Maternal physiological factors may influence the structure and function of the placenta's unique connection between mother and baby. To better understand the placenta, it is important to understand general periods of placental development:

Beginning of Gestation: Placental villi are multi-layered folds of tissue in which fetal and maternal blood vessels come in close contact (13). Villous and extravillous structures form as a result of the proliferation and differentiation of trophoblast cells, which arise during the earliest divisions of cells in the embryo. The villous and extravillous structures physically anchor the placenta into the uterus and allow gas and nutrient exchange and other functions.

First Half of Gestation: Trophoblasts undergo the most changes. For example, some trophoblasts replace endothelial cells that make up the uterine spiral arteries to ensure adequate fetal blood supply. This mechanism also serves to protect the placenta from potentially harmful fluctuations in oxygen levels (14).

Second Half of Gestation: Extensive angiogenesis and vascularization occurs. The formation of new blood vessels allows blood to enter the trophoblast cell-lined sinuses in the uterus and continue to meet the nutritional and other physiological needs of the growing offspring (15).

GESTATIONAL DIABETES

Abnormal placental morphology and function have significant impacts on fetal outcome. For example, gestational diabetes mellitus (GDM) has been linked to offspring risk for metabolic, cardiovascular, and neuropsychiatric problems including autism spectrum disorder (ASD), ADHD, depression, anxiety, and cognitive delay (16). Maternal diabetic abnormalities may have direct impacts on fetal metabolism, changing levels of specific nutrients and hormones. However, studies have also found in GDM models that maternal hyperglycemia leads to decreased placental glucose transporters (17). This in turn may lead to decreased fetal glucose, further leading to delays, as glucose is a critical nutrient (13). GDM also induces altered metabolism and placental development and function at early stages which may be responsible for excessive fetal growth (13). Specifically, inflammatory and cellular stress pathways in placental cells such as NF-κB signaling or ER stress likely play a role in GDM (18). Evidence suggests that abnormal maternal metabolism stimulates both adipose and placental cells, increasing production of inflammatory cytokines that then may influence the fetus in multiple ways (19).

MATERNAL INFLAMMATION

Other maternal factors, including bacterial or viral infections such as influenza, can elicit increased inflammatory cytokines which may alter placental function. Maternal immune activation (MIA) during pregnancy has been linked with an increased risk for neuropsychiatric risk in offspring, including ASD (20).

Animal models show that influenza infection during pregnancy alters the placenta in multiple ways. After maternal influenza, overall placental growth is reduced (21), as well as reduced labyrinth zone thickness, suggesting that disrupted vascular development of the placenta occurs and then likely reduces nutrient and oxygen exchange (22). Maternal influenza also alters the expression of a significant number of placental genes, mainly implicating inflammatory, immune, and hypoxia pathways but also overlapping with risk genes for neuropsychiatric disorders (23). In these studies, placentas also showed cellular abnormalities including thrombi and elevated immune cell number. Offspring brain showed persistent changes in some key neuronal genes many weeks after this maternal exposure (23). At the same time, no viral genes were found in placenta or offspring brain, suggesting indirect pathways for brain alterations such as placental morphological and functional changes.

FETAL ALCOHOL SPECTRUM DISORDER

Fetal Alcohol Spectrum Disorder (FASD) affects up to 1.5 of every 1,000 births in the United States (24). FASD includes low body weight, poor coordination, and cardiovascular complications. Children with FASD experience neurodevelopmental challenges such as hyperactive behavior, poor memory, and speech and language delays. As with other maternal physiological disruptions, the role of the placenta may be critical for impacts of gestational alcohol exposure.

Disruptions to placental vascular structure and function may be a mechanism involved in the origins of FASD. Increased glucocorticoid levels with alcohol consumption (25) may play a role in reduced blood flow, given known impacts of cortisol on placental angiogenesis (26). In human placentae from pregnancies in which women were prospectively assessed to have used alcohol, levels of two angiogenic proteins vascular endothelial growth factor receptor 2 (VEGFR2) and annexin-A4 (ANX-A4), were reduced (27). VEGFR2 and ANX-A4 both enhance proliferation, migration, and survival of the endothelial cells critical for placental blood vessels, suggesting that maternal and/or fetal blood flow that underlies many other placental functions may be dysregulated A trend increase of the pro-inflammatory cytokine, TNFα, in placenta after alcohol exposure also suggests that placental processes sensitive to inflammation, such as serotonin production, may be affected. This study reveals different aspects of placental abnormality than other assessments showing a higher rate of placenta accreta with gestational alcohol exposure (28). Placenta accreta occurs when trophoblast cells invade the uterine wall abnormally, which suggests altered initial placentation due to alcohol exposure. Placenta accreta can lead to complications during delivery and may be managed by pre-term cesarean delivery, both of which are linked to increased neurodevelopmental risk for children (29).

The aforementioned studies demonstrate examples of common maternal conditions with placental abnormalities that have also been linked to neurodevelopmental abnormalities of offspring. There are many other factors that can influence placental changes and therefore development of offspring; for example, regardless of maternal physiology, infant neurodevelopment has also been correlated with placental epigenetic variation (30). What is apparent from these studies is that the placenta's role is more than a waystation for the fetal brain to be exposed to molecules from maternal circulation. The impact of placenta nutrient transport, serotonin production, and hormone regulation are significant, as general development of the fetus has been found to be negatively influenced when these functions are abnormal (31). Additionally, a range of maternal physiological stresses impact placenta function (gestational diabetes mellitus, maternal infection). These forms of maternal stress and others may have overlapping neurodevelopmental impacts on offspring because of overlapping placental abnormalities.

LINKING PLACENTA AND NEURODEVELOPMENT

In the examples discussed above, maternal conditions during pregnancy are linked both with improper function of the placenta as well as with increased likelihood of neurodevelopmental problems in offspring. The critical nature of the placenta for neurodevelopmental changes is hypothesized from such descriptive studies, but few studies have been able to causally connect placental abnormalities directly to altered brain development.

The impact of alcohol on placental growth factor (PLGF) has been explored for its mechanistic role in disruptions of fetal brain vasculature in mice. With CRISPR-Cas9 mediated over-expression of the PLGF gene in placenta, the reduced proportion of cerebrocortical radial vessels with maternal alcohol consumption is corrected to normal levels (32).

Maternal immune activation (MIA), a risk factor for ASD, involves elevation of maternal IL-6, a proinflammatory cytokine responsive to infections. Sophisticated work has shown that the impact of IL-6 on the placenta is the critical mediator of MIA effects on offspring neurodevelopment (20). A specific transgenic removal of placental IL-6 receptor, through the CYP19Cre driver (a transgene under the control of the aromatase cytochrome P450 19), resulted in offspring protected from the impacts of MIA in brain and behavior. Other findings from this work suggest that IL-6 signaling may play this critical role because it impacts placental angiogenesis and vascular permeability, hormone production, or further inflammatory signaling cascades in fetal circulation.

Another study has implicated the placenta's adaptation to nutrient deprivation (i.e., maternal starvation) as a way to protect offspring hypothalamus neurodevelopment—a critical brain region for hormonal regulation. During mouse gestation, expression of the gene *Peg3* is coordinated in the fetal

hypothalamus and placenta, coordinating multiple other genes that regulate both placental and hypothalamic development (33). Following food deprivation, this pattern is uncoupled, with *Peg3* expression increased in fetal hypothalamus and decreased in placenta. This study demonstrates also that this maternal starvation stress advances hypothalamic cell growth, despite the reduction in nutrients due to breakdown of placental cells from decreased *Peg3* expression. Poor neurodevelopmental outcomes in the setting of maternal starvation may represent failure of this protective mechanism.

Maternal inflammation can be linked through the placenta to disrupted serotonin development in the fetal brain, particularly in the forebrain where serotonin plays a role in emotional regulation circuits. Specifically, MIA disrupts placental tryptophan metabolism, including altered expression of genes that synthesize serotonin [tryptophan hydroxylase 1 (TPH1) and indoleamine 2,3-dioxygenase 1]. Interestingly, controlled investigations of isolated placental and brain tissue after MIA also shows that TPH1 activity is increased only in placenta, but serotonin levels increase in fetal brain. Ultimately this increase in serotonin delivered to fetal brain suppresses normal outgrowth of fetal serotonin axons (34).

CONCLUSION

The placenta has a temporary role in development during gestation—despite its time-limited presence, impacts of its function are clear well into adulthood. The impact of maternal illness or exposures during pregnancy on neuropsychiatric functioning of the next generation may well be heavily influenced by the health and performance of the placenta. If prenatal brain developmental changes from maternal physiological stresses that contribute to ASD, cognitive delays, or other neuropsychiatric problems originate in the placenta, finding ways to protect its structure and function are critical. Moreover, the placenta is a much more accessible biological target than the developing fetal brain for such interventions. Therefore, understanding mechanisms by which it influences the fetal brain, such as inflammatory signaling and serotonin production, will allow for more protective measures to be developed for healthy brain development in children.

AUTHOR CONTRIBUTIONS

SK and HS conceived the manuscript. SK, HS, and SM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Untangle the Multi-Facet Functions of Auts2 as an Entry Point to Understand Neurodevelopmental Disorders

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Neurodevelopmental disorders are psychiatric diseases that are usually first diagnosed in infancy, childhood and adolescence. Autism spectrum disorder (ASD) is a neurodevelopmental disorder, characterized by core symptoms including impaired social communication, cognitive rigidity and repetitive behavior, accompanied by a wide range of comorbidities such as intellectual disability (ID) and dysmorphisms. While the cause remains largely unknown, genetic, epigenetic, and environmental factors are believed to contribute toward the onset of the disease. Autism Susceptibility Candidate 2 (Auts2) is a gene highly associated with ID and ASD. Therefore, understanding the function of Auts2 gene can provide a unique entry point to untangle the complex neuronal phenotypes of neurodevelpmental disorders. In this review, we discuss the recent discoveries regarding the molecular and cellular functions of Auts2. Auts2 was shown to be a key-regulator of transcriptional network and a mediator of epigenetic regulation in neurodevelopment, the latter potentially providing a link for the neuronal changes of ASD upon environmental risk-factor exposure. In addition, Auts2 could synchronize the balance between excitation and inhibition through regulating the number of excitatory synapses. Cytoplasmic Auts2 could join the fine-tuning of actin dynamics during neuronal migration and neuritogenesis. Furthermore, Auts2 was expressed in developing mouse and human brain regions such as the frontal cortex, dorsal thalamus, and hippocampus, which have been implicated in the impaired cognitive and social function of ASD. Taken together, a comprehensive understanding of Auts2 functions can give deep insights into the cause of the heterogenous manifestation of neurodevelopmental disorders such as ASD.

Keywords: autsim spectrum disorder, transcriptional regulation, epigenetic regulation, synapse formation, Auts2

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INTRODUCTION

Neurodevelopmental disorders are a group of diseases characterized by deficits in the functions of the brain that affect a child's behavior, memory, or ability to learn, including intellectual disability (ID), autism spectrum disorder (ASD), attention-deficit hyperactivity disorder (ADHD) and so on (1). The symptoms of these disorders typically start during early childhood, when the nervous

system undergoes intensive development and maturation, indicating that perturbed neurodevelopment contributes to the onset of diseases (2). In a patient, more than one neurodevelopmental disorder often appears as comorbidities, suggesting common neurological etiology shared by them (3). ASD is one of neurodevelopmental disorders, affecting ∼1% of the population (4). The patients often showed impaired social communications, restricted interests and repetitive behaviors (5, 6). Although, patients with autism usually have these core symptoms in different degree, they also display a wide range of comorbidities, including ID, ADHD, motor control deficit, epilepsy, anxiety, and sleep disorders (3). Moreover, ∼20% of the patients show dysmorphic features, varying from hypertelorism, strabismus, microcephaly and/or macrocephaly (7, 8).

Despite the wide spectrum of phenotypes observed in patients, the etiology of ASD remains largely unclear. It was proposed that the clinical manifestations of ASD patients are the sum-up effect of genetic, environmental factors, and gene-environment interactions (9). According to the summary of AutismKB, ∼3,000 genes and ∼4,900 copy-number variants were associated with the risk of ASD. Among them, 171 autism-related genes were considered to have high confidence for the causality link (http://autismkb.cbi.pku.edu.cn). The proteins encoded by the high-risk genes include synaptic receptors and cell adhesion molecules such as NRXN, scaffolding proteins and the actin cytoskeleton such as SHANKs, as well as transcription factor such as *Auts2* (10). However, the autism patients in which a genetic cause could be identified only account for ∼25% of the total number of cases. The etiology of vast majority (∼75%) cases remain undefined (11).

Recent studies have shown that epigenetic regulations such as, DNA methylation and histone modification are associated with ASD (12, 13). Furthermore, prenatal exposure to chemicals such as endocrine-disrupting compounds (EDCs) or maternal stress have been linked to higher risk of neurological and psychiatric disorders in the offspring including ASD (14, 15).

Due to the heterogeneity of the phenotypes displayed in ASD, the complexity of genetics and epigenetic risk factors, as well as the mostly unknown pathophysiological mechanisms of the disease, there is still lack of effective pharmacological treatment for ASD. Intriguingly, Balovaptan, a selective small molecule antagonist of the vasopressin receptor showed specific effect in improving social interaction and communication of ASD male patients with normal IQ level, according to its phase II clinical trial (16). Therefore, to understand the function of a single ASD risk-gene remains to be an important entry point in order to untangle the complexed neurological mechanisms during the onset and progressing of ASD.

Autism Susceptibility Candidate 2 (Auts2, also named as the "activator of transcription and developmental regulator" by the HUGO gene Nomenclature Committee (HGNC), #14262) was first identified as the genetical cause ASD in a pair of monozygotic twins (17). In addition, the chromosome structural rearrangement or copy-number variance of the Auts2 locus has been associated with a wide range of neurological and psychiatric conditions such as ID, epilepsy, bipolar disorders, alcohol abuse, ADHD, and schizophrenia (18–23). Notably, 80∼100% patients with Auts2 disruption were diagnosed with ID or developmental delay, and ∼40% patients had ASD (18, 24–26). Some patients displayed dysmorphic features such as short stature, microcephaly, and facial changes (18, 26, 27). Thus, the complexity of phenotypes caused by Auts2 mutations or genetic variations are consistent with the broad range of symptoms manifested by neurodevelopmental disorders, indicating its comprehensive roles in the development of brain functions (**Figure 1**, **Table 1**).

Auts2 gene is located on chromosome 7q11, span 1.2 Mb. It has 19 exons, coding for a protein with 1,259 amino acids in humans. The sequence is 62% conserved between zebrafish and human, and 93% between mouse and human. Sequence analysis showed that the Auts2 protein has a PY (Pro-Tyr) motif, eight CAC (His) repeats, two proline-rich domain, several putative SH2, and SH3 domains, kinase phosphorylation sites, as well as N-glycosylation sites (17, 41). Similar PY domain is also present in several transcription factors. The CAC repeats could be signals for locating at a subnuclear compartment, which associated with RNA splicing machinery (42). Therefore, the sequence of Auts2 indicated its role in transcriptional regulation. The multiple SH2 and SH3 sites and the proline-rich domain could be potential protein-protein interaction regions (41, 43).

AUTS2 IS A KEY-REGULATOR OF THE COMPLEX TRANSCRIPTIONAL NETWORK DURING BRAIN DEVELOPMENT

Auts2 gene has two transcription start sites, conserved in both mouse and human. One is in front of exon 1, and the other one is in exon 9, resulting in a full-length transcript (∼140kDa) and short isoforms (18, 28, 40). Additional alternative splicing exons include exon 10 and 12 in human, corresponding to exon 11 and 13 in mouse. All of them were found in the developing brain, but only two of the variants were found in adult human and mouse brain (28). In mouse embryonic stem cells (mESCs), the expression of short isoform becomes dominant when neurons start to differentiate, in replacement of the long isoforms. Overexpression of the long isoforms *in vitro* resulted in delayed neuronal differentiation, and mutations in the sequence specific to the long isoforms led to accelerated differentiation. The short isoform was also more expressed than the long one in embryonic human brain (28). In developing mouse brain, the long and short isoforms are expressed in cerebral cortex at embryonic stages, but the expression of short isoforms continuously decrease since E14.5, and is barely detectable 7 days after birth. In juvenile mouse cerebral cortex, only the full-length Auts2 transcripts were detected (40). Therefore, the temporal expression pattern of Auts2 isoforms is tightly regulated, indicating its multi-facet functions during neurodevelopment. But how exactly the expression of Auts2 isoforms is orchestrated need to be further investigated.

Interestingly, Auts2 has one of the highest intronic RNA level in fetal brain (44). Moreover, mutations in the non-coding region of Auts2 gene in both zebrafish and human were associated with developmental deficit (45–47). Candidates for the enhancers of

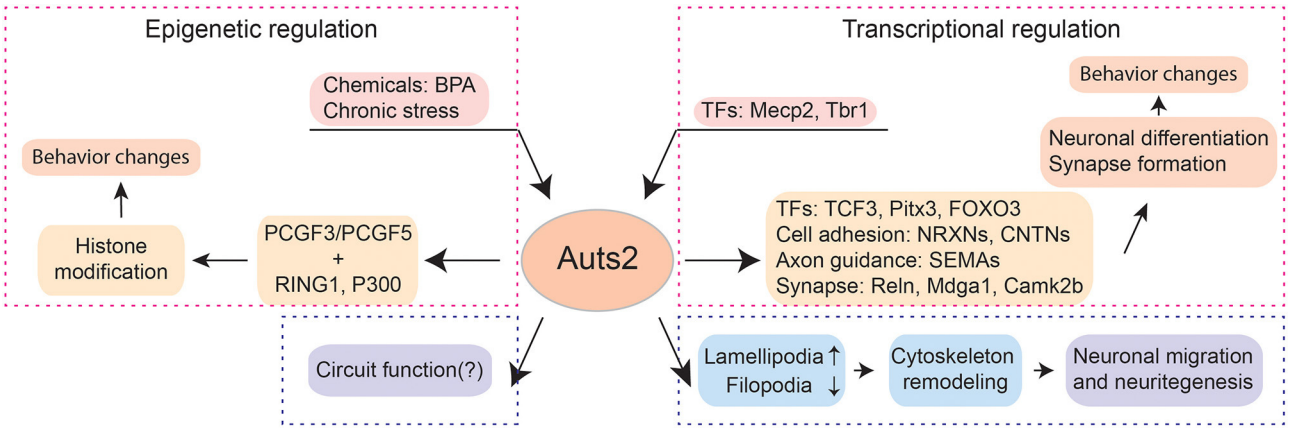

FIGURE 1 | The multi-facet functions of Auts2. Auts2 participates in transcriptional and epigenetic regulations, neuronal differentiation, neuronal migration, neuritegenesis as well as synapse formation. In the aspect of transcriptional regulation, the expression of Auts2 is controlled by transcriptional factors (TFs) such as Mecp2 and Tbr1. Moreover, Auts2 is a transcriptional regulator itself. It can control the expression of other TFs (e.g., TCF3, Pitx3, and FOXO3), cell adhesion molecules (e.g., NRXNs and CNTNs), axonal guidance molecules (e.g., SEMAs) and proteins involved in synaptic functions (e.g., Reln, Mdga1, and Camk2b). The precise coordinate of transcription by Auts2 is critical for neuronal differentiation and synapse formation. The deletion of Auts2 gene can lead to behavior impairment in social interaction, cognition, and ultrasonic vocalization, which captures the features observed in patients with neurodevelopmental disorders such as ASD. Auts2 also plays important roles in epigenetic regulations. After binding PCGF3/PCGF5, RING1 and P300, the Auts2-containing complex can bind to the promotor regions of various genes and modify histones. Through epigenetic regulation, Auts2 can mediate the effect of environmental factors, e.g., chemicals and maternal health factors on the nervous system. Cytoplasmic Auts2 regulates the neuronal migration and neuritegenesis through inducing lamellipodia and suppressing filopodia. The function of Auts2 in circuits remains to be further explored.

TABLE 1 | Summary of key findings about the multi-facet functions of Auts2.

Domains	Key findings about the functions of Auts2	References
Transcriptional regulation	Long isofroms of Auts2 delayed neuronal differentiation in mouse embryonic stem cells.	(28)
	Auts2 bound to regulatory elements of Pitx3, TCF3, FOXO3, NRXN1, CNTN4, RBFOX1, and ATP2B2.	(29)
	In Auts2 knock-out mice, genes such as, Reln, Mdga1, Camk2b, Cacnalc and C1ql-family were differentiately expressed.	(30)
	The transcription of Auts2 was regulated by Mecp2 and Tbr1.	(31–34)
Epigenetic regulation	The short isoforms of Auts2 bound to two polychomb proteins, PCGF3, and PCGF5.	(28, 35)
	Auts2-containing PRC1 suppressed gene expression, but after recruiting P300, it became a transcriptional activator.	(28, 35)
	Auts2-containing PRCs modified histone H3 acetylated at lysine 27 and trimethylation at lysine 4.	(29, 35)
	Auts2 activated the transcription of ganglioside-producing enzyme.	(36)
	Prenatal exposure to BPA decreased expression of Auts2 in neonatal mice.	(37)
	Maternal thermal stress in zebrafish induced down-regulation of Auts2 in the eggs.	(38)
	Chronic stress induced a DNA adenine modification at Auts2 and its regulator Tbr1.	(39)
Synapse formation	Auts2 controlled the number of excitatory synapses and synchronized the balance between excitation and inhibition in the brain.	(30)
Cytoskeleton regulation	Auts2 induced lamellipodia *via* activation of Rac1 and suppressed filopodia *via* down-regulations of Cdc42.	(40)
	Auts2 knock-out mice showed impairment in both migration and axon elongation of cortical neurons.	
Regions expressed	The expression of Auts2 in the brain was strong by E16, then decrease to a low level after P21.	(34, 40)
	In cerebral cortex, the expression of Auts2 exhibited a strong rostral-caudal gradient.	
	In hippocampus, Auts2 was expressed in the denta gyrus, CA1 and CA3 from E14 onwards, and Auts2 was located to granule cell layer and subgranular zone by P21.	
	In cerebellum, Auts2 was in granule neurons, precursor of Purkinje cells and deep nuclei at early developmental stage. By P21, Auts2 was expressed in Purkinje cells only.	(34)
	On E14, Auts2 was expressed in dorsal thalamus. By P21, Auts2 was in ATN, and VL/VM.	

Auts2 have been mapped to several introns, and showed specific expression in embryonic fish and mouse brain (45). Studies have shown that the intron retention is an important slicing program for transcriptional regulation in neurons, and it is modulated by neuronal activity (48, 49). Thus, the abundance of intronic Auts2 RNA suggests that Auts2 might be actively participating in, as well as subjected to various transcriptional regulation during development.

The function of different isoforms can be executed through the interaction with diverse partners during transcriptional regulation, as well as other cellular process. The short isoform of Auts2 was found to be exclusively expressed in the nucleus (28, 40). Moreover, Auts2 was found to localize at the promotors and non-promotor regulatory elements related to neurodevelopment, including transcription, splicing of RNAs, proliferation of cells and migration of neural precursor cells. Auts2-associated regions also included binding motifs of transcription factors involved in neurodevelopment, such as, paired-like homeodomain 3 (Pitx3), transcription factor 3 (TCF3) and forkhead box O3 (FOXO3). In addition, Auts2 was found to occupy functionally active enhancers of neurexin 1 (NRXN1), contactin 4 (CNTN4), RNA-binding protein fox-1 homolog (RBFOX1) and ATPase Ca++ transporting plasma membrane 2 (ATP2B2) (29). A genome-wide association study (GWAS) has shown that Auts2 is a regulatory element for semaphorin 5A (SEMA5A), an axon-guidance molecule (50). Both NRXN1 and SEMA5A belong to the core ASD-risk genes (51). Selective removal of Auts2 in excitatory neurons leads to the down-regulation of NRXNs and SEMAs in hippocampi (30). In addition, many genes coding for proteins implicated in synaptic functions, e.g., Reln, Mdga1, Camk2b, Cacna1c, and C1ql-family genes are differentially expressed (30). Therefore, it is possible that Auts2 regulate synaptogenesis through modulating the transcription of relevant genes.

The transcription of Auts2 is regulated by several genes indicated in ASD. For example, methyl CpG binding protein 2 (Mecp2), a gene implicated in Rett-syndrome and ASD, represses the expression of Auts2 in both mouse and human brain (31, 32). T-Box Brain Protein 1 (Tbr1), a transcription factor that assign regional and laminar identity of postmitotic neurons, can activate the expression of Auts2 (33, 34). Thus, Auts2 is a key-regulator of the complex transcriptional network during brain development. Indeed, perturbing Auts2 expression in both zebrafish and mouse brain can both lead to various neurodevelopmental phenotypes. The zebrafish Auts2 morphants show microcephaly and smaller-jaw size, similarly to the observations from human patients (17, 29). Auts2 knock-out mice also showed a general developmental delay, including lower body weight and impaired motor skills (30, 35, 52).

NUCLEAR AUTS2 IS A MEDIATOR OF THE EPIGENETIC REGULATION DURING NEURODEVELOPMENT

Auts2 was found to regulate neuronal differentiation through histone modifications, an epigenetic mechanism. The short isoform of Auts2 can bind to two polycomb proteins, PCGF3, and PCGF5, and be part of polycomb-repressive complex 1 (PRC1) for suppressing gene expression (28, 35). After recruiting P300, the Auts2-containing PRC1 were converted into a transcriptional activator from a repressor (28, 35). The histone modifications by Auts2-containing PRCs include histone H3 acetylated at lysine 27 (H3K27ac) and trimethylation at lysine 4 (H3K4me3) (29, 35). This can be one of the mechanisms that controls the metabolic reprogramming during the differentiation from stem cells towards the neural lineages. In neuronal stem-cells differentiation period, glycosphingolipid production was switched from a globo-series to ganglio-series (53). Auts2 was found to activate the transcription of rate-limiting ganglioside-producing enzyme, and thereby control the glycosphingolipid reprogramming (36). This study linked the Auts2-mediated epigenetic regulation with critical lipid metabolism during neuronal differentiations. Many questions remain to be elucidated. For example, what is the exact role of this metabolic glycosphingolipid switch for neuronal differentiation *in vivo*, as well as how this could contribute to the etiology of ASDs caused by Auts2-mutation?

Environmental chemicals and maternal health factors have been associated with increased risk of ASD (54). Exposure to ambient pesticide during prenatal period and infancy elevates the occurrence rate of Autism (55). Maternal use of selective serotonin reuptake inhibitors (SSRIs) as a result of diagnosed depression also increase the risk of ASD (56). The epigenetic regulation participated by Auts2 might be associated with environmental chemicals and maternal stress during pregnancy. Prenatal exposure to bisphenol A (BPA) lead to decreased expression of Auts2 in the hippocampi from neonatal male mice (37). In zebrafish, maternal thermal stress induced down-regulation of Auts2 in the eggs, as well as fear-related locomotor responses to a novel environment in the offspring (38). In mouse prefrontal cortex, chronic stress induced a DNA adenine modification at genes including Auts2 and its regulator Tbr1 (39), which led to a comprehensive gene expression changes in the brain and thereby might be the cause of neuropsychiatric disorders. However, there is no direct evidence so far to show that Auts2 mediated neuronal dysfunction induced by environmental factors. It is highly interesting to investigate the role of Auts2 as an epigenetic linkage for the etiology of ASD upon environmental risk-factor exposure.

AUTS2 REGULATES THE NUMBERS OF EXCITATORY SYNAPSES AND THUS SYNCHRONIZES THE BALANCE BETWEEN EXCITATION AND INHIBITION

The loss of Auts2 was shown to result in excessive excitatory synapses *in vitro* and *in vivo* (30). When Auts2 was selectively deleted in hippocampal neurons postnatally, the dendritic spine formation increased significantly, and more excitatory synapses were formed onto these spines. The number of inhibitory synapses were unchanged. Therefore, postnatal expression of Auts2 in the brain can modulate the balance between excitation and inhibition by limiting the number of excitatory synapses. Interestingly, the phenotype of excessive excitation in Auts2 knockout neurons could be rescued by re-expression of full-length transcripts. It could neither be rescued by the C-terminal Auts2 short isoforms, nor full-length transcripts tagged with the nuclear export sequence (30). This indicated that the function of balancing synaptic inputs by Auts2 depends on its genetic and/or epigenetic regulation onto the expression of relevant genes. The

authors proposed some candidate genes such as, Reln, Mdga1, Camk2b, and Cacna1c (30), but the exact pathway through which Auts2 controls the formation of excitatory synapses remains to be further explored.

The balance of excitation and inhibition (E/I balance) within neural circuits have been proposed as one of the causes of autistic behaviors (57, 58). Increased excitation in the mouse medial prefrontal cortex (mPFC) leads to great impairment in their social behaviors (59). In mice lacking contacting associated protein-like 2 (CNTNAP2), a gene strongly associated with autism, reduced excitation, or elevated inhibition in mPFC could rescue their deficits in social behaviors (60). Besides impaired social communication, ASD patients often manifest sensory abnormality (1), which may also be due to the imbalanced E/I in sensory cortices. The mice carried specific deletion of shank3 in parvalbumin interneurons of primary somatosensory cortex exhibited higher sensitivity in relevant behavior task (61). In mice lacking of Auts2, the social interactions reduced, and the sensitivity for startle response as well as nociceptive response increased (30). Whether re-expression of Auts2 in a circuit-specific manner could rescue the social deficit and hypersensitivity remained to be answered.

CYTOPLASMIC AUTS2 CAN DIRECTLY REGULATE CYTOSKELETON DURING NEURONAL MIGRATION AND NEURITEGENESIS

In addition to transcriptional regulation, Auts2 is present in cytoplasm and can regulate cytoskeleton through Rho family GTPases. It induces lamellipodia *via* activation of Rac1 and suppresses filopodia *via* down-regulations of Cdc42. Genetic ablation of Auts2 *in vivo* impairs both migration and axon elongation of cortical neurons (40). These observations revealed a novel function of Auts2 in neuronal migration and neurite outgrowth. The correct assembly of cortical structure is essential for the establishment of microcircuits, and the execution of sensory, motor, social, and cognitive functions (62, 63). During development, nuclear Auts2 can modify transcription according to genetic and epigenetic cues, whereas, cytoplasmic Auts2 join the fine-tuning of actin dynamics in neuronal processes. Thus, the multi-tasking of Auts2 in early development may attribute to the complexity of phenotypes upon Auts2 mutations.

AUTS2 IS EXPRESSED IN BRAIN REGIONS RELATED TO COGNITIVE FUNCTIONS

In developing mouse brain, Auts2 mRNA is highly expressed in the frontal cortex, olfactory bulb, hippocampus, dorsal thalamus, inferior colliculus, and substantia nigra. The expression of Auts2 mRNA in the brain was very strong by E16, then decrease to a low level after postnatal day 21 (34, 40). In cerebral cortex, the expression of Auts2 exhibits a strong rostral-caudal gradient, with a much higher expression in the frontal areas, suggesting its role in assigning regional identity of the cortex (33, 34). In hippocampus, Auts2 is expressed in the denta gyrus, CA1, and CA3 from E14 onwards, and the expression is located to granule cell layer and subgranular zone by P21, indicating a potential neurogenesis role (34). In cerebellum, the expression of Auts2 was in granule neurons, precursor of Purkinje cells, and deep nuclei at early developmental stage. By P21, it is expressed in Purkinje cells only. On E14, Auts2 was expressed in dorsal thalamus. By P21, the thalamic expression is restricted in the anterior thalamic nuclei (ATN), and in ventrolateral/ventromedial nuclei (VL/VM) (34). In human fetal brain, Auts2 was found in frontal, parietal and temporal lobes of the neocortex, as well as the ganglionic eminence, caudate nucleus, putamen nuclei, and cerebellum (41, 64).

From circuit level, the frontal cortex, hippocampus, and cerebellum have all been implicated in the impaired cognitive function of ASD (65–67). ATN and VL/VM thalamic nuclei have been found to form dense reciprocal connections with the frontal part of the cortex, particularly with the prefrontal cortex, prelimbic cortex, and anterior cingulate cortex (68, 69). Cerebellar nuclei can forward information to mPFC through VM, as well as to striatum through intralaminar thalamic nuclei (67, 70). These pathways provide rich regulations onto the brain areas that play central role in executing cognitive and social behaviors (67, 71, 72). For example, the deep layer mPFC neurons which project to the subcortical areas were recruited during social exploration. Altering the activity of these neurons could modulate the social performance in ASD mouse models (73). Moreover, activation of the Purkinje cells in crus1 could alleviate the social deficit and repetitive behaviors through modulating a multi-synaptic circuit targeted to mPFC (70). Auts2 mutant mice showed decreased social interaction as well as vocal communication (30, 35) [but see (52)]. How exactly Auts2 could implement its function upon neural circuits in these regions are still unknown. A detailed mapping of Auts2 function in a circuit-specific manner would greatly increase our knowledge for the behavioral abnormalities of neurodevelopmental disorders, and maybe lead to a novel treatment strategy.

CONCLUSIONS AND PERSPECTIVE

One of the main challenges in the field of neurodevelopmental disorders is to precisely link the genetic cause with the pathophysiological changes of the nervous system. To understand the function of each gene that associated with neurodevelopmental disorders is a unique entry point to elucidate the progress of the diseases. Patients with Auts2 mutation or variations showed a broad range of symptoms, including ID and ASD, consistent with the heterogeneity of neurodevelopmental disorders. Auts2 is a key-regulator of the transcriptional network during brain development. It can control the expression of transcription factors, cell adhesion, and axon guidance molecules, as well as proteins important for synaptic functions. Moreover, through binding Polycomb proteins and forming functional complex, Auts2 participates in the epigenetic modulations, which may mediate the effect of environmental or maternal factors on brain development. In addition, Auts2 regulates the neuronal migration and neuritegenesis by cytoskeleton remodeling. Furthermore, Auts2 controls the number of excitatory synapses to achieve precise

E/I balance in the central nervous system (**Figure 1**). A few key questions remain to be answered. For example, how the temporal expression of different Auts2 isoforms is orchestrated remains unknown. The downstream molecular pathways through which Auts2 regulates the synapse formation need to be elucidated. How exactly Auts2 exerts its influence onto cognitive and social behaviors should be best addressed in a circuit-specific manner. Thus, a comprehensive understanding of Auts2 function will help us to search for treatment strategies of neurodevelopmental disorders.

AUTHOR CONTRIBUTIONS

LX and WX designed the study. LX, WX, and PW wrote the paper. XY, LLi, and LLiu provided insightful discussion for the manuscript. All authors contributed to the article and approved the submitted version.

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Differential Metabolites in Chinese Autistic Children: A Multi-Center Study Based on Urinary ^{1}H-NMR Metabolomics Analysis

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Background: Autism spectrum disorder (ASD) is a group of early-onset neurodevelopmental disorders. However, there is no valuable biomarker for the early diagnosis of ASD. Our large-scale and multi-center study aims to identify metabolic variations between ASD and healthy children and to investigate differential metabolites and associated pathogenic mechanisms.

Methods: One hundred and seventeen autistic children and 119 healthy children were recruited from research centers of 7 cities. Urine samples were assayed by ^{1}H-NMR metabolomics analysis to detect metabolic variations. Multivariate statistical analysis, including principal component analysis (PCA), and orthogonal projection to latent structure discriminant analysis (OPLS-DA), as well as univariate analysis were used to assess differential metabolites between the ASD and control groups. The differential metabolites were further analyzed by receiver operating characteristics (ROC) curve analysis and metabolic pathways analysis.

Results: Compared with the control group, the ASD group showed higher levels of glycine, guanidinoacetic acid, creatine, hydroxyphenylacetylglycine, phenylacetylglycine, and formate and lower levels of 3-aminoisobutanoic acid, alanine, taurine, creatinine, hypoxanthine, and N-methylnicotinamide. ROC curve showed relatively significant diagnostic values for hypoxanthine [area under the curve (AUC) = 0.657, 95% CI 0.588 to 0.726], creatinine (AUC = 0.639, 95% CI 0.569 to 0.709), creatine (AUC = 0.623, 95% CI 0.552 to 0.694), N-methylnicotinamide (AUC = 0.595, 95% CI 0.523 to 0.668), and guanidinoacetic acid (AUC = 0.574, 95% CI 0.501 to 0.647) in the ASD group.

Combining the metabolites creatine, creatinine and hypoxanthine, the AUC of the ROC curve reached 0.720 (95% CI 0.659 to 0.777). Significantly altered metabolite pathways associated with differential metabolites were glycine, serine and threonine metabolism, arginine and proline metabolism, and taurine and hypotaurine metabolism.

Conclusions: Urinary amino acid metabolites were significantly altered in children with ASD. Amino acid metabolic pathways might play important roles in the pathogenic mechanisms of ASD.

Keywords: autism spectrum disorder, ^{1}H-NMR analysis, metabolomics, urine, amino acid metabolite

INTRODUCTION

Autism spectrum disorder (ASD) is a group of early-onset neurodevelopmental disorders characterized by social communication difficulties, narrow interests, and repetitive stereotyped behaviors (1). In the United States, the prevalence of ASD has increased in recent years, ranging from 1.57% in 2009 to 1.85% in 2020 (2, 3). The prevalence of ASD among children aged 6–12 years is ∼0.70% in China (4). The large cost associated, mainly consisting of special education services and parental productivity loss, has caused a heavy burden to society and families (5). Despite the lack of effective drug treatments, several studies highlight the potential benefits of early diagnosis and parent-mediated interventions, which have to some extent improved children's social and communicative abilities (6, 7). The detection rate of genetic etiology of ASD is about 10–15% (8–10). However, early diagnosis remains a challenge for non-genetic ASD, which is mainly based on combining clinician observation with caregiver reports (11). Currently, there is an urgent need to find valuable biomarkers for the early diagnosis of ASD.

Some metabolomic studies have indicated the presence of elevated biomarkers in blood and urine samples from ASD patients, and these biomarkers include pyruvate, lactate, and mitochondrial-related enzymes (12–14). Major analytical techniques for metabolomics are nuclear magnetic resonance spectroscopy (NMR) and chromatography, including gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–mass spectrometry (LC-MS). The advantages of ^{1}H-NMR are that the sample preprocessing is simple and non-destructive and that the detection of metabolites is comprehensive (15, 16). In 2010, Yap et al. first used urinary ^{1}H-NMR analysis to detect potential biomarkers for ASD (17). Over the last few years, with urinary ^{1}H-NMR analysis, some discriminating metabolites have been identified between ASD and healthy people. Studies indicated that ASD group showed higher levels of hippurate, glycine, tryptophan and D-threitol and lower levels of glutamate, creatine, valine, betaine and 3-methylhistidine. Further analyses indicated possible pathogenic mechanisms involving gut microbial metabolism, oxidative stress conditions and amino acid metabolism (18, 19). Overall, ^{1}H-NMR analysis shows great potential for the identification of biochemical signatures and investigation of the disease mechanisms of ASD. However, previous ^{1}H-NMR analyses lack large-scale sample sizes to confirm the significance and connection between metabolites and ASD. We aimed to conduct a large-scale and multi-center study to identify metabolic variations between ASD and control groups through urinary ^{1}H-NMR metabolomics analysis and to investigate potential biological mechanisms related to differential metabolites.

Abbreviations: ASD, Autism spectrum disorder; PCA, Principal component analysis; OPLS-DA, Orthogonal projection to latent structure discriminant analysis; NMR, Nuclear magnetic resonance spectroscopy; DSM-5, Statistical manual of mental disorders 5; ADOS, Autism diagnostic observation schedule; ADI-R, Autism diagnostic interview-revised; SW, Spectral width; RD, Recycle delay; FID, Free induction decay; CV, Cross-validation; CV-ANOA, Variance analysis of the cross-validated residuals; ROC, Receiver operating characteristics; AUC, Area under the curve; FNR, False-negative rate; FPR, False-positive rate; PPV, Positive predictive value; NPV, Negative predictive value; LR+, Likelihood ratio positive; LR–, Likelihood ratio negative; MSEA, Metabolite sets enrichment analysis.

METHODS

Participants

The study was conducted from January 2014 to December 2016. All the participants were recruited from research centers of 7 cities (Shanghai, Guangzhou, Changsha, Chongqing, Chengdu, Wenzhou, and Beijing). Participants were drawn from ASD and control group.

Autistic children from both hospitals and local autism rehabilitation of each research center were enrolled in the ASD group. The inclusion criteria for ASD group were: (a) children aged 2–18 years; (b) no limitation on the gender; (c) the diagnosis of ASD was based on the Diagnostic and Statistical Manual of Mental Disorders 5 (DSM-5) criteria (1) and confirmed with the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview-Revised (ADI-R) criteria by trained clinical psychiatrists from each research center; (d) urine sample was available. Exclusion criteria for ASD group were: (a) symptomatic autism (such as Rett syndrome and fragile X syndrome); (b) other mental illness (such as attention-deficit hyperactivity disorder); (c) other neurological disorders (such as epilepsy and central nervous system infections); (d) inherited metabolic diseases; (e) history of brain injury; (f) taking non-essential drug or dietary supplement before (72 h) and during sample collection.

Healthy children from the health examination center of each research center were enrolled in the control group. The

inclusion criteria for control group were: (a) children with no abnormality in health examination and typical development; (b) age- and sex-matching with ASD group; (c) urine sample was available. Exclusion criteria for control group were: (a) siblings of ASD group; (b) clinical evidence of ASD diagnosis; (c) mental illness (such as attention-deficit hyperactivity disorder); (d) neurological disorders (such as epilepsy and central nervous system infections); (e) inherited metabolic diseases; (f) history of brain injury; (g) taking non-essential drug or dietary supplement before (72 h) and during sample collection.

After all eligible participants and their parents provided informed consent, they were invited to participate in the study. The study was approved by the institutional ethics committee at the Children's Hospital of Fudan University.

Sample Collection

First morning urine specimens were collected from all participants during the research period. Samples were collected in 15 mL urine collection tubes without preservative. Each sample was centrifuged and aliquoted into 1.5 mL EP tubes. Afterwards, samples were numbered ("1" represents the ASD group, and "2" represents the control group) and stored at −80°C immediately until ^{1}H-NMR analysis.

^{1}H-NMR Spectroscopy Experiments and Data Processing

Sample Preparation

A 500 μL urine sample was added to a 1.5 mL EP tube prefilled with 14 μL KF (5 M) solution. After vortexing, the sample was allowed to rest for 10 min, followed by centrifugation (12,000 rpm, 4°C) for 10 min. A total of 450 μL liquid supernatant was added to an NMR tube preloaded with 10 μL EDTA-d12 (0.1 M). The NMR tube cap was covered and mixed by hand. Finally, 45 μL Na^+/K^+ buffer (1.5 M, pH = 7.40) was added to the NMR tube, which was mixed by hand and then placed in the NMR spectrometer for data collection. The sample preparation process for ^{1}H-NMR analysis is shown in **Supplementary Figure 1**.

^{1}H-NMR Spectroscopy Experiments

All ^{1}H-NMR spectroscopy experiments were performed at 298 K using a Bruker AVIII 600 MHz NMR spectrometer (Bruker BioSpin, Germany) with a proton resonance frequency of 600.13 MHz.

The NOESYGPPR1D pulse sequence (RD-90°-t1-90°-tm-90°-ACQ) was used to collect the spectra. The parameters were set as follows: spectral width (SW) was 20 ppm, recycle delay (RD) was 2 s, mixing time (t_m) was 80 ms, t_1 was 4 μs, 90° pulse length was 14.8 s, data time was 1.36 s, data points were 32 K, and free induction decay (FID) accumulation was 64 times.

Data Processing

Spectra were processed using MestReNova software (MestReNova 8.1, Spain). The ^{1}H-NMR FID signals were multiplied by an exponential function equivalent to a line broadening of 1 Hz before performing an automatic Fourier transformation. The phase distortion and baseline of each spectrum were manually adjusted. The internal standard trimethylsilyl propanoic acid (TSP, δ = 0 ppm) was used as the baseline to calibrate the chemical shifts. The concentration of TSP was 0.261 mmol/L, and the spectrum of TSP represented 12 ^{1}H. The regions of the ^{1}H-NMR spectra (δ 0.3–9.5 ppm) were divided into consecutive integrated spectral regions of equal width (δ 0.004 ppm). The spectral region of the water (δ 4.71–5.055 ppm) and urea (δ 5.6–6.12 ppm) peaks were removed from each spectrum to minimize variations caused by the presaturation of the residual water and urea resonances. Mnova software was used to correct the spectra with obvious chemical shifts after the integration. Metabolites were assigned by referencing the values for chemical shifts in J-resolved (JRES), COZY, TOCSY, HSQC, and HMBC spectra and literature reports. A series of 2D NMR spectra were acquired for selected samples.

To eliminate the instrument differences of sensitivity and stability and to reduce the analysis errors caused by the concentration differences of the samples, two normalization methods were performed. Creatinine normalization: the ^{1}H-NMR spectra were normalized by using the creatinine methylene resonance (δ = 4.05 ppm) as a reference. Total area normalization (20): the integrated area in each bucket was normalized by the total sum of peak intensities to eliminate the effects of variable concentration among different samples.

Data and Statistical Analysis

Clinical Characteristics of Participants

Difference in age between the ASD and control groups was evaluated by Student's *t*-test when the distribution was normal or the Mann-Whitney U test when it was skewed. Difference in sex was investigated using chi-square test. Statistical analyses were performed by using the SPSS statistical package program (version 20, SPSS Inc., Chicago, IL, USA), and $P < 0.05$ was considered statistically significant.

Multivariate Analysis

The normalized data were imported into the SIMCA-P+ software package (version 13.0, Umetrics, Sweden) for multivariate statistical analysis, including principal component analysis (PCA) and orthogonal projection to latent structure discriminant analysis (OPLS-DA) (21–24). PCA was first used to observe the overall distribution among samples and the stability of the whole analysis process. Abnormal data, which related to sample contamination or improper sampling, were removed according to the overall aggregation trend in all the samples. After which OPLS-DA was used to distinguish the overall difference in the metabolic profile and to find differential metabolites between the groups.

To prevent model overfitting, an internal validation method was used to verify the validity of the model. The OPLS-DA model was validated by a 7-fold cross-validation (CV) (25). R^2 and Q^2 were two parameters to assess the quality of the model. OPLS-DA was further validated by variance analysis of the cross-validated residuals (CV-ANOVA) (26), and the model was considered valid at $P < 0.05$.

After multiplying the loading value of each variable with its standard deviation, backtracking conversion of the data was performed. Then, the data were assessed by multivariate analysis and imported into mapping software based on MATLAB (version 7.1, USA) to plot the loading diagram of the correlation

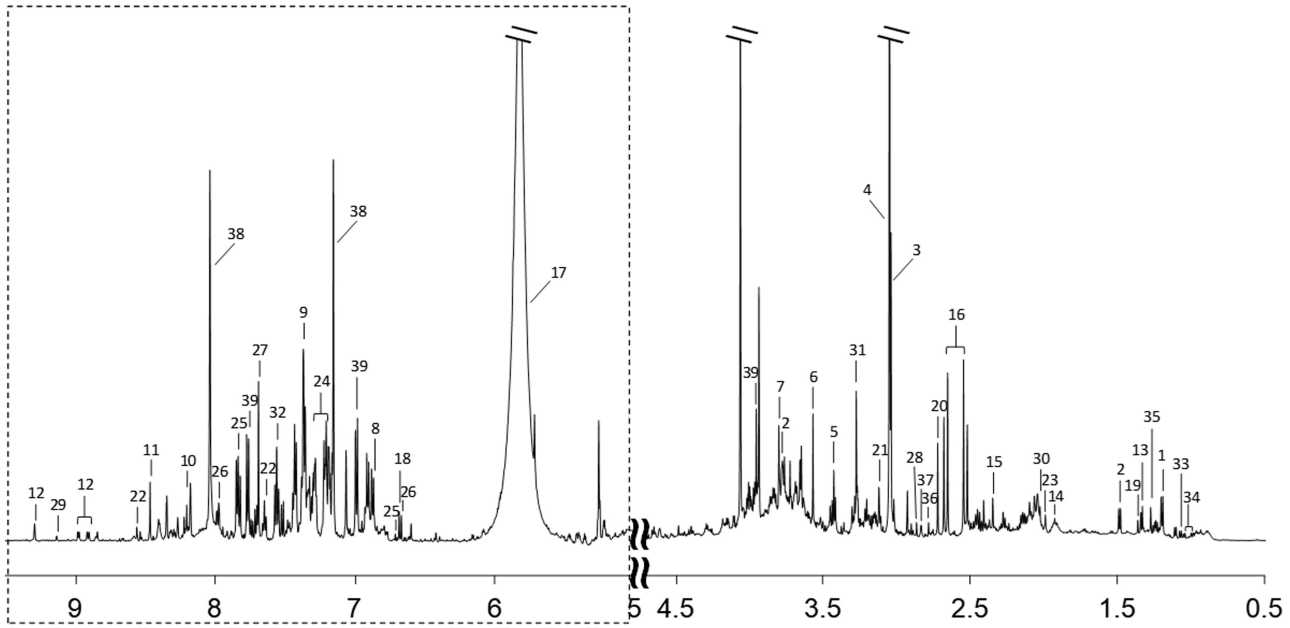

FIGURE 1 | A typical 600 MHz ^{1}H-NMR spectrum of a urine sample. Region of dashed box is vertically magnified 8 times. Metabolites corresponding to each number are listed in **Supplementary Table 1**.

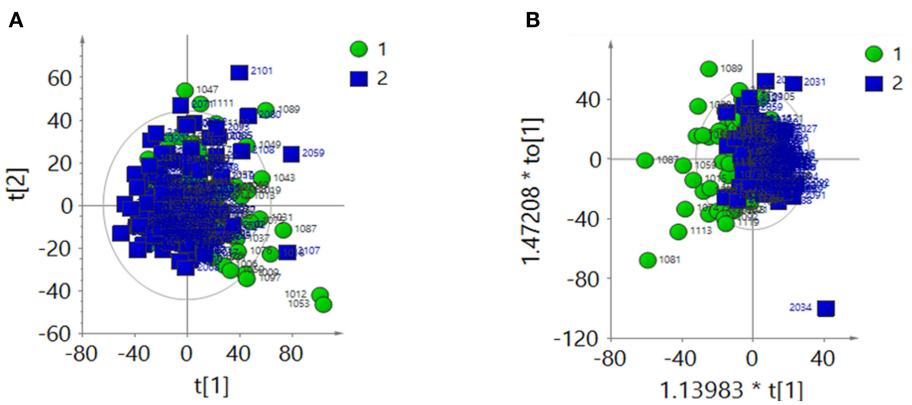

FIGURE 2 | After the creatinine normalization, multivariate analysis results of ^{1}H-NMR spectra of urine samples from the ASD group (green dots, 1) and control group (blue squares, 2). **(A)** PCA score plot: $R^2X = 0\ 403$, $Q^2 = 0.135$. **(B)** OPLS-DA score plot: $R^2X = 0.257$, $Q^2 = 0.0138$, CV-ANOVA $P = 0.523$.

coefficient. The Pearson correlation coefficient represented the linear correlation between the variable and the first principal component score value of the OPLS-DA model and was used to determine whether the variation contributed significantly to the intergroup differentiation. The significance was evaluated by the threshold value of the absolute correlation coefficient, which was determined according to the confidence interval of the sample size. If the absolute value of the Pearson correlation coefficient of the variable was higher than the threshold value ($P < 0.05$), the content of the variable was considered significantly different between groups.

Univariate Analysis

Differential metabolites between the two groups were selected for univariate analysis. Data from the total area normalized peak area are expressed as the mean ± SD. An independent sample *t*-test was used for comparing the two groups when the distribution was normal. The non-normal distribution data were evaluated by Mann-Whitney U test. $P < 0.05$ was considered statistically significant. The false discovery rate (FDR) was used to correct multiple hypothesis testing. FDR $P < 0.1$ was considered statistically significant. We applied correlation analysis to detect the relationship between differential metabolite levels and age.

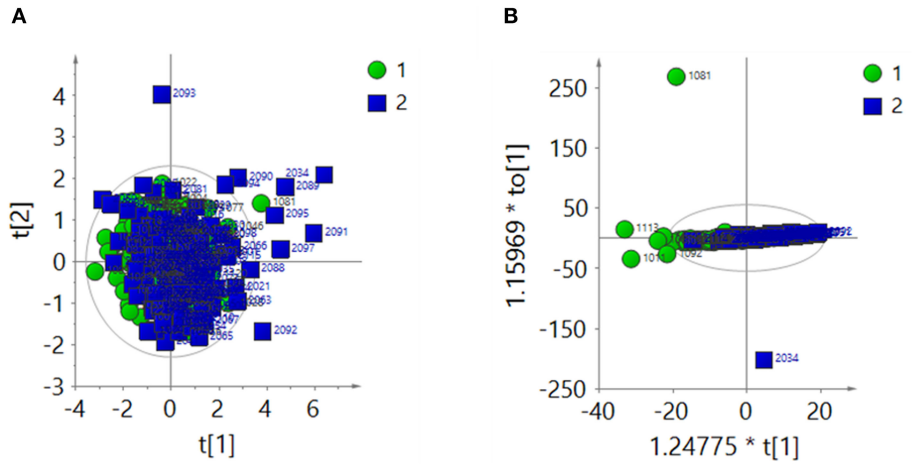

FIGURE 3 | After the total area normalization, multivariate analysis results of ^{1}H-NMR spectra of urine samples from the ASD group (green dots, 1) and control group (blue squares, 2). **(A)** PCA score plot: $R^2X = 0.366$, $Q^2 = 0.0656$. **(B)** OPLS-DA score plot: $R^2X = 0.274$, $Q^2 = 0.0565$, CV-ANOVA $P = 0.009$.

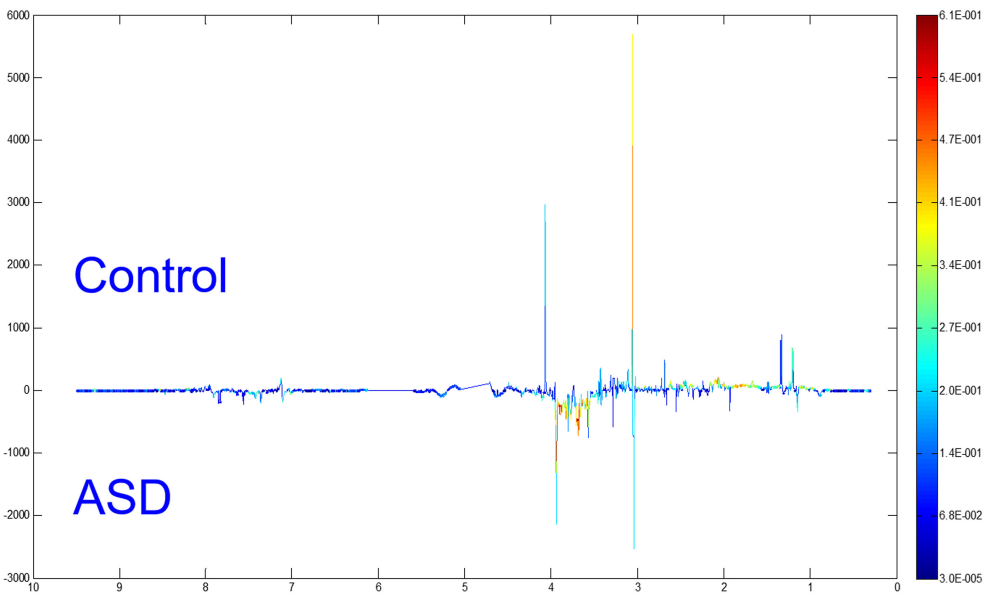

FIGURE 4 | OPLS-DA model: a polychromatic correlation coefficient loading plot.

Receiver Operating Characteristics (ROC) Curve Analysis

The sensitivity and specificity of metabolites with significant differences between the two groups in the diagnosis of ASD were evaluated using a ROC curve analysis. ROC curves were generated by using MedCalc statistical software (version 19.1.7, Belgium). The area under the curve (AUC) was used to measure the overall degree of identification power. An AUC > 0.7 was considered acceptable. Optimal cut-off points were determined by maximizing the Youden's J index (J = sensitivity + specificity – 1). The sensitivity, specificity, false-negative rate (FNR), false-positive rate (FPR), positive predictive value (PPV), negative predictive value (NPV), likelihood ratio positive (LR+), and likelihood ratio negative (LR–) were calculated to compare the diagnostic accuracy of the metabolites. Further analysis was performed after each group was stratified by sex and age. Logistic regression was used to analyse the combined metabolites.

Metabolic Pathways and Network Analysis

MetaboAnalyst (version 4.0, https://www.metaboanalyst.ca/) was used for pathway and network analyses (27). All differential metabolites were imported into the pathway analysis module to obtain matched pathways according to the *P*-values from the pathway enrichment analysis and pathway impact values from the pathway topology analysis. Significantly affected metabolic pathways associated with differential metabolites were identified

by metabolite set enrichment analysis (MSEA). A metabolite-gene-disease interaction network was established to detect the connections of differential metabolites and associated pathways.

RESULTS

Characteristics of the ASD and Control Groups

A total of 117 children with ASD were enrolled in the ASD group, and 119 healthy children were enrolled in the control group. The mean ages of the ASD and control groups were 10.12 ± 2.60 and 9.90 ± 1.73, respectively, with no significant difference between them ($P = 0.468$). The male to female ratios were 4.82:1 in the ASD group and 3.58:1 in the control group, with no significant difference between them ($P = 0.388$).

^{1}H-NMR Spectrum of Urine Samples

^{1}H-NMR spectra of urine samples from all participants were collected. A typical ^{1}H-NMR spectrum is shown in **Figure 1**. The keys for metabolites in **Figure 1** are given in **Supplementary Table 1**. A total of 39 metabolites were identified in the ^{1}H NMR spectra of urine samples.

Multivariate Analysis of ^{1}H-NMR Spectra of Urine Samples

Creatinine Normalization Analysis

After creatinine normalization, PCA was performed on ^{1}H-NMR spectra of urine samples. Discriminant variables obtained from the PCA score plot (**Figure 2A**) were $R^2X = 0.403$ and $Q^2 = 0.135$. The OPLS-DA score plot (**Figure 2B**) showed $R^2X = 0.257$ and $Q^2 = 0.0138$. CV-ANOVA of the OPLS-DA model indicated that the model was not valid ($P = 0.523$).

TABLE 1 | Pearson's correlation coefficient of discriminant metabolites.

Metabolites	Pearson's correlation coefficient*
3-Aminoisobutanoic acid	0.208
Alanine	0.220
Taurine	0.242
Glycine	−0.237
Guanidinoacetic acid	−0.424
Creatine	−0.278
Creatinine	0.268
Hydroxyphenylacetylglycine	−0.190
Phenylacetylglycine	−0.233
Hypoxanthine	0.370
Formate	−0.248
N-methylnicotinamide	0.348
Unknown	−0.296

**The negative value indicates that the metabolite content in the urine samples of the control group is significantly lower than that of the ASD group. Conversely, the positive value indicates that the metabolite of the control group is significantly higher than that of the ASD group.*

Total Area Normalization Analysis

The ^{1}H-NMR spectra of the two groups were analyzed by PCA after total area normalization. The parameters of the PCA score plot (**Figure 3A**) were $R^2X = 0.366$ and $Q^2 = 0.0656$. The OPLS-DA score plot (**Figure 3B**) showed $R^2X = 0.274$, $Q^2 = 0.0565$ and CV-ANOVA $P = 0.009$. These results suggested that the model was valid.

Differential Metabolites Between the ASD and Control Groups

To distinguish the metabolic differences between the two groups, a polychromatic correlation coefficient loading plot (**Figure 4**) was drawn. The color of the polychromatic loading plot was encoded by the absolute value of the correlation coefficient. The warmer the color is, the higher the absolute value of the correlation coefficient and the greater the contribution to the intergroup differentiation. The threshold of the absolute value of the Pearson's correlation coefficient was determined to be 0.182. The variables corresponding to the correlation coefficient with an absolute value > 0.182 contributed significantly to the intergroup differentiation ($P < 0.05$). Differential metabolites identified by the Pearson's correlation coefficient were showed in **Table 1**. Compared with the control group, the ASD group showed higher levels of glycine, guanidinoacetic acid, creatine, hydroxyphenylacetylglycine, phenylacetylglycine and formate and lower levels of 3-aminoisobutanoic acid, alanine, taurine, creatinine, hypoxanthine and N-methylnicotinamide.

The fold changes of differential metabolites are summarized in **Figure 5**. A total of 6 metabolites, including 3-aminoisobutanoic acid ($P = 0.0425$), creatine ($P = 0.0009$), creatinine ($P < 0.0001$), hypoxanthine ($P < 0.0001$), formate ($P = 0.0267$), and N-methylnicotinamide ($P = 0.0149$), showed significant differences in the normalized peak areas between the two groups. The differences of above 6 metabolites remained significant after correction for multiple hypothesis testing (FDR $P < 0.1$)

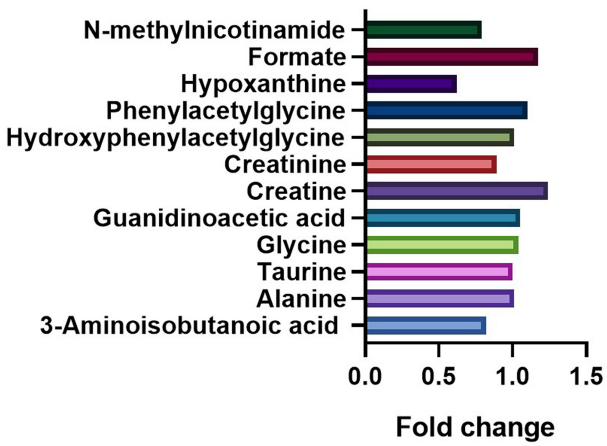

FIGURE 5 | Comparation of the fold changes of the differential metabolites between the ASD and control groups. The fold change represents the ratio of average peak area between ASD group and control group.

(**Table 2**). Correlation analysis showed that the level of creatinine was positively correlated with age in the ASD group (r = 0.215, 95% CI 0.013 to 0.401, $P = 0.0370$). In the control group, the level of creatinine was also positively correlated with age (r = 0.215, 95% CI 0.036 to 0.380, $P = 0.0190$), and creatine was negatively correlated with age (r = −0.277, 95% CI −0.435 to −0.102, $P = 0.0023$).

Differential Metabolites and Potential Biological Mechanisms

The Sensitivity and Specificity of Metabolites in the Diagnosis of ASD

The diagnostic accuracies of differential metabolites in the two groups were evaluated by ROC curve analysis (**Table 3**, **Supplementary Figure 2**). The ROC curve showed relatively significant diagnostic values of hypoxanthine (AUC = 0.657, 95% CI 0.588 to 0.726), creatinine (AUC = 0.639, 95% CI 0.569 to 0.709), creatine (AUC = 0.623, 95% CI 0.552 to 0.694), N-methylnicotinamide (AUC = 0.595, 95% CI 0.523 to 0.668) and guanidinoacetic acid (AUC = 0.574, 95% CI 0.501 to 0.647) for ASD (**Supplementary Figure 3**). The AUC of ROC analysis of the creatine/creatinine ratio was 0.6480 (95% CI 0.579 to 0.718). For each metabolite, there was no significant difference in AUCs between males and females (**Supplementary Table 2**). Compared with age stratification of 7–9 years old, the metabolites guanidinoacetic acid and creatine showed significantly higher diagnostic accuracy for ASD in the age stratification of 13–15 years old (AUC of guanidinoacetic acid = 0.802, 95% CI 0.566 to 0.944, $P = 0.0282$; AUC of creatine = 0.823, 95% CI 0.589 to 0.955, $P = 0.0344$. Results shown in **Supplementary Table 3**). By combining the metabolites of creatine, creatinine and hypoxanthine, the AUC of the ROC curve reached 0.720 (95% CI 0.659 to 0.777), with a sensitivity of 80.34% and specificity of 52.94%.

TABLE 2 | Peak areas of differential metabolites after the total area normalization.

Metabolites	ASD group		Control group		*P*-value*	FDR *P*-value*	Fold change
	Mean	SD	Mean	SD			
3-Aminoisobutanoic acid	0.77	0.61	0.94	0.68	**0.0425**	**0.0921**	0.82
Alanine	0.52	0.16	0.51	0.17	0.8440	0.9975	1.01
Taurine	0.63	0.21	0.62	0.17	0.9162	0.9162	1.00
Glycine	1.24	0.39	1.19	0.44	0.3430	0.4459	1.04
Guanidinoacetic acid	1.42	0.32	1.36	0.36	0.1446	0.2350	1.05
Creatine	1.78	0.86	1.43	0.72	**0.0009**	**0.0039**	1.24
Creatinine	4.68	0.90	5.26	1.21	**<0.0001**	**0.0012**	0.89
Hydroxyphenylacetylglycine	0.15	0.06	0.15	0.06	0.8860	0.9598	1.01
Phenylacetylglycine	0.48	0.20	0.44	0.21	0.1097	0.2037	1.10
Hypoxanthine	0.02	0.01	0.04	0.03	**<0.0001**	**0.0007**	0.62
Formate	0.06	0.04	0.05	0.02	**0.0267**	**0.0694**	1.17
N-methylnicotinamide	0.01	0.01	0.01	0.01	**0.0149**	**0.0484**	0.79
Unknown	0.26	0.34	0.22	0.18	0.3109	0.4491	1.16

**Bold value indicate that the difference was considered statistically significant (P < 0.05, FDR P < 0.1).*

TABLE 3 | Diagnostic accuracies of differential metabolites between the ASD and control groups.

Metabolites	AUC	Cut-off	Sensitivity (%)	Specificity (%)	FNR (%)	FPR (%)	PPV (%)	NPV (%)	LR+	LR–
3-Aminoisobutanoic acid	0.568	0.5890	67.52	50.42	32.48	49.58	57.2	61.2	1.36	0.64
Alanine	0.508	0.4190	73.50	31.93	26.50	68.07	51.5	55.1	1.08	0.83
Taurine	0.502	0.5683	44.44	61.34	55.56	38.66	53.1	52.9	1.15	0.91
Glycine	0.560	1.2698	41.88	70.59	58.12	29.41	58.3	55.3	1.42	0.82
Guanidinoacetic acid	0.574	1.2573	76.07	40.34	23.93	59.66	55.6	63.2	1.27	0.59
Creatine	0.623	1.8805	44.44	77.31	55.56	22.69	65.8	58.6	1.96	0.72
Creatinine	0.639	4.6505	55.56	69.75	44.44	30.25	64.4	61.5	1.84	0.64
Hydroxyphenylacetylglycine	0.504	0.1710	26.50	78.99	73.50	21.01	55.4	52.2	1.26	0.93
Phenylacetylglycine	0.571	0.2742	87.18	26.89	12.82	73.11	54.0	68.1	1.19	0.48
Hypoxanthine	0.657	0.0359	88.03	36.13	11.97	63.87	57.5	75.4	1.38	0.33
Formate	0.529	0.0768	19.66	91.60	80.34	8.40	69.7	53.7	2.34	0.88
N-methylnicotinamide	0.595	0.0104	74.36	49.58	25.64	50.42	59.2	66.3	1.47	0.52

AUC, area under the curve; FNR, false-negative rate; FPR, false-positive rate; PPV, positive predictive value; NPV, negative predictive value; LR+, likelihood ratio positive; and LR–, likelihood ratio negative.

Correlated Metabolic Pathways and Networks

The main metabolic pathways associated with differential metabolites are shown in **Supplementary Figure 4**. According to the bubble plot of the metabolic pathway impact, there were significant metabolite changes in the glycine, serine and threonine metabolism, glyoxylate and dicarboxylate metabolism and taurine and hypotaurine metabolism pathways (**Figure 6A**). The plot of metabolite set enrichment analysis (MSEA) listed the significant enrichment pathways of differential metabolites (**Figure 6B**). Glycine, serine and threonine metabolism, primary bile acid biosynthesis and aminoacyl-tRNA biosynthesis were the pathways with the most significant enrichment. The metabolite-gene-disease interaction network provides a global view of the connection of the differential metabolites and the potential functional relationships among metabolites, connected genes, and target diseases (**Supplementary Figure 5**).

DISCUSSION

Complex etiologies and atypical symptoms pose very large challenges to the early diagnosis of ASD. Urinary ^{1}H-NMR analysis provides a fast and comprehensive assessment to detect potential biomarkers of ASD (28). Our study was based on the ^{1}H-NMR analysis of urine samples from ASD and control groups to identify candidate metabolites and associated pathogenic mechanisms. Compared with other studies of urinary ^{1}H-NMR metabolomics analysis of ASD, our study sample was large size, multi-center, and representative. Besides, our study used two normalization methods, creatinine normalization and total area normalization. The analysis using creatinine normalization showed no significant differences between the two groups, though the total area normalization did detect a difference. The variation of creatinine was confirmed by the total area normalization. Whiteley et al. also found that excretion of urinary creatinine in the group of pervasive developmental disorders, which included ASD, was significantly lower than controls (29). The abnormal creatinine metabolism might be caused by rigidity in food choice and various exclusion diets associated with ASD (30). Therefore, total area normalization was more suitable for our study.

The results obtained from the ^{1}H-NMR analysis revealed that the levels of glycine, guanidinoacetic acid, creatine, hydroxyphenylacetylglycine, phenylacetylglycine and formate were higher in the ASD group than those in the control group. Moreover, the levels of 3-aminoisobutanoic acid, alanine, taurine, creatinine, hypoxanthine and N-methylnicotinamide were lower in the ASD group than those in the control group. The normalized peak areas of 3-aminoisobutanoic acid, creatine, creatinine, hypoxanthine, formate and N-methylnicotinamide differed significantly between the two groups. The metabolite levels of glycine, taurine, creatine and creatinine were consistent with those from previous reports (19, 31). Our study is the first to detect variations in the metabolites of hydroxyphenylacetylglycine in urine samples from children with ASD. The results of ROC analysis indicated that creatine, creatinine and hypoxanthine have the potential to be biomarkers for the diagnosis of ASD. The creatine/creatinine ratio slightly improved the diagnostic accuracy. Recent research has reported significant female-related alterations of creatine and creatinine, and the creatinine/creatine ratio might be a good predictor of

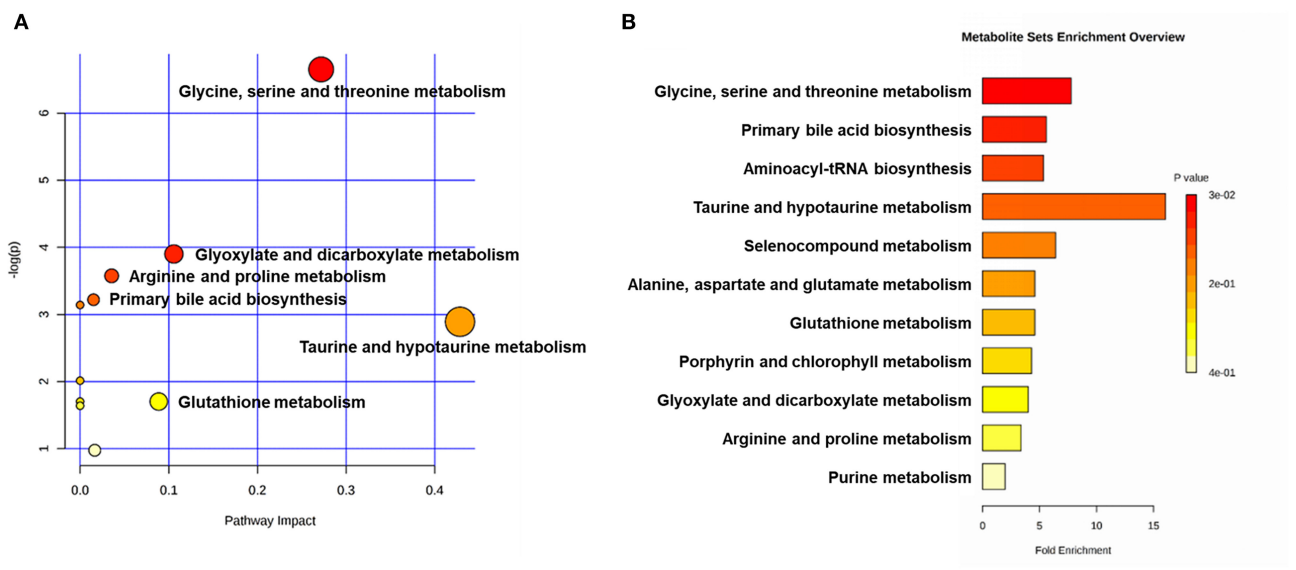

FIGURE 6 | Metabolic pathway analyses utilizing the MetaboAnalyst functional interpretation tools. **(A)** Bubble plot of metabolic pathway impact. The metabolic pathways are shown as bubbles. The X coordinate and size of the bubble represent the value of pathway impact in the topology analysis. The Y coordinate and color of the bubble represent the *P*-value of the enrichment analysis. The darker red color and larger size indicate a more significant metabolite change in the corresponding pathway. **(B)** Metabolite set enrichment analysis (MSEA) plot. Significantly enriched pathways are represented by bars. The color and length of the bar are based on the *P*-value and fold enrichment, respectively.

ASD in female subjects (32). We further found that combining the metabolites creatine, creatinine and hypoxanthine as a potential diagnostic indicator can largely improve the diagnostic accuracy for ASD.

The extension study of the metabolic pathway analysis demonstrated a possible imbalance of amino acid metabolism in ASD children. Differential metabolites between the ASD and control groups involved glycine, serine and threonine metabolism, arginine and proline metabolism, taurine and hypotaurine metabolism, and glutathione metabolism pathways (**Supplementary Figure 6**). In accordance with previous observations, amino acid metabolism disorder plays an important role in the pathogenesis mechanism of ASD (33–37). Creatine and creatinine, which show significant metabolism alterations in ASD, play an essential role in maintaining a high level of energy supply for the brain (38). Studies have indicated that creatine deficiency occurs in some ASD cases, and creatine may be a therapeutic target for ASD (38–41). Abnormalities in creatinine might closely linked to abnormalities in creatine. Creatine is biosynthesized from glycine and arginine with an intermediate metabolite of guanidinoacetic acid. Glycine acts as an excitatory neurotransmitter in the early developmental stage. As the nervous system matures, it transforms to the major inhibitory neurotransmitter. If the transformation does not occur, an abnormal level of glycine may result in neural disorders, including ASD (42–44). The taurine and hypotaurine metabolism pathway also differed significantly between the two groups. Oxidative stress imbalance is considered to be important components of the pathophysiology of ASD, and antioxidant therapy may improve the prognosis of ASD (45, 46). Park et al. reported that taurine, as an antioxidant and regulator of inflammation, might be a valid biomarker for ASD (47). Combined with vitamin D3, taurine showed benefits in the treatment of ASD (48, 49). All metabolic pathways interact with each other and constitute a complex network with related genes and diseases (**Supplementary Figure 5**).

Many studies have reported that ASD is associated with abnormal gut microbial metabolism (50–52). Gut microbiota metabolites, including phenylalanine, tyrosine, hippurate and tryptophan, have been reported to be factors in the development of ASD (53–56). In our study, phenylacetylglycine, a gut microbial co-metabolite, had a slight variation between the two groups. Phenylacetylglycine is the end product of the phenylalanine metabolism pathway (57). However, there are no studies on the relationship between phenylacetylglycine and the pathogenesis of ASD.

LIMITATIONS

There are some limitations in our study. At the cellular level, biochemical processes such as oxidative phosphorylation, redox reaction, and oxidative stress are regulated by circadian rhythms (58). About 50% of metabolites are thought to be rhythmic, involving metabolic pathways such as nucleotides, energy, oxidation, and carbohydrates (59). Studies indicated that disrupted circadian rhythm is closely related to ASD (60–62). Thus, in our study, inconsistent urine collection times may have affected the quality of metabolic analyses due to the restriction of research centers. A few small-scale sample studies have set healthy sibling group as control to remove the effects of confounding factors, such as heredity and environment (17, 19). However, our study lacks of healthy siblings as controls.

Though ^{1}H-NMR analysis is the regular NMR method, some studies have used 2D HSQC-NMR to improve urinary screening in ASD. Compared to ^{1}H-NMR analysis, ^{1}H-^{13}C HSQC-NMR analysis shows the advantage of improving the metabolite detection accuracy and the discrimination ability (18, 31). Moreover, it is necessary to compare metabolite levels that vary with ASD severity to better clarify the pathogenesis of ASD.

CONCLUSIONS

In our study, ^{1}H-NMR metabolomic analysis was used to investigate urinary metabolism patterns in the ASD and control groups. We revealed that urinary amino acid metabolites were significantly altered in children with ASD. A series of variations in amino acid metabolism pathways, including glycine, serine and threonine metabolism, arginine and proline metabolism, and taurine and hypotaurine metabolism might play important roles in the pathogenic mechanisms of ASD. Further studies of differential metabolites are needed to improve the understanding of ASD pathogenesis.

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 ethics committee at the Children's Hospital of Fudan University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YW conceived of the study. YM and HZ contributed to the analysis, synthesis and interpretation of the results, and wrote the manuscript. CL, LZ, and TW contributed to the sample collection. XZ, XL, LW, TL, XC, MM, YH, and EL contributed to the diagnosis of ASD and sample collection from each research center. YA, XX, WY, and YJ provided guidance for the study. All of the authors contributed to the preparation of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.624767/full#supplementary-material

Supplementary Figure 1 | Urine sample preparation process.

Supplementary Figure 2 | ROC curve of the metabolites. AUC, area under the ROC curve; SEN, sensitivity; SPE, specificity.

Supplementary Figure 3 | ROC curve of the metabolites with relatively significant diagnostic values.

Supplementary Figure 4 | Metabolic pathways associated with the differential metabolites.

Supplementary Figure 5 | Metabolite-gene-disease interaction network.

Supplementary Figure 6 | Differential metabolites associated with amino acid metabolism disorders.

Supplementary Table 1 | Main metabolite attributions in the ^{1}H-NMR spectra of urine samples.

Supplementary Table 2 | Sex stratification analysis of the AUC of differential metabolites.

Supplementary Table 3 | Age stratification analysis of the AUC of differential metabolites.

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Estimating the Prevalence and Genetic Risk Mechanisms of ARFID in a Large Autism Cohort

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This study is the first genetically-informed investigation of avoidant/restrictive food intake disorder (ARFID), an eating disorder that profoundly impacts quality of life for those affected. ARFID is highly comorbid with autism, and we provide the first estimate of its prevalence in a large and phenotypically diverse autism cohort (a subsample of the SPARK study, $N = 5{,}157$ probands). This estimate, 21% (at a balanced accuracy 80%), is at the upper end of previous estimates from studies based on clinical samples, suggesting under-diagnosis and potentially lack of awareness among caretakers and clinicians. Although some studies suggest a decrease of disordered eating symptoms by age 6, our estimates indicate that up to 17% (at a balanced accuracy 87%) of parents of autistic children are also at heightened risk for ARFID, suggesting a lifelong risk for disordered eating. We were also able to provide the first estimates of narrow-sense heritability (h^2) for ARFID risk, at 0.45. Genome-wide association revealed a single hit near *ZSWIM6*, a gene previously implicated in neurodevelopmental conditions. While, the current sample was not well-powered for GWAS, effect size and heritability estimates allowed us to project the sample sizes necessary to more robustly discover ARFID-linked loci via common variants. Further genetic analysis using polygenic risk scores (PRS) affirmed genetic links to autism as well as neuroticism and metabolic syndrome.

Keywords: autism, eating disorders, genetics, ARFID, heritability

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1. INTRODUCTION

Parents and caretakers of young children often report "picky eating" as a major concern. Food selectivity (eating from a small range of foods) and food neophobia (refusing to try new foods), are commonly seen in children under the age of 6 and are associated with socioeconomic factors (1) as well as individual factors like food preferences, appetite, and parental feeding strategies (2). Currently, there is no consensus among clinical and research settings on the definition of picky eating, and multiple terms for feeding challenges are used in the pediatric feeding literature (3). As a result, there are differences in assessment and estimates of prevalence, which can affect treatment recommendations. Prevalence studies show about half (46%) of the general population of young

children struggle with typical picky eating, as reported in a 2015 Dutch study (4), while roughly one in ten children are considered to have extreme picky eating: 13% in the Netherlands (4), 14% in the United States (5).

Although picky eating is common in the general population of young children, increased prevalence rates are seen in children with developmental disorders–particularly autism spectrum disorders (autism) (6). Specifically, a meta-analysis of prospective studies found that children with autism are 5 times more likely to have an eating concern than their typically developing (TD) peers (7), although clinically relevant negative eating behavior rates do begin to decrease by age 6 (8). The most common presentations of eating concerns in children with autism include eating from a very narrow range of foods, or only eating foods with a specific presentation or sensory characteristic (pickiness), and avoiding eating new foods (food neophobia) (6). Bandini and colleagues have specifically defined food selectivity in this population as defined by food refusal, a limited food repertoire, and high-frequency single food intake (having a diet overwhelmed by one particular food) (9). Although some feeding concerns are shown to wane with age (10), adolescents and adults with autism still report significantly higher eating/feeding concerns than their TD peers (11, 12).

Traditionally, the clinical relevance of restrictive meal patterns in the context of eating disorders has focused on the fear of fatness/drive for thinness, which excluded many patients without such concerns from diagnosis and treatment. In 2013, Avoidant Restrictive Food Intake Disorder (ARFID) was first included in the DSM-V as a feeding disorder, characterized by a persistent pattern of food avoidance, which leaves individuals unable to meet their nutritional needs. ARFID is distinct from anorexia, as individuals with ARFID typically do not fear weight gain (13), implicitly making it a broader, more inclusive diagnosis. ARFID is reported to be particularly comorbid with psychiatric disorders, including autism, ADHD, and anxiety disorders (13, 14). In adults with ARFID, the resulting eating behaviors may cause just as much (if not more) distress and impairment as eating disorders like anorexia and bulimia (15). Of children/adolescents either presenting for eating disorder evaluation or currently in treatment for eating/feeding behavior problems, 5–22% (depending on the study) meet diagnostic criteria for ARFID (16). Although no population-based prevalence estimates based on clinical assessment are available, a self-report questionnaire of primary school children in Switzerland estimated general prevalence of ARFID at 3.5% (14).

ARFID and autism have high comorbidity, with (16) finding that 13% of pediatric ARFID patients in their clinic had autism, despite the fact that *"…patients with longstanding feeding issues and autism are not typically admitted to our program…"*. Opportunistic estimates like this are critical to establish the over-representation of autism among individuals with ARFID, but they do not help provide an estimate of how many individuals with autism might be at risk for ARFID. Furthermore, relatively little remains known about how risk for each disorder might inform the other. For example, the primary drivers of ARFID are often categorized as: lack of interest in eating (appetite), avoidance due to sensory characteristics of food (pickiness), and anxiety over adverse consequences from eating like choking or vomiting (fear). Research on food selectivity in autism has typically focused on sensory sensitivities, but restricted and repetitive behaviors (RRBs) have also been found to strongly correlate with this phenomenon as well (17). While, the overlap of sensory sensitivities between autism and ARFID provides an obvious avenue for this comorbidity, how RRBs might increase risk for ARFID (or if they may make it more difficult to detect) remains understudied. For example, a narrative study found that because many parents believe eating problems are endemic in autism, they acquiesce to perceived "pickiness" (18), potentially failing to raise concerns until the effects of malnutrition are apparent. A clearer sense of how many individuals with autism are at risk for ARFID—as well as the particular behavioral patterns which may be indicative of this risk—may help clinicians and caregivers identify children with autism who should be referred for treatment.

Treatment of ARFID is complex, with a broad range of treatment options and settings (outpatient to intensive treatment programs), population (from neurotypical to primarily autism), and practitioner (psychologists, occupational therapists, speech and language pathologists, registered dietitian nutritionists). This heterogeneity can result in inconclusive diagnoses or inconsistent care plans (19). Treatment approaches also vary widely in part because there are very few randomized controlled trials with most clinical treatment based upon case reports/series or retrospective chart review (20). Common treatment approaches include: Cognitive Behavioral Therapy, Family Based Therapy, Responsive Feeding Therapy, Applied Behavioral Analysis, and Sequential Oral Sensory therapy. The lack of empirical treatments—coupled with the broad range of available options—can make it difficult for individuals and families to identify the most appropriate treatment. Therefore, further research at the intersection of AFRID and autism is warranted to better understand the root causes of feeding symptoms and to better inform treatment.

These prior studies beg several questions, which we set out to address in this investigation. First, what is the prevalence of ARFID in an autism sample and in their parents? Second, what is the eating profile of high risk ARFID individuals, and what core autism traits are most associated with ARFID risk? Lastly, what role does common genetic variation play in ARFID risk? This study was uniquely poised to address these questions due to the availability of genetic data and detailed phenotypic data on eating habits and problems in a large cohort of both individuals with autism ($N = 5{,}157$) and typically-developing parents ($N = 4{,}985$). The primary phenotypes utilized in assessing ARFID risk were the Nine-Item Avoidant/Restrictive Food Intake Disorder (ARFID) screen (NIAS) (21), as well as extensive questions on inflexible eating behaviors and sensory sensitivities, and familial history of ARFID and other eating disorders. This data, combined with genetic data and surveys related to core autism symptoms, allowed us to identify individuals at high-risk for ARFID and profile the associations of ARFID in the broader context of general eating habits, core autism traits, and common genetic variation.

TABLE 1 | Factor loadings on the NIAS.

Question	Picky factor		Appetite factor		Fear factor	
	Proband	Parent	Proband	Parent	Proband	Parent
I am a picky eater	**0.83**	**0.83**	0.17	0.18	0.09	0.10
I dislike most of the foods that other people eat	**0.81**	**0.85**	0.19	0.18	0.11	0.10
The list of foods that I like and will eat is shorter than the list of foods I won't eat	**0.83**	**0.74**	0.15	0.21	0.07	0.14
I am not very interested in eating; I seem to have a smaller appetite than other people	0.21	0.24	**0.81**	**0.80**	0.18	0.19
I have to push myself to eat regular meals throughout the day, or to eat a large enough amount of food at meals	0.21	0.22	**0.82**	**0.79**	0.18	0.21
Even when I am eating a food I really like, it is hard for me to eat a large enough volume at meals	0.13	0.16	**0.76**	**0.74**	0.27	0.30
I restrict myself to certain foods because I am afraid that other foods will cause GI discomfort, choking, or vomiting	0.12	0.12	0.12	0.13	**0.84**	**0.81**
I eat small portions because I am afraid of GI discomfort, choking, or vomiting	0.07	0.10	0.25	0.30	**0.85**	**0.80**
I avoid or put off eating because I am afraid of GI discomfort, choking, or vomiting	0.08	0.14	0.26	0.25	**0.84**	**0.83**

Bold values indicate the primary loadings of each question as previously identified by Zickgraf and Ellis (21).

2. RESULTS

2.1. Factor Analysis of NIAS

Zickgraf and Ellis (21) identified three latent factors within responses to the NIAS in a typically developing population: picky eating, low appetite and fear. Within the autism-enriched sample of SPARK, we identified the same three factors in both probands and parents. The loadings of these factors were very similar between probands and parents (see **Table 1**).

2.2. Identification of Individuals at High Risk of ARFID

Because ARFID is believed to be under-diagnosed, we sought to identify individuals at high-risk for ARFID using the three NIAS factors—as well as all additional survey questions—using a logistic regression model to predict the small number of individuals who indicated they or their dependent had an ARFID diagnosis (53 probands and 35 parents). The fitted values from the models including the NIAS and survey questions (referred to hereafter as "ARFID Score") performed better than a naïve predictor using only age and sex, as well as the NIAS questions alone (**Supplementary Figure 1**), with a balanced accuracy of 0.87 for parents and 0.80 for probands. To classify undiagnosed individuals as "high-risk" for ARFID (ARFID Risk Group), we set a cutoff on the ARFID Score corresponding to the point on the ROC curve which was closest to a perfect predictor (0,1) (indicated by the dotted line in **Supplementary Figure 1A**). Under this heuristic, 17% of parents and 21% of probands were predicted as being at high-risk for ARFID.

2.3. Profile of Individuals at High Risk of ARFID

The survey responses from parents and probands at high risk for ARFID exhibited distinct profiles, with some notable similarities, as seen in **Figure 1**, and described below. Unless otherwise stated, all reported associations had FDR $p < 0.05$.

2.3.1. Nine-Item ARFID Screen (NIAS)

Although not directly included in either sub-cohort's ARFID risk model, the individual questions of the NIAS served as the foundation of both models. In both probands and parents, all nine items were significantly ($p < 0.05$, see **Figure 1**) associated with an endorsed ARFID diagnosis, though the questions underlying the "fear" factor was the least associated compared to the "picky" and "appetite" factors. In probands, three questions underlying the Picky Factor were the most enriched in high-risk individuals (Wilcox test location shift 95% CI range: 0.90–1.49 SD), while the questions underlying the Appetite Factor generally ranked higher in parents (Wilcox test location shift 95% CI range: 0.28–0.91 SD).

2.3.2. Inflexible Eating Behaviors (INFLEX)

Anxiety over eating ("The idea of eating a food [parent/proband] does not like fills [her/him/me] with anxiety."), most distinguished parents and probands identified as high risk from those at low risk for ARFID, (Wilcox test location shift 95% CI range: 0.70–0.77 SD for parents, 1.66–1.73 SD for probands). In fact, this item was included in both ARFID Score models (indicated by an asterisk in **Figure 1**). Inflexible behaviors were generally more enriched in high-risk probands compared to parents. This difference is particularly stark when it comes to presentation of food ("If a food that [parent/proband] usually likes is not presented in a certain way, [she/he/I] prefer(s) not to eat it.").

2.3.3. Sensory Sensitivities (SENS)

Sensory sensitives were found to be more broadly enriched in parents at high risk for ARFID than probands. In probands, sensitivity to taste was most pronounced in high-risk individuals (Wilcox test location shift 95% CI range: 0.64–0.88 SD). The opposite trend was found in parents, although the difference in enrichment was smaller than that observed in children.

2.3.4. Gastrointestinal Issues (GIH)

Food sensitivities and problems with bowel movements were weakly enriched in high-risk probands (Wilcox test location shift

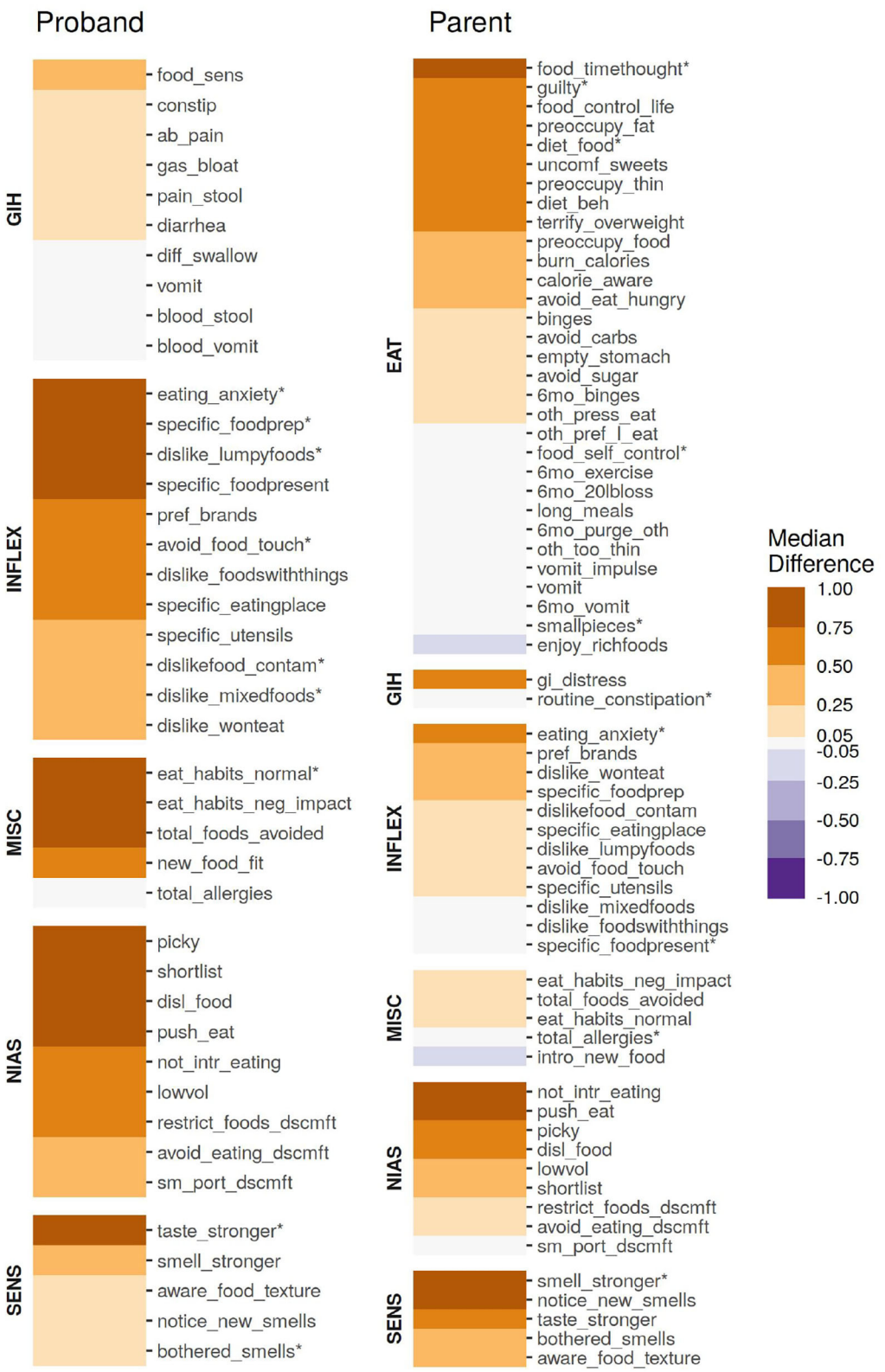

FIGURE 1 | The association of individual age-corrected and scaled items from the parent or proband surveys, quantified as the median difference (location shift) from a Wilcox rank-sum test. All items were scaled to have a unit variance of 1 prior to quantification. Items marked with an asterisk (*) were included in the ARFID Score model.

95% CI range: 0.09–0.49 SD). Meanwhile, issues with choking and vomiting were some of the weakest enrichment in high risk probands (Wilcox test location shift 95% CI range: 0.03–0.05 SD), which may be reflective of the slightly weaker enrichment of the items underlying the Fear Factor in this sub-cohort. Despite receiving a far less granular set of questions related to GI issues, general GI distress showed substantial enrichment in high-risk parents (Wilcox test location shift 95% CI range: 0.47–0.61 SD).

2.3.5. Supplementary Questions (MISC)

Unsurprisingly, both high-risk parents and probands were more likely to have "abnormal" eating habits which negatively impacted day-to-day life—although only ("How much of a departure from the 'norm' do you consider [proband/parent's] eating habits to be?") was informative enough to be included in the proband ARFID risk model.

2.4. Association of NIAS Factors and ARFID Risk With Core Autism Symptoms

The NIAS factors and ARFID score were found to be strongly associated with several measures of core autism symptomology (RBS-R, SCQ, and DCDQ) as well as adaptive behaviors (VABS). Unless otherwise stated, all associations reported below were FDR $p < 0.05$. Compared to the other NIAS factors, the Appetite Factor was found to have notably fewer—and weaker—associations (**Figure 2**).

Of the assessments covering core autism symptoms, restrictive and repetitive behaviors (RBS-R) had the strongest and broadest association, particularly with the ARFID Score (RBS-R total score $\rho = 0.30$, 95% CI: 0.28–0.32). Communication ability (SCQ) had more modest association with the ARFID Score (SCQ summary $\rho = 0.18$, 95% CI: 0.16–0.20), and had a stronger association with the Picky Factor (SCQ summary $\rho = 0.15$, 95% CI: 0.12–0.17), compared to the Fear Factor (SCQ summary $\rho = 0.11$, 95% CI: 0.09–0.13) or Appetite Factor (SCQ summary $\rho = 0.02$, 95% CI: −0.01 to 0.04, not significant). Developmental Coordination (DCDQ) had generally weaker correlations, the strongest of which were with the Fear Factor (DCDQ total $\rho = 0.12$, 95% CI: 0.09–0.15) and ARFID Score (DCDQ total $\rho = 0.10$, 95% CI: 0.07–0.13).

All of the adaptive skills assayed by the VABS—save for writing—were significantly associated with at least one NIAS factor or with ARFID risk. The strongest associations across all domains were with internalizing problems, (ARFID Score $\rho = 0.30$, 95% CI: 0.26–0.33, Fear Factor $\rho = 0.21$, 95% CI: 0.17–0.24, Picky Factor $\rho = 0.17$, 95% CI: 0.13–0.21, Appetite Factor $\rho = 0.11$, 95% CI: 0.07–0.15).

2.5. PRS

The three NIAS factors and ARFID Score demonstrated genetic overlap with several neuropsychaitric and morphological traits, as measured by correlation with polygenic risk scores (PRS) for those traits (**Figure 3**). The ARFID Score of parents had significant positive association with PRS for metabolic syndrome ($\rho = 0.070$, FDR $p = 0.03$) and neuroticism ($\rho = 0.072$, FDR $p = 0.03$), and a nominal positive association with autism ($\rho = 0.058$, FDR $p = 0.094$). The Appetite Factor had significant negative correlations with BMI ($\rho = -0.067$, FDR $p = 0.022$) and basal metabolic rate ($\rho = -0.066$, FDR $p = 0.022$) in probands, while the Fear Factor was nominally positively correlated with basal metabolic rate ($\rho = 0.052$, FDR $p = 0.194$) and anorexia ($\rho = 0.055$, FDR $p = 0.138$) in parents. The Picky Factor was found to be negatively associated with PRS for educational attainment in both probands ($\rho = -0.059$, FDR $p = 0.045$) and parents ($\rho = -0.062$, FDR $p = 0.057$) and positively correlated with PRS for birth weight ($\rho = 0.075$, FDR $p = 0.007$) and nominally with major depression ($\rho = 0.046$, FDR $p = 0.194$) in probands alone.

2.6. Heritability

The size of each sub-cohort was too small detect narrow-sense (SNP-based) heritabilities of modest effect sizes, with a minimal detectable heritability (at 80% power) of 0.28 in the probands, and 0.40 in the parents (**Supplementary Figure 2A**). Despite this, the proband ARFID Score was estimated to have significant SNP h^2 of 0.45 (95% CI: 0.13–0.76) (**Figure 4A**). The ARFID score for parents had a SNP h^2 of 0.25 (95% CI: −0.19 to 0.69).

In the combined cohort, the h^2 estimate for the ARFID score had little-to-no excess pedigree heritability (**Figure 4B**).

2.7. ARFID Score GWAS

One SNP on chromosome 5, rs13177031 ($p = 1.6 \times 10^{-8}$), reached genome wide significance (**Figure 4C**, **Table 2**). The gene nearest to this SNP is *ZWIM6* (13 kb downstream of SNP), which has been previously implicated in intellectual disability (22) and schizophrenia (23). A locus on chromosome 17 with the lead SNP rs73984121 ($p = 5.0 \times 10^{-7}$) is near *ULK2* (105 kb upstream of SNP) and *ALDH3A1* (17 kb downstream of SNP). *ULK2* has been shown to regulate axon growth in mice (24). A locus on chromosome 7 with the lead SNP rs78495856 ($p = 4.9 \times 10^{-7}$) is in an intron of the *THSD7A* gene. A previous genome-wide association study of BMI found an association with *THSD7A* (25).

The current sample is likely underpowered for effective gene discovery, which is supported by the discordance between the appreciable estimated SNP heritability and finding only one locus that exceeded the genome-wide significance threshold. Indeed, in a follow-up analysis that used the effect size and allele frequency of 162 independent lead SNPs from the GWAS, most would not be detectable as genome-wide significant without a cohort size of approximately 10,000 (**Supplementary Figure 2B**).

3. DISCUSSION

The essential contributions of this work to our understanding of ARFID include the estimation of risk for ARFID in individuals with autism (21% of probands and up to 17% of their parents) and the demonstration of its heritability, estimated at 0.45 (95% CI: 0.13–0.76). We further implicated autism, metabolic syndrome, and neuroticism as genetically linked to ARFID via common variants, and identified *ZSWIM6*, a known neurodevelopment gene, as the first putative genetic association with ARFID. Our ARFID GWAS also suggested other genes implicated in BMI and axon growth, such as *ULK2* and *THSD7A*. Finally, although our GWAS was underpowered, our estimates of heritability

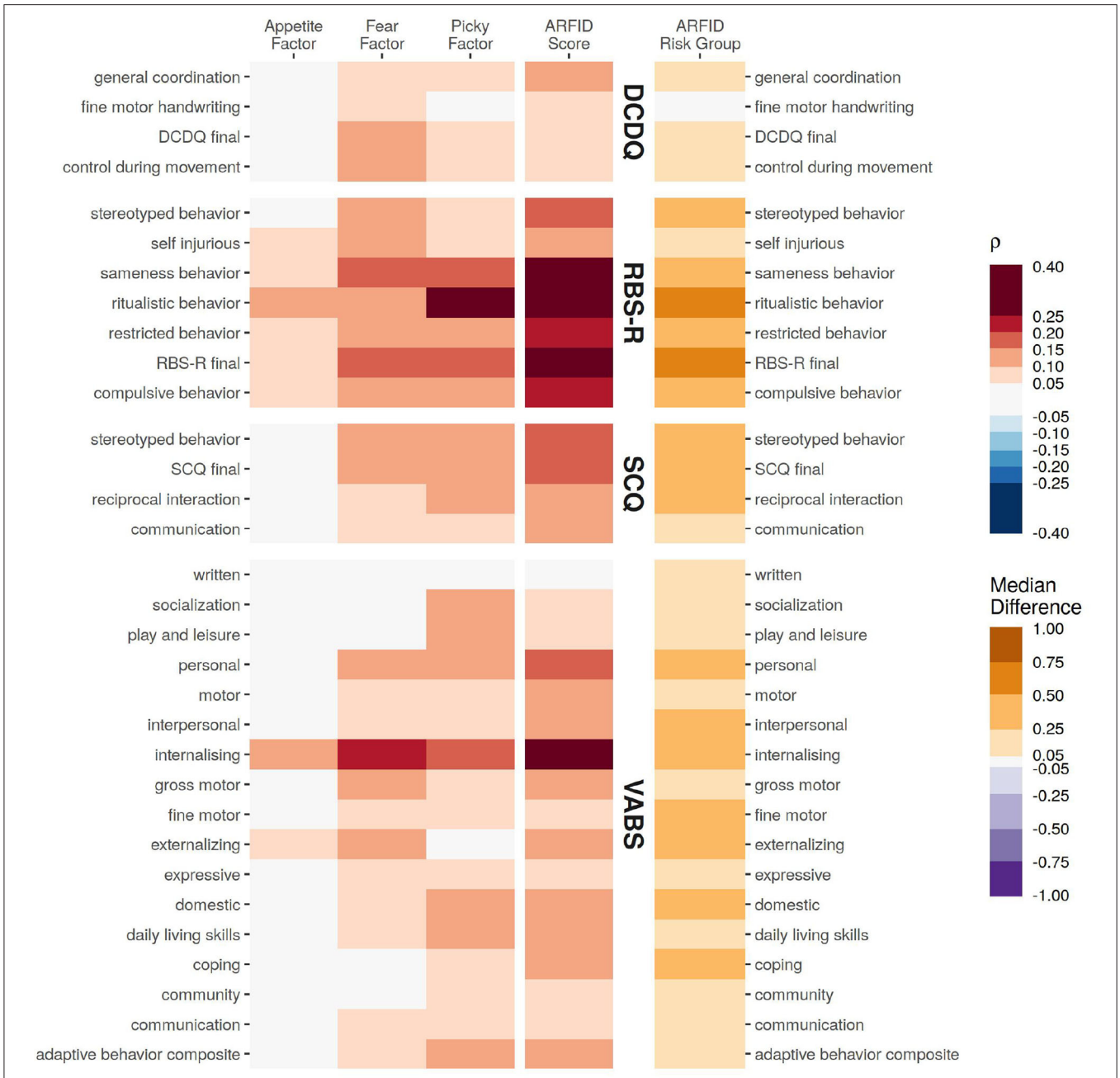

FIGURE 2 | The association of various assessments from the probands with the NIAS factors and ARFID Score, quantified as Spearman correlation coefficient. For differences for those identified as high risk for ARFID (ARFID Risk Group), the association is quantified as the median difference (location shift) from a Wilcox rank-sum test.

allowed us to project the number of participants needed (N = 10,000) to achieve sufficient power for further ARFID gene discovery.

In line with previous work, three latent factors (appetite, fear, and pickiness) were found to underlie the NIAS in both the parent and proband sub-cohorts (21). These factors, along with other individual items from the parent and proband survey were useful in predicting ARFID diagnosis, resulting in an ARFID Score (**Supplementary Figure 1**), with a balanced accuracy of 0.85 in parents and 0.80 in probands. These estimates lead us to estimate that 17% of parents and nearly 21% of autism probands may be at high-risk for ARFID. These estimates are notably higher than one estimate of ARIFD rates in the general population of children at 3.5% (14), but are in line with an estimated ARFID prevalence of 22.5% among children undergoing treatment for eating disorders (16).

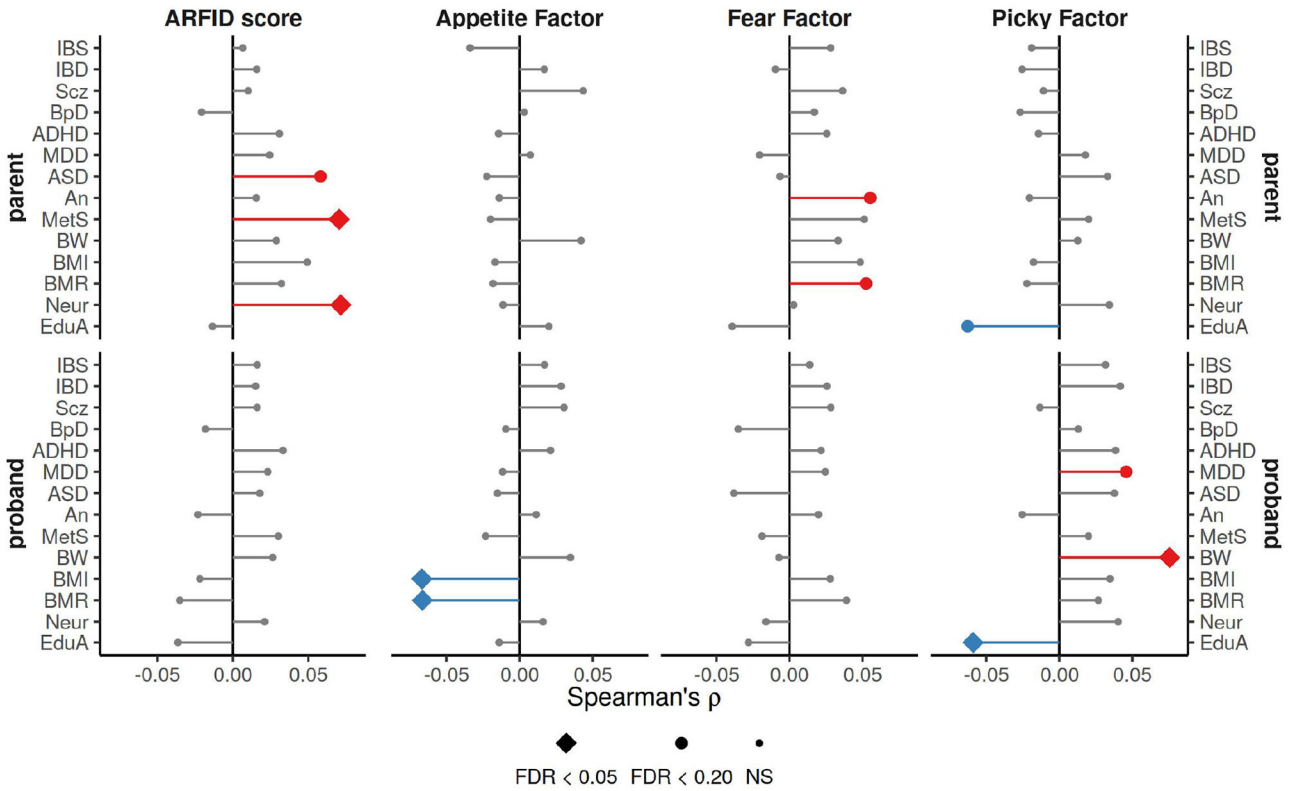

FIGURE 3 | Spearman correlation coefficients for ARFID Score and NIAS factors with PRS for a variety of neuropsychiatric and morphological traits. The strongest association across three *p*-value thresholds (0.0005, 0.05, and 0.5), is shown here. FDR correction applied across all tests ($n = 336$). IBS, irritable bowel syndrome; IBD, inflammatory bowel disease; Scz, schizophrenia; BpD, bipolar disorder; ADHD, attention deficit hyperactivity disorder; MDD, major depression disorder; autism, autism; An, anorexia; MetS, metabolic syndrome; BW, birth weight; BMI, body mass index; BMR, basal metabolic rate; Neur, neuroticism; EduA, educational attainment.

In contrast to previous studies of ARFID, which noted anxiety due to fears of choking, vomiting, or GI issues as the predominate drivers in a typically developing population, (16), our data indicate that RRBs and sensory sensitivities play a larger role, at least in autism. In probands, RRBs had stronger associations with ARFID risk than social skills, developmental coordination, and adaptive behaviors (**Figure 2**). RRBs also had the broadest association across all three of the NIAS factors, suggesting primacy in all domains of ARFID risk. Internalizing problems—a maladaptive subscale on the Vineland Adaptive Behavior Scales featuring questions about anxiety, worry, fear, and eating problems—had the next strongest association across all domains of that instrument. This raises the prospect that therapies targeted at RRBs or anxiety/fear may alleviate the factors underlying ARFID in some individuals with autism. Despite the strong inter-relationships of core autism symptoms (e.g., RRBs) with the fear and pickiness NIAS factors, the appetite NIAS factor lacked strong associations with any of the other adaptive, social, and coordination measures. This suggests a weaker interaction of low appetite with core autism symptoms, and consequently, less potential for inroads to treating ARFID via behavioral therapies when low appetite is the driving factor.

Given this apparent elevated risk for ARFID in autism, patterns of behavior associated with this risk—whether they involve eating or not—may be useful hallmarks for clinicians as they consider diagnoses. We found sensory sensitivities—particularly related to taste and food texture—to be over-represented in individuals at high risk for ARFID (**Figure 1**). This difference was less pronounced in autism probands than parents, perhaps due to the higher baseline amount of sensory sensitivity in individuals with autism. Thus, while these sensitivities clearly indicate ARFID risk, the presence of core autism symptoms may make them less salient.

Most neurodevelopmental conditions are heritable, with autism's SNP-based heritability estimated at 0.12 (95% CI: 0.10–0.14) (26) based on a cohort of 35,740 individuals. While, the continuous predictor of ARFID risk (ARFID Score) showed significant heritability in probands, the sample here was smaller by an order of magnitude (3,142 for probands and 2,205 for parents), leading to a less precise estimate: 0.45 (95% CI: 0.13–0.76) in probands and 0.25 (95% CI: −0.19 to 0.69) in parents. This indicates that common genetic variation likely plays a significant role in ARFID, especially in those with autism. We identified little excess pedigree heritability for the ARFID

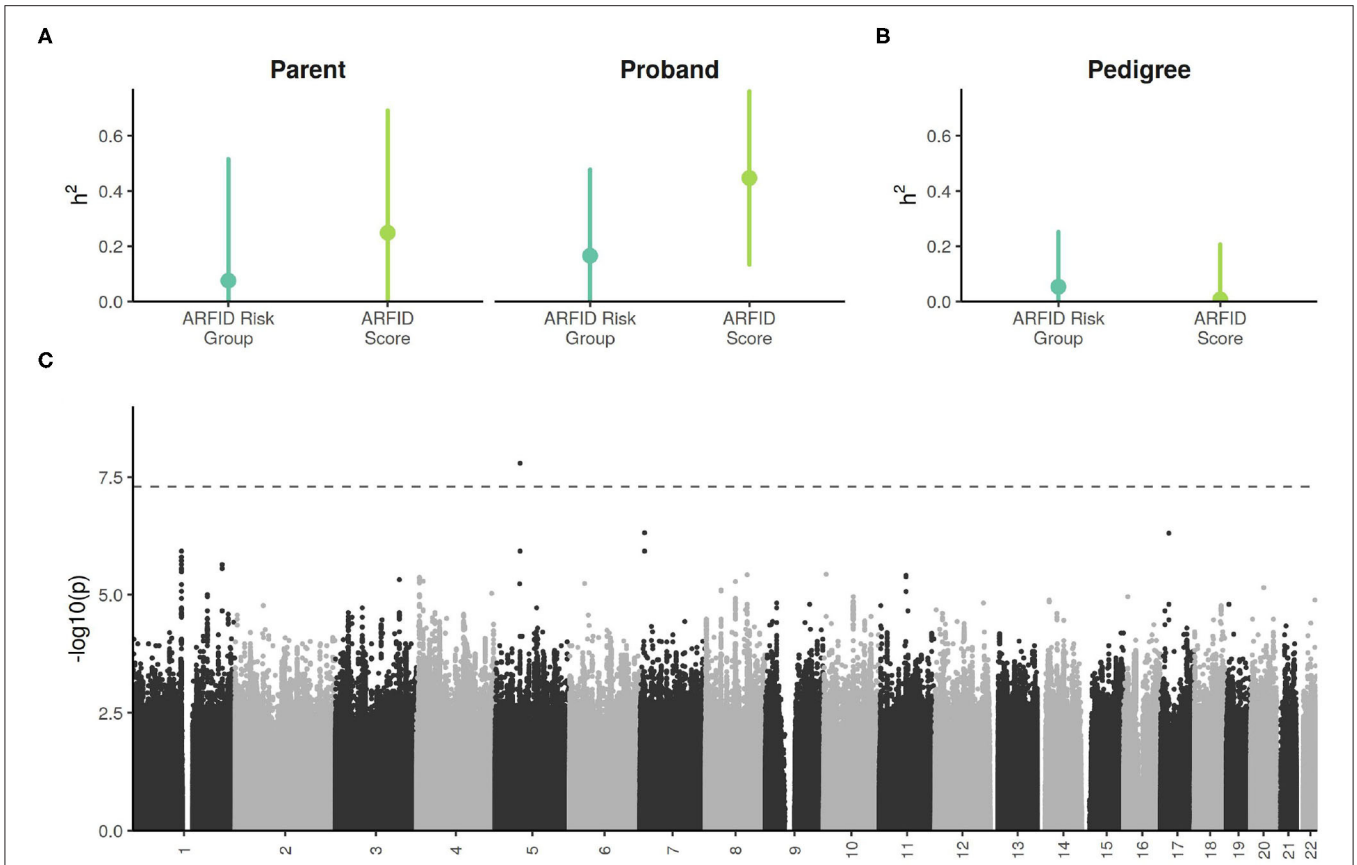

FIGURE 4 | (A) SNP-based (i.e., narrow sense) heritability estimates of ARFID Score and ARFID Risk Group (high vs. low), with 95% confidence intervals. **(B)** Excess pedigree heritability estimates (beyond what is explained by SNP h^2) ARFID Score and ARFID risk group, with 95% confidence intervals. **(C)** GWAS Manhattan plot of the proband ARFID Score. The dashed line indicates the genome-wide significance threshold of $5e^{-8}$.

TABLE 2 | Top 5 lead SNPs from proband ARFID Score GWAS.

rsID	Chr	Effect allele	Allele frequency	INFO score	*P*-value	Beta	SE	Nearest genes
rs13177031	5	A	0.35	0.83	1.6e-08	0.17	0.03	*ZSWIM6*
rs78495856	7	T	0.04	1.00	4.9e-07	0.32	0.06	*THSD7A*
rs73984121	17	T	0.03	0.95	5.0e-07	0.41	0.08	*ULK2, ALDH3A1*
rs1575620	1	C	0.50	0.96	1.2e-06	−0.13	0.03	*GDAP2, WDR3, SPAG17, TBX15, WARS2*
rs78624779	1	T	0.04	0.85	2.3e-06	−0.33	0.07	*GPATCH2, SPATA17*

Score, suggesting that common genetic variation dominates environmental factors for ARFID risk. The GWAS and PRS analyses implicated both neurodevelopment and metabolism as significant factors involved in ARFID. The higher heritability in probands increased the power of GWAS, revealing one genome-wide significant SNP near the neurodevelopmental gene *ZSWIM6*. PRS analyses also revealed pleiotrophic associations of ARFID risk with neuroticism, autism, and metabolic syndrome in the parents. These associations may not have been detectable in probands because that sub-cohort was already enriched for autism and neuroticism risk. Further dissection of the ARFID score phenotype by the PRS associations of the NIAS factors showed the Appetite and Fear Factors to be more associated with metabolism, while the Picky Factor showed more associations with neurodevelopment (**Figure 3**).

3.1. Limitations

A primary limitation of nearly all studies in the SPARK cohort is the constraint of self- and parent-report surveys, rather than clinical assessment, to collect data. This means the length of a survey must be balanced with the burden on participants, often resulting in limitations of resolution and specificity. For example, because this study was focused specifically on a population-level study of ARFID, it was not well-situated to conclusively distinguish ARFID risk factors from those of other eating disorders. Regardless, this limitation is generally offset by the

large sample sizes that can be recruited from within SPARK, and this work remains by far the largest study the authors are aware of to examine ARFID in the context of autism. Additionally, it should be noted that the NIAS (21), while designed as an adult self-report instrument, was adapted for use as a parent-report measure in this study due to a lack of empirically validated screening tools for ARFID in children at the time this research was conducted. The current study did demonstrate similar factor loadings of the three latent factors (picky eating, low appetite, and fear) in the NIAS across proband and parent subsamples. However, there may be error introduced in the probands' NIAS scores due to our reliance on parents' observation and interpretation of their children's eating habits.

A secondary limitation to reliance on self- and parent-report surveys in the context of eating problems is the effect of environment on the perception of eating habits. Parents in particular have extensive control over their environment, and may do so in order to minimize the adverse effects caused by sub-clinical eating problems, resulting in a bias of self-perception. There is also potential for ascertainment bias in reported ARFID diagnoses as a result of how some participants are recruited into SPARK. Although SPARK actively recruits across the entire United States, many participants are enrolled through partnerships with clinical sites (typically co-located with large research institutions). Due to data anonymization, we were unable to determine if self- and parent-reported ARFID diagnoses came from a small subset of clinical sites, where practitioners might be more aware of ARFID. However, such a scenario would simply reflect the overall under-diagnosis of ARFID, which was a major motivator for this study.

Particular features of the SPARK cohort also complicated comparisons between probands and parents due to representation of difference sexes in each. The Research Match survey for this study was sent to "primary" participants (independent adults with autism, or the parent who initiated enrollment into SPARK). In the data release use in this study, 88.6% of such "primary" parents are the biological mothers of probands. It is therefore possible that—although sex was accounted for in all tests performed here—the signal detected in parents is somewhat female-specific, just as the male-skewed proband subsample may lead to poor detection of patterns specific to female probands.

As a genome-wide genetic investigation, the current study had low power, though some important insight was obtained. We found that the current cohort was only suitable to detect substantial narrow-sense heritability (SNP $h^2 = 0.28–0.40$; **Supplementary Figure 2A**). Similarly, the SNP-level associations found by GWAS were mostly below the detection threshold for the current cohort size (**Supplementary Figure 2B**). However, performing these analyses is an important first step in understanding the genetics of ARFID, as it allowed us to project that sample sizes of 10,000 and more would begin to yield returns, based on the effect sizes observed in our study.

3.2. Future Directions

This study showed the first evidence of significant SNP heritability of ARFID risk, and potential genetic connections to other traits, such as metabolic syndrome, autism, and neuroticism. Although the data yielded one genome-wide significant hit, this study is clearly underpowered, and future work will focus on expanding the sample size. Further, since previous studies have found evidence of rare variant burden in eating disorders (27–29), this is a logical next step in this cohort. Together, rare and common variant association studies may lead to a better understanding of the molecular pathways that underlie ARFID risk, potentially exposing new therapeutic opportunities. Finally, our estimators of ARFID do not explain all the variation in eating and gastrointestinal problems in our sample or in SPARK generally. As such, future work will concentrate on the identification of robust and recurrent patterns of eating and gastrointestinal phenotypes distinct from ARFID.

TABLE 3 | Demographic summary of the cohort.

Role	Proband	Parent
Total *N*	5,157	4,985
Mean age (SD)	11.1 (5.87)	41.63 (8.24)
Male	81%	16%
Race: Asian	4%	–
Race: African American	7%	–
Race: Native American	3%	–
Race: Native Hawaiian	1%	–
Race: White	85%	–
Race: Other	5%	–
Cognitive impairment	17%	–
Mean BMI (SD)	–	29.84 (7.97)
Genotype *N*	3,142	2,205

Variables with the mean reported have the standard deviation in parenthesis. Nine probands did not have data for cognitive impairment. Race was self-reported endorsement with none and/or multiple selections allowed.

4. MATERIALS AND METHODS

4.1. Cohort Description

Participants were recruited from the nation-wide SPARK study (30) via a research match. All respondents provided informed consent. This study was approved by the IRB of University of Iowa (IRB# 201801821) and SPARK is approved by the Western IRB (IRB# 20151664). In total, 5,686 independents responded to survey questionnaires for themselves and one proband. After merging the survey responses with available basic demographic and medical data, 5,157 probands and 4,985 adults without autism were used for analyses (**Table 3**). For analyses, these SPARK-collected data were combined with research match participants' survey responses on measures of eating behaviors and GI symptoms. A subset of 3,142 probands and 2,205 parents of European ancestry had SNP genotyping data available for analysis.

4.2. Phenotypic Measures

In addition to the measures assessed as part of this study (see below), all families also had available demographic, medical,

and core autism behavioral data collected through SPARK. Except where noted, each primary respondent was a parent who answered the measures in our study for both self and their dependent (proband). Independent adults with autism were given the same survey as parents and answered the measures for themselves, but were not analyzed as part of this study.

4.2.1. Nine-Item Avoidant/Restrictive Food Intake Disorder (ARFID) Screen (NIAS)

At the time this study was conducted, there was no validated measure for assessing symptoms of ARFID in children, so the NIAS (21) was adapted in the surveys to collect responses for parents as well as probands. The 9 items in this measure assess three categories of eating disturbance that can lead to ARFID symptoms, as described in the DSM-5: avoidance of many foods based on sensory properties, low appetite or limited interest in eating, and fear of negative consequences (i.e., choking, vomiting) from eating. The scale demonstrated high internal consistency (alpha ranging from 0.79 in an undergraduate sample to 0.90 in a sample with familial eating problem behaviors). One item in this measure simply asks respondents to self-identify picky eating behavior, which has been shown to distinguish picky from non-picky eaters in and of itself (31–34).

4.2.2. Inflexible Eating Behavior

An Inflexibility Index was created by Zickgraf et al. (15) to measure the rigid eating behavior associated with picky eating severity, with items such as: "the thought of eating a food I do not like fills me with anxiety" and "I avoid letting different foods touch on my plate, even when they are both foods that I like." An unrotated principal components analysis (PCA) indicated that all items loaded on a single factor, with all loadings > 0.50, and the internal reliability for all 12 items was excellent (alpha $= 0.92$).

4.2.3. Sensory Sensitivities

Additionally, Zickgraf et al. (15) developed 11 sensory sensitivity-related questions based upon questionnaires such as the SensOR Assessment (35) and the Glasgow Sensory Questionnaire (36). In an attempt to reduce the question load on participants of this study, we selected questions only related to food sensitivities (e.g., "my sense of taste/smell is stronger/more sensitive than other people's"; "I am very aware of the texture of food in my mouth"). These questions are designed to assess the sensory component of eating that might be affecting eating behavior in the study cohort.

4.2.4. Eating Attitudes Test (EAT-26)

This shortened (26-item) version of the Eating Attitudes Test (originally 40 items) was developed in 1982 by Garner and colleagues, and has been used popularly as a screening measure for disordered eating behaviors since. Internal consistency of the EAT-26 is high (alpha $= 0.90$ in a cohort with anorexia nervosa and 0.83 for a group of female controls), and higher scores (> 20) are indicative of potentially disordered eating (37). This measure was only included in the survey sets for parents.

4.2.5. Gastrointestinal History (GIH)

The GIH assesses the frequency of 10 current gastrointestinal symptoms (within past 3 months). Assessing GI symptoms in individuals with autism can be very challenging due to potential lack of communicative abilities, and thus assessments so far have been varied, and none have yet been validated, according to a comprehensive literature review (38). However, of the 5 GI measures designed with an autism population in mind, Holingue and colleagues describe the CHARGE GIH as one of the simplest to take, while assessing a comprehensive range of GI symptoms and their frequencies. The GIH was only included in probands' survey sets; parents were simply asked to report the frequency with which they experience constipation or other gastrointestinal upset.

4.2.6. Miscellaneous

Several standalone items were included in survey sets in order to assess some potentially important factors in eating behavior patterns: food allergies, foods avoided by preference, family history of eating disorders, and the subjective negative impact of eating behavior on daily life. The family history of eating disorders included the responding parent and proband (i.e., "self" history of eating disorders). ARFID diagnosis was based on this report by the responding parent.

4.2.7. Measures of Broad Autism Symptoms

These data were released as part of SPARK Data Collection 5 in December 2020. For a breakdown of the summary and sub-scores from individual instruments, including sample number, see **Supplementary Table 4**. All data from SPARK, including autism diagnosis, are parent/caregiver or self-reported through online surveys.

4.2.7.1. Social-Communication Questionnaire (SCQ)

The SCQ (39) was administered to probands and control siblings (when available), between the ages of 2 and 18 years. The final score was nullified when a surplus of missing items affected validity.

4.2.7.2. Developmental Coordination Disorder Questionnaire (DCDQ)

The DCDQ (40) was administered for probands between the ages of 5 and 15 years. Scores were reversed so that higher scores indicated worse coordination. The DCDQ was not completed if parents/caregivers indicated the proband was unable to use his/her hands or to ambulate. The final score was nullified if one or more items were incomplete across an entire measure (in concordance with publisher recommendation).

4.2.7.3. Repetitive Behavior Scale-Revised (RBS-R)

The RBS-R (41) was administered to dependent probands age 3 and older. Subscale scores were nullified if more than two component items were missing. The final score was nullified if more than 5 items total were missing.

4.2.7.4. Vineland Adaptive Behavior Scales (VABS)

The Vineland 3rd Edition Parent-Caregiver Comprehensive rating form (42) was administered to parents or caregivers of probands using Pearson Q Global. Scores for all non-maladaptive scales were revered so that higher scores indicated more problems.

4.3. Phenotype Data Cleaning

Ordinal survey items were imputed to the median. 0.6% ordinal data was missing and imputed in the proband data, and 0.3% was missing in the parents. Ordinal survey items were corrected for non-linear effects of age via local regression. First, the empirical cumulative distributions of individual items were mapped to a standard normal distribution via `ecdf()` and `qnorm()` functions in R. Age was then residualized from responses using R's `loess()` function with `span = 0.5`.

4.4. Identification of Individuals at High Risk for ARFID

We performed a two-step process to create a quantitative score estimating every individual in the cohort's risk for ARFID and used this score to classify individuals as high or low risk. Both of these steps were carried out in parallel for parents and probands. The demographics, mean NIAS factor values, and median model items stratified by low risk and high risk ARFID groups are shown in **Supplementary Table 2** for probands and **Supplementary Table 3** for parents.

4.4.1. Factor Analysis

Previous studies have reported three clear factors underlying the nine questions of the NIAS (21). Factors were calculated on the individual NIAS items using the `factanal()` function in R. Factor scores using Thompson's method were calculated for each individual. These factor scores were then scaled within each age decile (10 separate age bins for parents and probands), and these age-corrected factor scores were used as the phenotypes for all association analyses.

4.4.2. Logistic Regression

To leverage the additional granularity of the survey questions described above, we fit logistic regression models predicting ARFID diagnosis with `glm()` in R, serially adding terms to the model to reduce the Akaike information criterion (AIC). The three NIAS factors were used as a baseline model and each additional survey item was added to the model, with the single item which reduced the AIC the most carried forward (**Supplementary Table 1**). This process repeated until none of the remaining survey questions reduced the models' AIC.

4.5. Common Genetic Variation

The SNPs used in this study were based on the combined SPARK 2019 Version 3 release and the SPARK 2020 Version 4 release.

4.5.1. SNP Processing and Imputation

SNPs were merged using PLINK (43), then lifted from hg38 over to hg19. The SNP QC process was based on the recommendations by (44) using PLINK (43) and R (45). First, 25,840 SNPs and 86 individuals were removed due to global missing rate greater than 20%. Second, the more stringent threshold of 5% global missing rate was used again which removed an additional 30,845 SNPs and 711 individuals. Third, 102,530 SNPs were removed because the minor allele frequency was less than 1%. Fourth, 47,825 were removed due to the HWE p-value less than 1×10^{-10}. Fifth, 1,180 individuals were removed because of missing rate greater than 5% on any autosome. Sixth, 1,115 individuals were removed due to their heterozygosity rate not within 3 standard deviations of the cohort mean heterozygosity. After this QC, the remaining SPARK cohort was merged and clustered with the 1,000 Genomes Phase 3.

Clustering was based on the first 10 components from multi-dimensional scaling of the combined kinship matrix of the cohort and 1,000 Genomes. This combined cohort was clustered into 5 groups, representing the 5 distinct super-populations. Three thousand nine hundred and sixty-three individuals were removed due to being more than 3 standard deviations away from any of the 5 mean multi-dimensional scaling components. In total, 36,154 individuals and 409,281 SNPs remained. The top 10 principal components of each of the 5 clusters of the SPARK cohort were calculated separately to be used as covariates in heritability, polygenic risk score, and genome-wide association analyses. These remaining individuals and SNPs were imputed to the 1,000 Genomes Phase 3 reference of their respective cluster using the Genipe pipeline (46). Genipe performed LD calculation and pruning with PLINK (43), genotype phasing with SHAPEIT (47), and genotype imputation by IMPUTE2 (48) using default parameters.

4.5.2. Polygenic Risk Scores

Polygenic risk scores (PRS) were calculated using PRSice (49) from the following base GWASes: metabolic syndrome (UK Biobank 2019) (50), body mass index (UK Biobank 2019) (51), birth weight (UK Biobank 2019) (51), basal metabolic rate (UK Biobank 2019) (51), neuroticism (SSGAC 2016) (52), educational attainment (SSGAC 2018) (53), schizophrenia (PGC 2018) (54), major depression disorder (PGC 2018) (55), bipolar disorder (PGC 2018) (54), autism spectrum disorder (PGC 2019) (26), anorexia (PGC 2017) (56), attention deficit hyperactivity disorder (PGC 2019) (57), inflammatory bowel disease (58, 58), and irritable bowel syndrome (59). PRSes were calculated at three p-value thresholds: 0.0005, 0.05, and 0.5. To control for the possible effects of population stratification, the first 10 common genetic principal components were regressed out of each PRS using the `lm()` function in R. To control for any systematic differences in phenotype between sexes, all phenotypes were scaled separately by sex before association testing. The association of the NIAS factors and ARFID Score with each PRS was quantified as non-parametric (Spearman's) correlation using the `cor.test()` function in R. False discovery rate correction was applied to the p-values of these test across all PRS thresholds and scores/factors using the `p.adjust()` function in R.

4.5.3. SNP Heritability

GCTA (60) was used to calculate SNP-based heritability, and the GCTA power calculator (61) was used to estimate the power of each sub-cohort (**Supplementary Figure 2A**). The genetic relationship matrix (GRM) was created for $N = 29{,}443$ individuals in the European population cluster. SNPs were pruned for a final set of 192,500 autosomal SNPs to generate the GRM. This resulted in $N = 3{,}142$ probands and $N = 2{,}205$ parents being used for GCTA. SNP-based heritabilities were calculated separately for parents and probands using the GREML method (62). The ARFID Score was scaled separately for males and females, and the first 10 scaled genetic principal components

were used as covariates. Pedigree-based heritabilities on the combined phenotypes were calculated with both the original GRM and an additional GRM with a relatedness cutoff of 0.05 using the GREML method (63).

4.5.4. Genome-Wide Association Study

The GWAS on the proband ARFID Score was performed on the directly observed and imputed SNPs using BOLT (64), a mixed-model analysis which accounts for population stratification and relatedness. The ARFID Score was scaled separately for males and females. The first 10 scaled genetic principal components were used as covariates. After the imputation quality control filtering performed by BOLT, 3,142 individuals were used for the GWAS. The summary statistics were filtered on 1% minor allele frequency and INFO imputation score of 0.8 or greater. 8,275,942 SNPs remained after filtering. Lead SNPs were identified using the default clumping parameters of plink (43). Estimates of the minor allele frequency and effect size detection threshold (at 80% power) in the proband sub-cohort was calculated using the `genpwr` package (65), assuming an additive genetic model (**Supplementary Figure 2B**).

4.6. Visualization and Data Processing

All visualizations were generated in R (45) with the ggplot2 (66) and patchwork (67) packages. Unless otherwise noted, data processing was performed in R using the tidyverse family of packages (68). When reported, 95% confidence intervals for Spearman correlations were based on 1,000 bootstrap permutations.

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://base.sfari.org, SFARI SPARK Research Match, RM0018. The code can be found below: https://research-git.uiowa.edu/michaelson-lab-public/spark-arfid-2021.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Iowa IRB #201801821. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

TT and TK performed all analyses and contributed equally to this publication. NP and JM conceived the study design and received all regulatory approvals. ML and NP performed background research. All authors drafted and provided revision of the manuscript for intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.668297/full#supplementary-material

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REVIEW
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The Prenatal Hormone Milieu in Autism Spectrum Disorder

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Though the etiology of autism spectrum disorder (ASD) remains largely unknown, recent findings suggest that hormone dysregulation within the prenatal environment, in conjunction with genetic factors, may alter fetal neurodevelopment. Early emphasis has been placed on the potential role of *in utero* exposure to androgens, particularly testosterone, to theorize ASD as the manifestation of an "extreme male brain." The relationship between autism risk and obstetric conditions associated with inflammation and steroid dysregulation merits a much broader understanding of the *in utero* steroid environment and its potential influence on fetal neuroendocrine development. The exploration of hormone dysregulation in the prenatal environment and ASD development builds upon prior research publishing associations with obstetric conditions and ASD risk. The insight gained may be applied to the development of chronic adult metabolic diseases that share prenatal risk factors with ASD. Future research directions will also be discussed.

Keywords: autism, autism spectrum disorders, steroid hormone (progesterone, testosterone, estradiol), perinatal, prenatal, risk factors

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INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by persistent deficits in social interaction and communication in addition to stereotyped, repetitive behaviors (1). While the etiology of ASD remains largely unknown, multifactorial contributors such as genetics, neuroanatomical abnormalities, and the environment likely play a role (2). Prenatal factors associated with elevated ASD risk include maternal conditions that contribute to a suboptimal prenatal environment, particularly as it relates to inflammation, metabolism, and steroid hormone regulation (3, 4).

Prenatal stress (5, 6), maternal immune dysfunction (5, 7–9), pre-existing/gestational diabetes (3, 10), pre-pregnancy obesity (11), weight gain during pregnancy (12), pre-existing/gestational hypertension (13, 14), polycystic ovarian syndrome (PCOS) (4, 15) and prenatal complications such as low birth weight (3) and pre-term birth (16) have all been associated with ASD in offspring. How these conditions may alter prenatal mechanisms promoting ASD pathogenesis has yet to be clearly established, though several physiological processes that link these conditions have been implicated including direct insults (e.g., oxidative stress, hypoxia, inflammation) and adaptive responses (e.g., epigenetic changes, fetal programming) (17–20).

Studying the relevance of prenatal risk factors in ASD pathogenesis inherently involves understanding the remarkable, well-coordinated interaction among the mother, fetus, and placenta—referred to as the maternofetoplacental unit (21, 22). The interdependence across the maternofetoplacental unit for steroid hormone production, immune response mediation, and

nutrient transfer is particularly relevant to sustain pregnancy and ensure newborn viability. The placenta, together with the amnion and chorion, create a unique immune environment that permits co-existence of the fetal allograft within the mother while accessing her rich nutrient and oxygen supply for development, growth, and survival.

Immune response and steroid production are intrinsically linked during pregnancy with the placenta acting as the key mediator, particularly regarding estradiol and progesterone synthesis. Estradiol augments both cell- and antibody-mediated immune responses (23). Rising levels of estradiol during pregnancy promote an immunologic shift from an inflammatory state to a regulatory response (23–26). The dramatic increases in progesterone enhance maternal-fetal tolerance (27) and promote anti-inflammatory factors through progesterone-induced binding factor (PIBF) (28, 29). Placental progesterone, estradiol, and human chorionic gonadotropin (hCG) facilitate blastocyst implantation and spiral artery formation, which are essential early gestational events to sustain pregnancy. These hormones support implantation by recruiting immune cells to the maternal-fetal interface (30–33). Immune cells subsequently secrete angiogenic factors such as transforming growth factor beta (TGF-β) and vascular endothelial growth factor (VEGF) (31) to promote spiral artery formation (34) and placental vascularization.

The influence that placental steroid hormone production exerts across the maternofetoplacental unit—involving inflammatory responses, oxygen/nutrient exchange, and neuroendocrine functioning—cannot be overstated. This review article will describe the current understanding of the *in utero* steroid-related environment associated with ASD and explore its potential role in ASD pathogenesis. Existing literature that is relevant to the prenatal hormone milieu in ASD largely appears to fall within the following four domains: obstetric conditions, fetal programming, sex differential, and steroid-related biomarkers. The literature on each domain will be summarized.

OBSTETRIC CONDITIONS

Hypertensive Disorders

Hypertensive disorders during pregnancy, regardless of subset (i.e., chronic, gestational, *de novo* or superimposed preeclampsia), have been correlated with elevated ASD incidence among offspring (13, 14, 35–37). Placental insufficiency is one of the most concerning complications of hypertension during pregnancy (22, 38, 39). Common indicators or complications of placental insufficiency that overlap with ASD risk factors include small for gestational age/*in utero* growth restriction, prematurity, maternal infection, and maternal metabolic syndrome (16, 38–42). Histologic signs of placental insufficiency, such as trophoblastic inclusions, are also more commonly found in placentas of children who develop ASD (43). Steroid hormone dysregulation, altered immune function, and placental insufficiency are intertwined, as maternal serum inflammatory markers (e.g., atypical cytokine profiles, leukocytosis, and elevated platelet counts) are associated with gestational hypertension (44), while the maternal cardiovascular adaption to pregnancy is influenced by placental estrogen and progesterone production (45).

Gestational Diabetes/Insulin Resistance

Maternal diabetes, both pre-existing and gestational onset, is also an established ASD risk factor (46, 47). While the exact mechanism underlying the relationship between ASD and maternal diabetes is unknown, maternal diabetes can lead to several obstetric complications affecting the mother (e.g., gestational hypertension, pre-eclampsia) and baby (e.g., high or low birth weight, shoulder dystocia, hypoglycemia, hyperbilirubinemia, hypocalcemia, and respiratory distress) (48, 49). These conditions result from the amplification of the typical prenatal metabolic state characterized by hypercortisolemia (50) and insulin resistance (51, 52). During uncomplicated pregnancies, relative maternal hypercortisolemia and insulin resistance facilitate adequate transfer of nutritional resources from mother to baby. As pregnancy progresses, a developmental switch prompted by rising fetal cortisol synthesis shifts resource allocation from tissue proliferation to maturation (53). In maternal diabetes, excess glucose supply to the fetus causes higher fetal insulin production to maintain glucose homeostasis, stimulating fetal overgrowth and delaying lung maturation (54). Diabetes during pregnancy also leads to higher placental release of pro-inflammatory cytokines (e.g., leptin, tumor necrosis factor-α (TGF-α), and interleukins) (55), with the potential consequence of reducing oxygen diffusion across the placenta by enhancing placental thickening (48).

Pre-pregnancy Obesity/Gestational Weight Gain

Epidemiologic studies identify increased ASD risk associated with pre-pregnancy obesity (46) and/or gestational weight gain (2, 12, 56). Obesity promotes both inflammation and endocrine dysfunction, perturbing the prenatal environment. As a pro-inflammatory state, obesity contributes to elevated lipids, leptin, and IL-6 during pregnancy (57). The bioavailability and synthesis of estrogens are impacted through the endocrine and metabolic function of adipose tissue (58). Furthermore, studies exploring the *in utero* steroid environment, primarily as it relates to hormone-sensitive cancer risk among offspring, have found elevated maternal serum estrogen, progesterone, and testosterone levels in pregnancies characterized by higher weight gain (58–61). Because the gestational weight gain associated with increased ASD risk (12) is not clinically relevant from an obstetric perspective (i.e., about three pounds), its link with ASD may relate to a shared etiology rather than a cause and effect relationship.

Maternal Stress

Epidemiologic studies have also found that heightened maternal stress during the 2nd trimester increases fetal vulnerability to adverse outcomes such as shortened gestational age, preterm birth, low birth weight, and small for gestational age (62). ASD has also been associated with intense maternal stress (e.g., life events and hurricanes) during this gestational window (5, 6, 63).

FETAL PROGRAMMING

Fetal programming, a concept also referred to as the "developmental origins of health and disease" hypothesis, explains that *in utero* disruption during critical developmental periods can relay health consequences to the fetus that persist throughout adulthood (64–68). Originally bolstered by epidemiological findings describing regional overlap in areas with high infant mortality and coronary heart disease (69), there has been increased recognition that *in utero* phenomena can lead to a wide range of chronic conditions. Supporting evidence includes the well-established relationship between prenatal exposure to maternal metabolic conditions [e.g., hypertension (70), diabetes (71–73), obesity (74–76)] and chronic metabolic disorders that begin in adolescence and adulthood (77, 78). Pre-pregnancy maternal obesity has been linked to poorer metabolic, endocrine, cardiovascular, and neurodevelopmental outcomes in offspring (79). Even after adjusting for pre-pregnancy obesity, maternal metabolic conditions such as gestational diabetes remain associated with increased risk of cardiovascular disease (80), obesity (81), type 2 diabetes (82), and early childhood metabolic syndrome development (83) among offspring.

Fetal programming of adolescent/adult metabolic disorders may occur through epigenetic changes (84). Telomeres are repetitive DNA tracts that protect against excessive chromosomal degradation (85). Shortened telomeres occur in fetuses exposed to gestational diabetes and are associated with higher cardiometabolic disease risk in adults (86, 87). Pre-eclampsia has been shown to induce epigenetic changes in offspring through decreased DNA methylation of *IGF2* (88), a mediator of cell proliferation and apoptosis (89). Aside from its influence on postnatal growth, aberrant *IGF2* expression is linked to subsequent development of hypertension, diabetes, and other metabolic disorders (88).

The proposed basis for fetal programming includes calibration of fetal regulatory systems in response to intrauterine nutrient availability to optimize extra-uterine survival. The subsequent mismatch between pre- and postnatal resources—whether involving excess or scarcity—predisposes offspring to develop one or more metabolic disorders. Fetal programming serves as a potential mechanism through which stress, metabolic disturbances, inflammation, and steroid dysregulation during pregnancy could predispose offspring to ASD. Altered fetal hypothalamic-pituitary-adrenal (HPA) axis development falls within the concept of fetal programming (90–92).

HPA Axis Functioning in ASD

The HPA axis modulates neural, endocrine, and immune responses to stress to maintain homeostasis (93). While HPA axis plays a critical role in coordinating short-term physiological stress responses (94), HPA axis dysregulation has been implicated in several psychological and physiological disorders (95–100). Some children with ASD demonstrate signs of HPA axis dysregulation (101–106) such as altered circadian rhythms (107) and abnormal cortisol stress responses (105). Collectively, these findings raise the possibility that aberrant HPA axis functioning in some ASD individuals may have originated during fetal life through fetal programming.

Fetal HPA Axis Maturation

Understanding fetal HPA axis development in the 2nd trimester (i.e., 13th to 26th week gestation) provides a context in which to interpret the association between ASD and steroid hormone levels (whether measured in maternal serum or amniotic fluid) during this gestational window. By the 12th week of gestation, the fetal hypothalamus releases corticotropin-releasing hormone (CRH) to signal the anterior pituitary gland to release adrenocorticotropic hormone (ACTH) (108–110). ACTH stimulates the fetal zone of the adrenal gland to synthesize dehydroepiandrosterone (DHEA) (110) and its conjugated form, dehydroepiandrosterone sulfate (DHEAS) (111). Although the fetal HPA axis is active by 12–18 weeks gestation (112), fetal adrenal gland development lags behind that of the hypothalamus and anterior pituitary. The fetal adrenal gland does not typically develop *de novo* cortisol synthesis capacity—referred to as fetal HPA axis maturation—until around 23–24 weeks gestation (91, 113–115). Because cortisol promotes the physiologic shift from somatic growth to organ maturation [e.g., lungs (116), gut (117), liver (116, 118)], fetal HPA axis maturation is essential for extra-uterine survival (119). Under duress, fetal HPA axis maturation can occur as early as 20 weeks gestation (113). This may be induced by increased placenta estradiol production (120).

The Placenta's Role in Fetal HPA Axis Maturation

Placental estradiol synthesis promotes fetal HPA axis maturation through multiple mechanisms. Estradiol influences cortisol transfer from the mother to fetus to establish a maternal-fetal cortisol gradient through its actions on placental 11β-hydroxysteroid dehydrogenase (11β-HSD) activity (121). 11β-HSD converts active maternal cortisol into inert cortisone. This gradient serves to protect the fetus from maternal cortisol overexposure (122, 123). Maternal cortisol in the fetal compartment exerts negative feedback on the fetal hypothalamus and anterior pituitary gland. As pregnancy progresses, 11β-HSD activity increases, less maternal cortisol reaches the fetal compartment, and negative feedback to the fetal HPA axis is reduced (115, 122, 124–126). The subsequent increase in fetal HPA axis activation coincides with the emergence of the fetal adrenal's *de novo* cortisol synthesis capacity (125) leading to fetal HPA axis maturation (see **Figure 1**). The placenta synthesizes estradiol from its precursor DHEA(S) which is derived from both fetal and maternal adrenal glands (124, 140). Excess fetal DHEA(S) supply can drive increased placental estradiol synthesis (110, 124, 141, 142). Because the placenta shunts over 90% of the estradiol it produces into the maternal circulation, maternal serum estradiol levels reflect placental estradiol synthesis (130).

The placenta also influences fetal HPA axis maturation through its production of CRH (pCRH). pCRH stimulates fetal adrenal DHEA(S) production by (1) signaling fetal pituitary ACTH release (143), (2) heightening fetal adrenal gland sensitivity to ACTH (144), and (3) stimulating DHEA(S) producing fetal adrenal cells directly (145, 146). By increasing

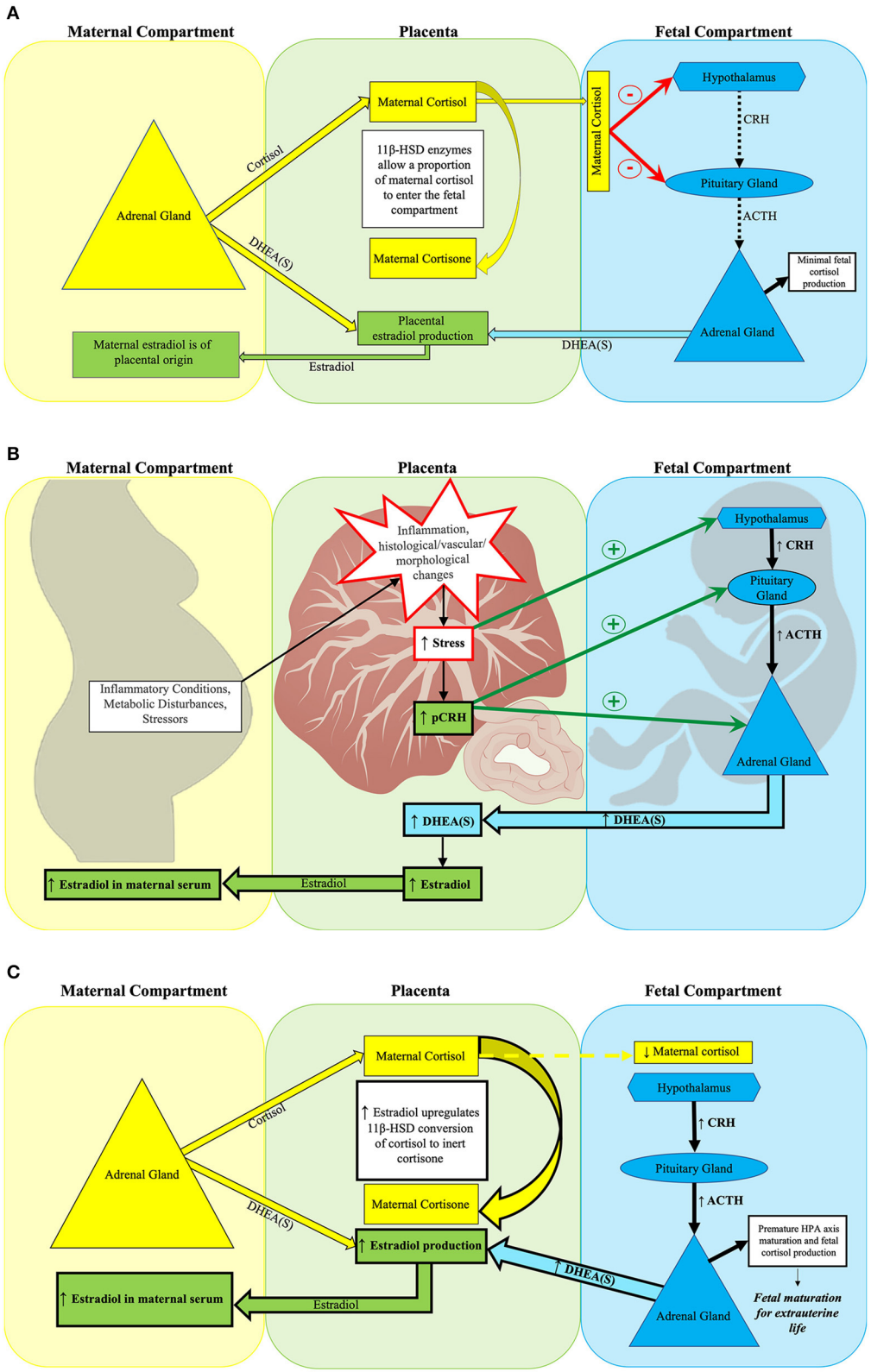

FIGURE 1 | Placental estradiol and the fetal HPA axis at mid-gestation. **(A)** Normal fetal HPA axis functioning in the setting of typical placental estradiol activity at mid-gestation. **(A)** Depicts normal suppression of fetal HPA axis activity during mid-gestation by maternal cortisol. The placental glucocorticoid barrier contains

(Continued)

FIGURE 1 | 11β-HSD enzymes, which control fetal exposure to maternal cortisol (110) by converting most of the maternal cortisol entering the placenta to its inert form, cortisone. While fetal cortisol levels are 5–10 times lower than maternal cortisol levels (127), the maternal cortisol that enters the fetal compartment suppresses fetal HPA axis activity through negative feedback on the fetal hypothalamus and pituitary gland (112). Typically, the fetal adrenal gland has not yet developed *de novo* cortisol synthesis capacity at this point in gestation (124). Rather, the fetal adrenal gland primarily produces DHEA(S) when stimulated by ACTH or pCRH. Fetal DHEA(S), along with maternal DHEA(S), subsequently serves as the substrate for placental estradiol production (115, 128, 129). The placenta shunts over 90% of estradiol produced into the maternal circulation, thus maternal serum estradiol levels by mid-gestation reflect placental estradiol production (130). **(B)** Obstetrical adversity increases placental estradiol production at mid-gestation. Maternal adversity [e.g., stressors (131), inflammation (132–134), and metabolic disorders (135–138)] can increase placental estradiol production by stimulating pCRH release through disrupting placental structure and function. Subsequently, fetal stress increases, which activates the fetal HPA axis. Both pCRH and fetal HPA axis activation increase fetal adrenal DHEA(S) synthesis, leading to higher placental estradiol production. **(C)** Early fetal HPA axis maturation precipitated by excess placental estradiol activity at mid-gestation. Estradiol acts on 11β-HSD placental glucocorticoid barrier enzymes to increase conversion of maternal cortisol to inert cortisone, thereby reducing the amount of maternal cortisol entering the fetal compartment (139). Less maternal cortisol in the fetal compartment reduces its negative feedback on the fetal hypothalamus and pituitary gland. The relative absence of negative feedback precipitates fetal HPA axis maturation, which is characterized by the onset of *de novo* cortisol synthesis capacity by the fetal adrenal gland (125). Higher placental estradiol production leads to elevated maternal serum estradiol concentrations.

fetal DHEA(S) synthesis, pCRH facilitates increased placental estradiol production. As estradiol acts on placental barrier enzymes that modulate fetal cortisol exposure, pCRH can indirectly facilitate precocious HPA axis maturation by decreasing negative feedback from maternal cortisol. pCRH also directly promotes adrenal *de novo* cortisol synthesis (147, 148). Following its maturation, activation of the fetal HPA axis signals adrenal production of both DHEA(S) and *de novo* cortisol.

Inflammation and Hypoxia Stimulate Early Fetal HPA Axis Maturation

Excess inflammation triggered by infection, tissue injury, autoimmunity, or disruption in immunogenic tolerance to the fetus (149) can provoke early fetal HPA axis maturation through increased fetal HPA axis activity (115). Likewise, hypoxia can also stimulate fetal HPA axis maturation through a similar mechanism (150). Considering estradiol's role in strengthening the maternal-fetal cortisol gradient, estradiol serves as a feasible intermediary through which inflammation and hypoxia contribute to precocious fetal HPA axis maturation.

Furthermore, pCRH synthesis is upregulated in response to signifiers of an adverse intrauterine environment, such as elevated cortisol levels, pro-inflammatory cytokines, catecholamines, and decreased uterine blood flow (115, 151, 152). As pCRH promotes ACTH release from the fetal pituitary gland (143) and expression of cortisol synthesizing enzymes (147), elevated pCRH from *in utero* stress can serve as an additional mechanism through which the fetal HPA axis matures precociously (153).

Sex Differential in ASD

One of the strongest risk factors for developing ASD is male sex. ASD prevalence in males is 3 to 4 fold higher than in females (154–156). In the absence of intellectual disability, this ratio increases further to 7:1 (157). Baron-Cohen et al. (158) has proposed the "extreme male brain" theory to explain the significant sex differential in ASD incidence. This theory suggests that excess prenatal androgen exposure contributes to the development of autistic traits in offspring by amplifying traits thought to be more typical of males, such as systemization, while diminishing traits commonly associated the femininity, such as empathy (158). Sex steroid hormones have long been thought to exert profound effects on the sexual dimorphism of the brain (159, 160); however, the exact mechanism has yet to be fully understood.

The "aromatization" hypothesis proposes that the roles of estradiol and testosterone in fetal neurodevelopment are intertwined (161), as masculinization of the rodent fetal brain is dependent upon the conversion of testosterone to estradiol by the enzyme aromatase (162, 163). In primates, reliance on this mechanism to explain masculinization of the human fetal brain remains unproven. Unlike primates, alpha-fetoprotein in rodents has a high binding affinity to circulating estradiol thereby sequestering estradiol and preventing it from entering and masculinizing the fetal rodent brain (164). Therefore, only the sex steroids produced by the fetal rodent influence brain masculinization. The assumption that estradiol's masculinizing role in the rodent fetal brain could be extrapolated to the human fetal brain is significantly flawed because it fails to account for the substantial difference between these mammals in regards to *in utero* estradiol sequestration by alpha-fetoprotein.

Zhao et al. (165) describes a multiple threshold liability model to explain the sex difference in ASD risk. This model theorizes that females require a higher genetic mutation load relative to their male counterparts to develop ASD thus contributing to ASD's male predominance. Werling and Geschwind (157) speculate that these mutations may interact with androgen-related mechanisms in families with affected females, as prior studies have found an association with ASD and genes that modulate sex-steroid function (166–168). As steroid hormone receptors exert epigenetic effects through DNA methylation and histone acetylation (169–172), it is possible that epigenetic modifications in response to *in utero* sex-steroid hormone exposure affect sex-specific neurodevelopmental processes.

Sex-Specific Fetal and Placental Responses to Adversity

Fetal growth, development, and HPA axis programming in the setting of obstetric adversity differ by fetal sex (173–175). These sex-specific responses serve as additional mechanisms in which to consider male ASD predominance (see **Figure 2**). As the placenta is derived from extra-embryonic tissues, the placenta has the same sex as the fetus (178). Evidence suggests that

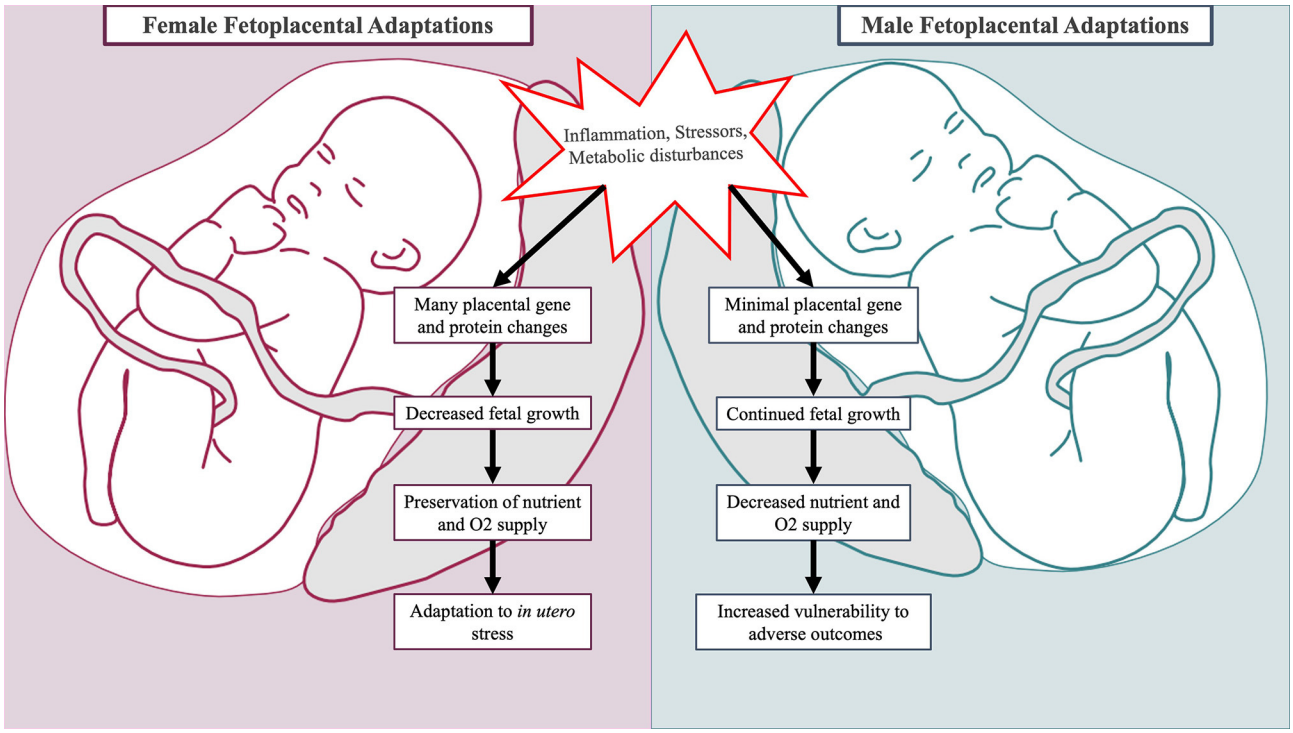

FIGURE 2 | Sex-specific fetal and placental adaptations to maternal adversity. Placentas of male and female fetuses respond differently to mild forms of maternal adversity. In the placenta of female fetuses, multiple changes in glucocorticoid barrier enzyme activity, gene expression, and protein synthesis occur leading to decreased growth (176, 177). This is advantageous as it preserves fetal oxygen and nutrient delivery. In the placenta of male fetuses, minimal changes in gene and protein expression occur, and the male fetus continues to grow incurring increased vulnerability to adverse outcomes (176).

the placentas of male and female fetuses differ in response to adverse prenatal environments through modulation of steroid pathways, placental genes, and protein synthesis (176). Placental growth and structure differ by sex, with male placentas being smaller in size but more efficient at nutrient and oxygen delivery (179, 180). Fetal growth depends upon the limited capacity of the maternal-placental interface to deliver oxygen and nutrients. Thus, greater placental efficiency among males precipitates faster somatic growth while increasing vulnerability to *in utero* perturbations (179, 181). This may have deleterious neurodevelopmental consequences, as fetal brain development relies on the availability of oxygen and nutrients such as fatty acids, glucose, and amino acids (131, 182, 183). In contrast, female placentas may have superior ability to buffer and adapt to suboptimal prenatal conditions (180).

Newborns differ by sex in regards to birth weight, morbidity, and mortality. This is attributable at least in part to sex-specific adaptions that regulate the balance between fetal growth and extra-uterine survival (184, 185). In the setting of mild physical adversity (i.e., chronic maternal asthma), changes in placental 11β-HSD glucocorticoid barrier enzyme activity, gene expression, and protein synthesis occur and coincide with reduced female fetal growth (176, 177). This prepares the female fetus for future adverse events through preservation of oxygen and nutrient delivery. In pregnancies complicated by mild pre-eclampsia, male and female fetuses differ in growth progression mediated by differential fetal microvascular (186) and placental inflammatory cytokine responses (187). In the setting of maternal hyperglycemia, males demonstrate greater hyperglycemic growth stimulation (188) and higher incidences of respiratory distress (175). The sex hormones estradiol and testosterone stimulate opposing processes on fetal lung development: estradiol facilitates surfactant production while testosterone promotes lung tissue proliferation at the expense of lung maturation (189). Collectively, sex-specific responses to obstetrical adversity leave males less physiologically prepared for survival, particularly if birth were to occur prematurely (176).

A growing body of evidence demonstrates a link between placental pathology and ASD. Anderson et al. (43) found increased trophoblastic inclusions in the placentas of fetuses who later developed ASD. Trophoblastic inclusions, a histological finding that results from atypical growth and folding of the placenta (190), are more common in genetically atypical gestations (191–194). Furthermore, studies of high ASD risk cohorts (defined by having at least one older sibling with ASD) also found increased placental trophoblastic inclusions (195) in addition to altered placental morphology (196) and placental chorionic surface vascular networks (197). The specific morphological changes found, such as increased thickness and roundness, may reflect decreased ability to adapt to variations in the prenatal environment (196). Notably, variations within the placental chorionic surface vascular networks may be

the result of atypical vasculogenesis and angiogenesis (197). Several conditions associated with ASD, such as pre-eclampsia (198), intrauterine growth restriction (41), and pre-term birth (16) are also attributed to placental vascular abnormalities (199–201). In ASD individuals, Straughen et al. demonstrated an association between placental inflammation, maternal vascular malperfusion, and ASD (9) that occurred more prominently among males.

Sex-specific placental signaling and epigenetic phenomena in fetal adaption to nutrient availability and maternal stress are exemplified by the X-linked gene O-linked N-acetylglucosamine transferase (OGT) (131, 202). OGT induces glucose-sensitive epigenetic changes that influence immune responses, steroidogenic activity, and fetal development. Because placental X inactivation spares OGT (20) such that the female placenta has two active gene copies while the male placenta has only one, OGT expression and interaction with steroid receptors demonstrate sex-specificity (203, 204). This provides an additional potential mechanism through which maternal metabolic conditions (i.e., hyperglycemia, insulin insensitivity) could influence the sex-differential observed in ASD (20).

STEROID-RELATED BIOMARKERS IN ASD

In utero steroidogenesis occurs through the well-coordinated interactions among the mother, placenta, and fetus (21, 22). The placenta is the main orchestrator of adaptions by the maternofetoplacental unit of steroidogenic activity in response to *in utero* and external environmental cues (205). As the transcriptome of the placenta changes throughout pregnancy (206), expression of placental genes involved in steroid hormone regulation consequentially shift (205). This section is a review of steroid-related biomarkers that have been associated with ASD accompanied by a brief summary of their potential connection to ASD.

Androgens

Multiple studies have demonstrated associations between ASD/ASD traits and amniotic fluid androgen levels (i.e., androstenedione and testosterone) (207–212) supporting the "extreme male brain" theory for ASD's etiology proposed by Baron-Cohen et al. (158). Amniocentesis is typically performed between the 15th and 20th week of pregnancy, overlapping the critical gestational window when the male fetus produces peak amount of androgens to promote genital differentiation (213). This testosterone surge occurs between 11 and 17 weeks gestation (214).

Elevated androgen levels (androstenedione and testosterone) in amniotic fluid collected from pregnancies with male offspring correlated with later ASD diagnoses (212). Decreased eye contact (207), poorer quality of social relationships (211), increased restricted interests (211), reduced empathy (210), and higher autism trait scores (208, 209) have also been associated with elevated amniotic fluid androgen levels. Researchers conducting these amniotic fluid studies applied ASD trait severity measures that they developed, including the Quantitative Checklist for Autism in Toddlers (Q-CHAT) (215), the Childhood Autism Spectrum Test (CAST) (216), and the Child Autism Spectrum Quotient (AQ-Child) (217). In contrast, Kung et al. (218) found no relationship between autistic traits and amniotic testosterone levels in pregnancies resulting in typically developing children using the CAST. Elevated amniotic androgen levels associated with ASD found by Baron-Cohen et al. (212) also did not appear to persist in a re-analysis published in Baron-Cohen et al. (219).

Maternal serum has also been analyzed to investigate the relationship between prenatal androgen levels and the emergence of ASD (19). Maternal serum drawn during early 2nd trimester did not identify significant associations between maternal androgen levels and ASD among offspring (19). Although gestational timing for the maternal serum and amniotic fluid collections overlap, differences in study findings between Baron-Cohen et al. (212) and Bilder et al. (19) may result in part from differences in study purposes and designs. For the maternal serum study, investigators were particularly interested in measuring steroid-related biomarkers as potential intermediaries between ASD outcome and the effects of obstetrical conditions associated with steroid dysregulation (i.e., hypertension and diabetes) which they referred to as "prenatal metabolic syndrome" (PNMS). As such, both the ASD case and non-ASD control groups were enriched (by 50%) for the presence of PNMS exposure. Extrinsic testosterone exposure to the fetus is measurable in maternal serum, though fetal testosterone production cannot be measured in maternal serum because androgen movement across the placenta is generally considered unidirectional from mother to fetus (220, 221). Testosterone levels in amniotic fluid, however, reflect both fetal testosterone exposure and production as the amniotic fluid collected is composed primarily of fetal urine.

In Park et al. (222), no association was found between androgen levels (testosterone, androstenedione, and DHEA) in umbilical cord blood and autistic traits at 12 and 36 months of age among the Early Autism Risk Longitudinal Investigation (EARLI) cohort using the Autism Observation Scale for Infants (AOSI) and Social Responsiveness Scale (SRS), respectively. The EARLI cohort were younger siblings of children with ASD. Several other studies have also failed to demonstrate an overall relationship between umbilical cord testosterone levels and ASD traits (223–225). Unlike the amniotic fluid and maternal serum studies described above, umbilical cord blood samples represent prenatal testosterone exposure at the time of parturition, far after the fetal testosterone surge occurs (214). Upon subdividing the sibling cohort into multiple groups based on proband and participant sex, umbilical cord blood testosterone levels correlated with ASD traits among siblings of female probands. The authors attributed their findings to the multiple threshold liability model (157, 165) suggesting a higher genetic load required for females to develop ASD.

Meconium androgen levels were also measured in the EARLI cohort (226). Findings demonstrated a positive correlation with some androgen levels (i.e., unconjugated testosterone and total DHEA) and SRS scores overall. Following stratification of the cohort by participant and proband sex, various associations were found between androgen levels and ASD traits at 12 and 36

months of age. Meconium begins accumulating in the 13th week of gestation, with the highest volume produced between 28 and 34 weeks gestation (227). As meconium represents cumulative exposures throughout gestation (228), it is difficult to link meconium steroid concentrations to a specific gestational time period of exposure.

Fetal testosterone in males is predominantly produced by the Leydig cells of the testes beginning around 8 weeks gestation (229). Placental human chorionic gonadotropin (hCG), in tandem with luteinizing hormone (LH) produced in the fetal pituitary gland, stimulates testosterone synthesis by the testes during early gestation (214, 230–233). Interestingly, LH and hCG share a common receptor transcribed from a single gene (234). Placental hCG production rapidly increases following implantation until it reaches its apex at the end of the first trimester (235). Following this, hCG levels progressively reach a nadir by 18–20 weeks gestation (236–238). As hCG levels decline, LH levels rise. From 8 to 24 weeks of gestation, the testosterone level in males substantially exceeds that of females (239), which is believed to impart neurodevelopmental effects resulting in sex-specific behavioral differences (240). However, the timing and mechanism in which this occurs in the human fetal brain has not been definitively determined. While the fetal adrenal gland can also synthesize testosterone, the amount is negligible (241, 242) and its contribution to amniotic fluid androgen levels is not clearly established.

hCG

Aside from stimulating testosterone synthesis, hCG serves a variety of functions, including pregnancy maintenance through stimulation of the corpus luteum to secrete progesterone (129, 243). Additionally, hCG has been associated with angiogenesis, mediation of immune tolerance, umbilical cord development, myometrial contraction suppression, and fetal organ growth and differentiation (244–246). To our knowledge, only one study has directly examined 2nd trimester maternal serum hCG levels in relation to ASD risk. Windham et al. (247) found a U-shaped relationship (i.e., both higher and lower levels) between hCG levels associated with increased ASD risk among offspring, particularly in males.

Elevated hCG levels during the 2nd trimester have also been linked to complications such as preeclampsia (248–250), intrauterine growth restriction (IUGR) (248), pre-term delivery (248, 250, 251), low birth weight (250), and fetal death (250, 251). As the placenta secretes hCG in response to stress hormones (252), an adverse prenatal environment may contribute to steroid dysregulation in ASD through elevated hCG exerting its influence on testosterone synthesis in male fetuses. This proposed mechanism of aberrations within the fetal steroid hormonal milieu aligns with findings of elevated 2nd trimester amniotic fluid testosterone levels associated with ASD described above. Interestingly, hCG can also stimulate fetal adrenal DHEA(S) secretion during the 2nd trimester (253). As DHEA(S) is a substrate for placental estradiol synthesis, abnormalities in 2nd trimester estradiol and testosterone linked to ASD development may share a common genesis involving the placental response to adversity.

Estrogens and Progesterone

While prior investigations have focused on androgens, recent research studies have explored the potential role that estrogen and progesterone may play in the development of ASD. Bilder et al. (19) found significantly higher estradiol levels in early 2nd trimester maternal serum associated with ASD among offspring. As described above, both ASD and comparison cohorts were enriched for PNMS exposure. Along with higher estradiol levels, lower SHBG levels were also identified, suggesting the potentiation of estradiol activity in pregnancies associated with ASD as SHBG binds biologically active estrogens rendering them inert (254). Estradiol, rather than estriol, was selected as the estrogen of interest for this study because maternal estradiol levels represent placental estrogen activity more accurately through estradiol's substantially higher potency (255), longer estrogen receptor binding duration (256), and higher maternal serum concentrations (257, 258). Because maternal serum estradiol levels result from, and reflect, placental estradiol production by this gestation window (130), Bilder et al. (19) interpreted these results as indicating increased ASD risk associated with elevated placental estradiol production and activity. Higher placental estradiol production could result from greater fetal steroidogenic activity, and increased placental estradiol activity could facilitate premature fetal HPA axis maturation in the early 2nd trimester. Bilder et al. (19) did not find a significant association between serum progesterone concentrations and ASD risk.

Baron-Cohen et al. (219) also identified higher estrogen levels (i.e., estradiol, estriol, and estrone), along with increased progesterone levels, in 2nd trimester amniotic fluid of male offspring with ASD relative to controls. Notably, elevated estradiol was the most significant predictor of subsequent ASD development. Baron-Cohen et al. (219) attributes the link between higher amniotic estrogens, progesterone, and ASD among offspring to increased fetal steroidogenic activity in ASD's etiologic pathway.

Elevated ASD risk associated with higher prenatal estrogen levels in maternal serum (19) and amniotic fluid (219) contrast with prior study results from Windham et al. (247), which demonstrated lower unconjugated estriol levels in 2nd trimester maternal serum. Estriol is a weak estrogen produced exclusively during pregnancy and requires fetal liver enzyme activity in its synthesis pathway (257, 258). Estriol levels in Windham et al. (247) were initially measured as a component of a prenatal integrated screening test for aneuploidy and neural tube defects. Low maternal serum estriol levels have been used for decades as a clinical indicator for obstetric complications (259), such as placental insufficiency (260), fetal growth restriction (261), pre-eclampsia (262), preterm birth (247), low birth weight (263), and pregnancy loss (260). Windham et al. (247) attributes study findings to the involvement of hormones in the development of ASD through their influence on fetal development and signaling activity in the CNS and immune system.

Cortisol

Baron-Cohen et al. (212) identified elevated cortisol levels in the amniotic fluid collected during the pregnancies of male

offspring who developed ASD, though this finding was not replicated in Baron-Cohen et al. (219). However, Baron-Cohen and colleagues (212, 219) conclude that the findings from both studies support the presence of increase fetal steroidogenic activity in ASD. The significant diurnal variation in serum cortisol concentrations preempts its use as a biomarker in banked maternal serum samples from most large obstetric studies because standardization of serum collection times for these studies is typically not implemented.

Sex Hormone-Binding Globulin (SHBG)

Bilder et al. (19) found an inverse relationship between maternal serum SHBG levels and risk for diabetes/hypertension exposure and ASD. The highest SHBG levels were measured for the cohort with neither PNMS exposure nor ASD. Conversely, the lowest SHBG levels occurred in the cohort with both PNMS exposure and ASD. In addition to binding estrogens and testosterone, SHBG also serves as a serum biomarker for insulin sensitivity that supersedes its relationship with sex hormones regardless of sex, age, and pregnancy status (264). Even among pregnancies without clinical PNMS manifestations, SHBG levels were lower in the ASD cohort compared to either non-ASD cohorts, suggesting the presence of exposure to a subclinical metabolic condition during gestation in these offspring who developed ASD. Unlike most serum biomarkers for insulin sensitivity, SHBG measures require no specific collection time (e.g., time of day or proximity to caloric consumption) to ensure meaningful results interpretation. SHBG has been studied as a predictive 1st and early 2nd trimester biomarker for the emergence of gestational diabetes (265–267). Thus, SHBG is a useful biomarker for investigating maternal insulin sensitivity as a prenatal risk factor within maternal serum samples banked for non-specific purposes.

"Estimated Fetal DHEA"

As a *post hoc* analysis, Bilder et al. (19) calculated a value from maternal serum measurements of estradiol, DHEA, and DHEAS to estimate the relative contribution by the fetal adrenal gland to the DHEA substrate supply for placental estradiol production (EF-DHEA). The area under the curve for EF-DHEA used to predict ASD among offspring exceeded those of its measured components. This was interpreted to indicate that the relationship between elevated maternal serum estradiol and increased ASD risk may in part be driven by excess fetal adrenal activity in early 2nd trimester. The EF-DHEA calculation was created specifically for this study and has not been validated in animal models nor have these findings been replicated. However, EF-DHEA findings are consistent with the amniotic fluid results, particularly elevated cortisol, from Baron-Cohen et al. (212) demonstrating an association between ASD among offspring and increased fetal steroidogenic activity during this gestational window.

Synthesis of Pertinent Biomarker Findings

Figure 3 links heightened *in utero* stress from inflammation, stressors, and metabolic disturbances to perturbation within the prenatal hormone milieu. Through pCRH stimulation, the placenta upregulates fetal HPA axis activity in response to *in utero* stress. Subsequently, the fetal adrenal glands increase DHEA(S) synthesis leading to elevated placental estradiol production. Higher placental estradiol and pCRH production promotes HPA axis maturation denoted by fetal adrenal *de novo* cortisol synthesis. In response to *in utero* stress, the placental also increases hCG production which stimulates fetal gonadal testosterone synthesis.

DISCUSSION

This review provides an up-to-date synopsis of the current evidence and supported theories regarding the role that the *in utero* steroid environment may play in ASD pathophysiology. Considering placental steroid hormone biosynthesis, metabolism, and transport (205), prenatal steroid dysregulation may be attributed to disruptions in vital components of placental structure and function in response to an adverse maternofetoplacental environment. In particular, stress, inflammation, and metabolic abnormalities can contribute to morphological and functional placental changes that affect nutrient and oxygen exchange, the protective transplacental barrier, and hormone synthesis (268, 269).

Obstetric conditions associated with ASD that may contribute to an adverse prenatal environment include hypertensive disorders of pregnancy (i.e., chronic, gestational, *de novo* or superimposed preeclampsia) (13, 14, 35–37), maternal immune dysfunction (5, 7–9), pre-existing/gestational diabetes (3, 10), pre-pregnancy obesity (11), gestational weight gain (12), and PCOS (4, 15). Notably, common indicators or complications of placental insufficiency also overlap with ASD risk factors, including small for gestational age/*in utero* growth restriction, prematurity, maternal infection, and maternal metabolic syndrome (16, 38–42). As the placenta is critical for fetal growth and development, adverse prenatal environments that impact the placenta may increase fetal stress and influence fetal programming, thereby contributing to ASD etiology.

The placenta shares the sex of the fetus as it is derived from extra-embryonic tissues (178). Numerous studies have documented that placentas of male and female fetuses differ in response to adverse prenatal environments (270–273). Specifically, placental signaling and gene expression influence sex-specific differences in fetal adaption to the *in utero* environment related to nutrient availability, maternal stress, and immune response (131, 202). In the setting of mild adversity, ongoing somatic growth of male fetuses confers increased vulnerability to subsequent adverse events (176, 177) that can influence neurodevelopment. Additionally, evidence of placental pathology appears more prominent from pregnancies of male offspring with ASD compared to those of their female counterparts. Further investigation of placental pathology in ASD is needed, particularly as it relates to fetal/placental sex differences.

Second trimester serves as a critical time for fetal steroidogenic activity in regards to fetal HPA axis maturation. Multiple ASD epidemiologic studies highlight the 2nd trimester as the period

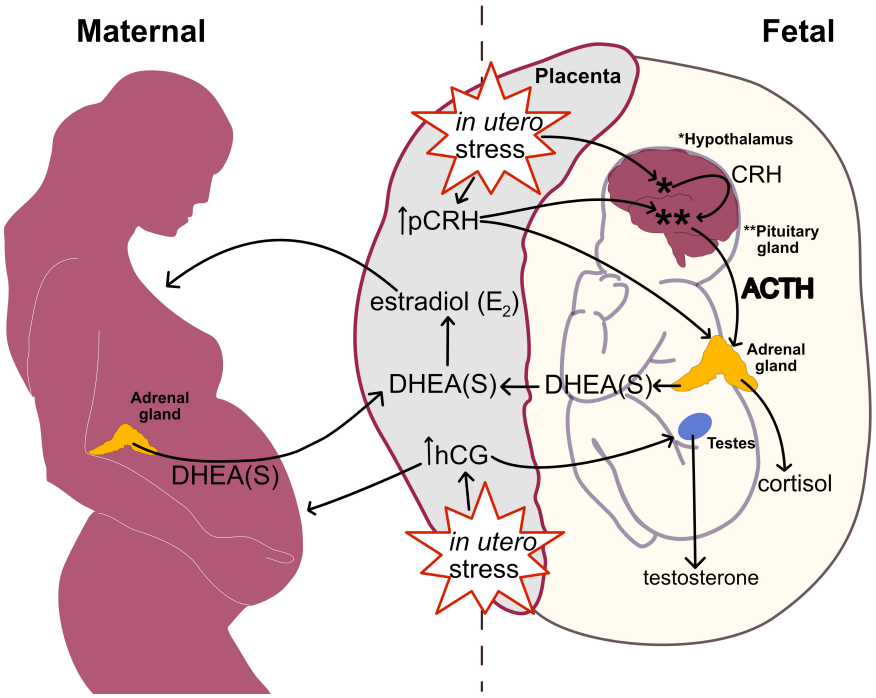

FIGURE 3 | Linking *in utero* stress to increased fetal steroidogenic activity and ASD biomarkers at mid-gestation.

of greatest fetal vulnerability to stressful maternal life events (5, 6, 63). Bilder et al. (19) and Baron-Cohen et al. (212, 219) interpret the link between higher estrogen levels and ASD risk as an indicator of elevated fetal steroidogenic activity in the 2nd trimester of offspring who develop ASD. While Baron-Cohen et al. (212, 219) conceptualize fetal steroidogenic activity more broadly, particularly in regards to fetal androgen production, Bilder et al. (19) focuses on excess fetal adrenal activity and premature fetal HPA axis maturation. By strengthening the maternal-fetal cortisol gradient, placental estradiol reduces the amount of maternal cortisol in the fetal compartment (139) thereby easing maternal cortisol suppression of the fetal HPA axis and promoting its maturation. Fetal steroidogenic activity is enhanced by CRH released by the fetal hypothalamus and placenta (124, 145, 274–276). CRH production is stimulated by stress, inflammation, and hypoxia providing a mechanism through which obstetrical adversity could contribute to increased fetal steroidogenic activity and early HPA axis maturation (115, 150, 277).

The relationship between higher 2nd trimester estrogen levels and increased ASD risk is intriguing as estrogen is widely seen as an indicator of maternal and fetal health. Estradiol acts to preserve fetal viability during *in utero* stress through suppression of inflammation (278, 279) and uterine artery dilation that increases oxygen and nutrient supply to the fetus (280, 281). Estradiol also mediates several critical factors in neurodevelopment providing an overall neuroprotective benefit (162). From the perspective of fetal viability, excess estradiol in the early 2nd trimester can stimulate precocious fetal HPA axis maturation in preparation for extrauterine life. As described above, low—rather than high—maternal serum estrogen levels predict several conditions related to maternal and fetal adversity. Therefore, it is possible that elevated estradiol in early 2nd trimester maternal serum and amniotic fluid may be the result of a compensatory rather than primarily pathologic mechanism. However, it must be noted that the historical obstetric practice of using a synthetic estrogen to foster healthy pregnancies was quite misguided. The use of the synthetic estrogen diethylstilbestrol (DES) in pregnant women during the mid-1900's was halted when exposed adolescent and young adult female offspring were found to develop a rare type of cancer, vaginal adenocarcinoma (282). Subsequent studies have found transgenerational adverse effects of DES exposure, as reproductive tract and immunologic abnormalities have occurred in exposed male and female offspring (283). Grandchildren of exposed women were also found to have a higher incidence of ADHD (284). To our knowledge, there have been no studies published that report on an epidemiologic investigation of ASD risk associated with DES exposure. Although DES exposure differs in many respects from increased placental estrogen synthesis, such a study could improve understanding of estrogen's potential role in ASD's etiology.

An early theory connecting the prenatal steroid environment with ASD conceptualizes ASD as an extreme manifestation of male cognitive traits and implicates excess *in utero* androgen exposure in ASD's etiology (158). Although elevated amniotic testosterone levels were associated with the presence of ASD traits in most (207–211), but not all studies (218), results regarding ASD diagnosis and testosterone concentrations have been more variable (212, 219). Two amniotic fluid studies and a maternal

serum study found no relationship between testosterone and ASD (19, 218, 219). Results from umbilical cord blood and meconium analyses were mixed (222, 226). Disparate findings on androgen biomarkers may be attributable to differences in sample type, gestational timing, ASD outcome measures, and cohort characteristics. Additional investigations are needed which implement multiple sampling strategies and gestational time points in a large obstetrical cohort to clarify the relationship between androgen levels throughout pregnancy and ASD risk.

Overall, prior research findings have established that an altered prenatal environment is a component of ASD pathogenesis. However, the exact process detailing the interaction between diverse prenatal factors and increased ASD risk remains unclear. While several studies link various aspects of perturbations *in utero* to ASD development, to our knowledge, there has been a lack of a unifying paradigm. Based on our current understanding, we propose that disruption within the maternofetoplacental unit initiates a causal sequence resulting in placental changes, thereby influencing fetal programming, steroid hormone modulation, and HPA axis development in ASD.

Factors that contribute to disruption within the maternofetoplacental unit include metabolic disturbances, inflammatory conditions, and stressors. These broad categories are manifested by several obstetric conditions that are associated with ASD development. The placenta reacts to changes *in utero* through adaptations that differ based on sex, which are disadvantageous to male fetuses. Fetal programming is affected, which may have neurodevelopmental implications. Considering the placenta's endocrine functions, steroid hormone modulation is also impacted which could alter HPA axis development and functioning. This may be relevant to HPA axis dysregulation found in some individuals with ASD.

Current literature findings support multidisciplinary research efforts to investigate the manner in which the *in utero* steroid milieu and fetal steroidogenic activity interact with genetic/epigenetic, environmental, inflammatory, and obstetrical ASD risk factors. Studies examining these interaction effects on fetal neuroendocrine development may identify feasible intervention targets that could reduce risk for neonatal morbidity/mortality and childhood neurodevelopmental disabilities. In particular, elucidating the link between the *in utero* steroid environment and immune response may provide an opportunity to optimize both immediate outcomes and long-term neurodevelopmental functioning.

AUTHOR CONTRIBUTIONS

WW conceptualized and designed all components of the paper, drafted the initial manuscript, reviewed, and revised the manuscript. SD conceptualized the obstetrical components of the paper, reviewed, and revised the manuscript. DB supported the conceptualization of all components of the paper, reviewed and revised the manuscript. All authors approve the final manuscript as submitted and agree to be accountable for all aspects of the work.

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microRNAs and Gene–Environment Interactions in Autism: Effects of Prenatal Maternal Stress and the *SERT* Gene on Maternal microRNA Expression

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Background: Genetics and environment both are critical in autism spectrum disorder (ASD), but their interaction (G × E) is less understood. Numerous studies have shown higher incidence of stress exposures during pregnancies with children later diagnosed with ASD. However, many stress-exposed mothers have unaffected children. The serotonin transporter (*SERT*) gene affects stress reactivity. Two independent samples have shown that the association between maternal stress exposure and ASD is greatest with maternal presence of the *SERT* short (S)-allele (deletion in the promoter region). MicroRNAs play a regulatory role in the serotonergic pathway and in prenatal stress and are therefore potential mechanistic targets in this setting.

Design/methods: We profiled microRNA expression in blood from mothers of children with ASD, with known stress exposure during pregnancy. Samples were divided into groups based on *SERT* genotypes (LL/LS/SS) and prenatal stress level (high/low).

Results: Two thousand five hundred mature microRNAs were examined. The ANOVA analysis showed differential expression (DE) of 119 microRNAs; 90 were DE in high- vs. low-stress groups (stress-dependent). Two (miR-1224-5p, miR-331-3p) were recently reported by our group to exhibit stress-dependent expression in rodent brain samples from embryos exposed to prenatal stress. Another, miR-145-5p, is associated with maternal stress. Across *SERT* genotypes, with high stress exposure, 20 significantly DE microRNAs were detected, five were stress-dependent. These microRNAs may be candidates for stress × *SERT* genotype interactions. This is remarkable as these changes were from mothers several years after stress-exposed pregnancies.

Conclusions: Our study provides evidence for epigenetic alterations in relation to a G × E model (prenatal maternal stress × *SERT* gene) in ASD.

Keywords: autism spectrum disorder, prenatal stress, miRNA, epigenetics, dopamine, gene x environment

INTRODUCTION

The developmental origins of health and disease (DOHaD) hypothesis proposes that environment experiences during development *in utero* influence health after birth (1). Numerous studies demonstrate that adverse environmental exposures affect neurological development, including those which are salient to autism spectrum disorder (ASD). Genetics is a critical factor in ASD (2–6). However, importance of environmental factors is being increasingly recognized (7), with heritability estimated at 0.87 by the latest, more conservative analysis (8). While progress has been made toward the understanding of genetic factors, including development of animal models based on these genes (9–12), environmental factors are less understood.

Psychological stress during pregnancy impacts behavioral and developmental outcomes in humans (13). Early personality development in the child, schizophrenia, and emotional disturbances in offspring are all associated with maternal stress (14–17). Relationships between maternal stress and a range of adverse offspring behavioral outcomes are reported in animal models, including abnormal behavioral fear responses, as well as abnormal physiological stress reactivity in offspring, which lasts into adulthood (18, 19). Recent evidence suggests that among environmental factors, maternal stress exposure is important in ASD (20–22).

Early studies surveyed for history and timing of prenatal psychosocial stressors corresponding to major life events on the Social Readjustment Rating Scale in mothers of children with ASD, Down syndrome, and typically developing controls. A higher overall incidence of stressors among the mothers who had a child with ASD was found compared to other groups, with a peak in stressors among mothers with a child with ASD at 25–28 weeks of gestation, but not in the other groups (21). This has subsequently been supported by other studies, showing a relationship between the incidence and severity of tropical storms in the United States in Louisiana during the 5th to 6th months of gestation and the incidence of ASD births (22). Larger epidemiological studies support this relationship between prenatal stress and ASD. One Danish national registry study suggested against the presence of an association between maternal bereavement and ASD (23), but the association between maternal bereavement and ASD was present when maternal psychological conditions were included (23). Another Danish national registry study found that maternal psychological conditions were one of the strongest prenatal risk factors for ASD (24). A Swedish registry study also confirmed a relationship between third-trimester prenatal stress and ASD (25). Furthermore, results from the Nurses' Health Study, another large registry study, showed that maternal exposure to partner abuse during pregnancy is strongly associated with ASD (26). Children *in utero* in New York City during the September 11 terrorist attacks were also found to be 7–9% more likely to be in special education classes (27), although no specific data was available regarding ASD diagnoses. A recent meta-analysis has further supported the relationship between prenatal stress and ASD (28). Finally, a recent study reported that children with ASD that were exposed to prenatal stress present with a more severe condition than those with no history of prenatal stress exposure (29). Therefore, a better understanding of the relationship between prenatal stressors and gene × environment (G × E) interaction in ASD would represent a significant breakthrough, as reviewed recently (30, 31).

A significant proportion of stress-exposed mothers have unaffected children. Several factors could interact with stress exposure to increase impact on neurodevelopment. For example, prenatal exposure to air pollution, which is associated with ASD (32–35), can impact microglia–neuron interactions in a sex-specific manner (36), which may interact with prenatal stress exposure and further impact development. A potential reason why prenatal stressors might result in neurodevelopmental effects only in some cases could be a G × E interaction. One candidate is the serotonin transporter (*SERT*) gene, which is well-studied for its role in stress susceptibility. The *SERT* gene encodes for the SERT protein, which transports serotonin from the synaptic cleft back to the presynaptic neuron (37). Variations in this gene can alter aspects of its function (38–42). The most widely studied variation is an insertion or deletion of 44 base pairs within the promoter region of the *SERT* gene, *SLC6A4*, resulting in a long (L) or short (S) allele, respectively (37, 38, 40). The relationship between the S-allele (SS or LS genotype) and increased risk of depression after exposure to stress has not reliably been demonstrated (43–45). However, presence of the S-allele is also related to suicidality (46), alcoholism (47, 48), and susceptibility to anxiety (40), and greater activation of the amygdala, a brain region which is critical for fear reactions (49). The S-allele of the *SERT* gene has not been consistently linked to ASD itself (50–54), but its role as a gene mediating stress susceptibility remains of interest. It has also been recently demonstrated that maternal serotonin concentrations affect core symptoms and cognitive ability in ASD, with the lowest maternal serotonin levels associated with the greatest severity (55). Linkage studies have also associated rigid-compulsive behaviors in patients with ASD with the region of the genome containing *SERT* (56). A variation in a single nucleotide on the gene, Gly56Ala, is also linked to ASD (42). However, the *SERT* polymorphisms most reliably associated with ASD result in overactivity, rather than a loss of function. Therefore, for a potential role of the S-allele in a G × E interaction in ASD, the superimposed effect of prenatal stress on the maternal *SERT* genotype in this case may be distinct from the mechanism of the *SERT* polymorphisms directly associated with ASD.

The clinical salience of a G × E interaction was explored for *SERT* and prenatal stress exposure, demonstrating that

Abbreviations: ANOVA, analysis of variance; ADHD, attention deficit hyperactivity disorder; ADI-R, Autism Diagnostic Interview-Revised; ADOS, Autism Diagnostic Observation Scale; ASD, autism spectrum disorder; DNA, deoxyribonucleic acid; DOHaD, developmental origins of health and disease; DE, differentially expressed; G x E, Gene x Environment; GATK, Genome Analysis Toolkit; IGFBP-1, insulin-like growth factor-binding protein 1; LOWESS, locally weighted scatterplot smoothing; L, long; miRNA and micro RNA, micro ribonucleic acid; MAF, minor allele frequency; PPARα, peroxisome proliferator-activated receptors α; OGT, O-GlcNAc transferase; PCR, polymerase chain reaction; *SERT*, serotonin transporter; S, short; SNP, single nucleotide polymorphisms; SNVs, Single Nucleotide Variants; WES, Whole Exome Sequencing; wt, wild type.

the relationship between prenatal stress exposure and ASD appears to be mediated by maternal genetic susceptibility to stress, specifically by maternal presence of the S-allele, which has been shown in two independent patient populations (57). Stress surveys were administered to two independent samples of mothers of children with ASD. The *SERT* gene was genotyped for the L-alleles and S-alleles in the mothers. Those individuals that have the LS or SS genotypes (∼64% of the population) are known from previous work (43, 45–49) to have increased stress reactivity and express lower levels of *SERT* than those individuals that have the L/L genotype. Thus, mothers were examined for the presence vs. absence of the S-allele as a genetic marker associated with stress reactivity and the presence vs. absence of prenatal stress according to stress surveys (57).

If the S-allele is a maternal risk factor for development of ASD with exposure to prenatal stress, one might expect that among mothers of children with ASD, a history of prenatal stress exposure during pregnancy would be observed more frequently in presence of the S-allele. In other words, if the association between prenatal stress and ASD was driven by the need for additional presence of maternal genetic susceptibility to stress, then this association between prenatal stress and ASD should be primarily observed in the pregnancies of mothers with this genetic susceptibility to stress. In both samples, the presence of the S-allele and the history of prenatal stress were found to significantly co-segregate in mothers of children with ASD within the critical period of pregnancy suggested in our previous work. To account for the possibility that the S-allele simply conferred increased recall of stress, or for some other reason greater exposure to during pregnancy, prenatal stress exposure history was obtained from the same mothers for the pregnancies of the proband's unaffected sibling. There was no increase in report of prenatal stress exposure regardless of genotype when these same mothers were queried about pregnancies of unaffected siblings. This suggests that the S-allele does not cause an overall increase in recall of stress or stress exposure during pregnancy. Rather, this provides support that the S-allele might serve as a genetic risk factor for increased maternal stress response in association with the development of ASD, and the effect is specific to exactly the same timeframe as reported by previous research using self-report measures and also the timeframe suggested by the Louisiana storms (21, 22, 57).

An animal model was developed to facilitate exploration of mechanism and experimental manipulations directed at developing treatment and prevention strategies. Social behavior was examined in the offspring of female *SERT*-heterozygous knockout (*SERT*-het) mice, whose SERT function is reduced 50%, comparable to that observed in humans with the S-allele and are known to have an increased susceptibility to stress (41, 58, 59), which are then exposed to chronic variable stress during gestation. In a 2 × 2 (stress × genotype) experimental design, *SERT*-wild-type (*SERT*-wt) and *SERT*-het dams were exposed to stress during gestation. A control group for each genotype had no stress exposure. This stress paradigm has previously been shown effective based on cortisol measurements but does not cause changes in feeding or body weight (60). Using the three-chamber social approach task (61), a significant maternal genotype × stress interaction was found, with unstressed offspring of wild-type mice spending significantly more time with the novel stranger than prenatally stressed offspring of *SERT*-het dams, supportive of a maternal gene/stress interaction in offspring behavior in the mouse model. These offspring of stressed heterozygous mothers did not demonstrate more general anxiety as assessed by elevated plus maze, suggesting specificity of this avoidance effect to the social domain (62).

To explore the mechanism of these effects, epigenetic markers were explored. Recent research revealed numerous gene expression changes associated with stress exposure. With maternal stress exposure in rodents, placental tissue showed increased expression in peroxisome proliferator-activated receptors α (PPARα), insulin-like growth factor-binding protein 1 (IGFBP-1), GLUT4, and HIF3α, specifically observed in placentas associated with male offspring (63), in addition to O-GlcNAc transferase (OGT) (64), of particular interest given the high percentage males with ASD. MicroRNAs (miRNAs) also play a significant regulatory role in serotonergic pathways (65, 66), immunity (67), and prenatal stress (68–70). Dysregulation of miR-103, miR-145, miR-219, miR-323, and miR-98 is associated with maternal stress (71). Inflammatory responses in the brain may be altered by miR-323 and miR-98 (71). MiR-135 regulates response to chronic stress through interaction with serotonergic activity (72). Furthermore, roles of specific miRNAs have been reported in regulating serotonergic genes (Let-7a) (66), *SERT* (miR-16 and miR-15a) (73, 74), and *SLC6A4* (miR-325) (75). Recent work has shown that parental stress effects on offspring are also mediated by miRNA changes (76, 77).

Previous work explored the miRNA gene profile, expression, and methylation profile in the brains of the offspring of this *SERT*-het/stress model in mice, revealing a striking attenuation of the gene expression and miRNA changes in response to stress in the brains of the *SERT*-het/stress offspring mice in contrast to response to prenatal stress in the brains of *SERT*-wt mice (78). Significantly increased global methylation was observed in *SERT*-het/stress offspring brains, and there were more upregulated miRNA in stressed control mice as compared to wt, but not for *SERT*-het/stress compared to *SERT*-wt. Similarly, there were fewer upregulated genes when *SERT*-het/stress was compared to *SERT*-wt than when stressed control mice were compared to wt. Therefore, with increased methylation (generally suppressing gene expression), and decreased miRNA and overall expression, it appears that the typical epigenetic response to stress in offspring brains is blunted in the presence of maternal *SERT*-het (78).

Given the findings in the G × E mouse model, we began to explore the potential for detecting epigenetic changes in the clinical G × E setting. As an initial investigation in this direction, we examined the miRNA profile in the samples from the mothers in the previously described clinical G × E study to determine if significant changes were detectable in those mothers of children with ASD exposed to stress during pregnancy and whether these were further impacted by the maternal *SERT* genotype. Additionally, we performed exome sequencing of the maternal samples in an exploratory manner to determine whether there

are other maternal factors that might be of interest for potential future exploration.

METHODS

Samples

Thirty-four maternal blood samples from one of the sites in the previous G × E study were examined in this study. We did not have any samples with LS genotype in the low-stress group. Therefore, the samples were divided into five groups based on *SERT* genotypes and prenatal stress level, as shown in **Table 1**. Families with a child diagnosed with ASD under the age of 10 years were contacted from the University of Missouri Thompson Center for Autism & Neurodevelopmental Disorders database. All participants with ASD were below 10 years of age (average age $= 6.8 \pm 1.8$) to maximize the parents' ability to recall information from the prenatal period. Families were invited to provide samples for genetic analysis and complete a questionnaire regarding the prenatal period. All ASD diagnoses were confirmed *via* Autism Diagnostic Interview-Revised (ADI-R) (79) and/or Autism Diagnostic Observation Scale (ADOS) (80) scores. Experimental procedures were approved and conducted in accordance with the University of Missouri Health Sciences Institutional Review Board. Blood was drawn *via* a standard venipuncture from the median cubital vein of the arm. Genomic DNA was obtained from the subjects' whole blood (FlexiGene Kit; Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR was performed as previously described (81). Briefly, the promoter region of the serotonin transporter gene was amplified using the Qiagen PCR kit from 25 ng genomic DNA to determine the *SERT* genotype, as described in a previous study (57), with 35% of mothers revealing the SS genotype, 32% the SL genotype, and 32% the LL genotype from the sample in the present study, where samples from mothers with at least three stressors were selected for the high-stress group, and samples from mothers with 0 or 1 stressor were selected for the low-stress group. At the time of the appointment with the experimenter, mothers completed questionnaires regarding their child with ASD as well as the gestational period in which the stressors occurred for that child. Details of the stress surveys to which the mothers replied are previously described (57). Survey questions obtained information on the child's birth date, pregnancy length, and the occurrence and subjective severity of major stressful events during the pregnancy. A list of common stressors was provided to the subjects to facilitate recall of events that may have occurred during the pregnancy. Severity of each stressor was also recorded using an established ranked scale of typical stressor intensities (21, 57).

TABLE 1 | Comparison groups.

Prenatal stress	SERT genotype	*N*	Group
High	SS	6	G1a
	LS	11	G1b
	LL	5	G2a
Low	SS	6	G2b
	LL	6	G3

Samples were divided into five groups based on SERT genotypes and prenatal stress level.

miRNA Expression Profiling

Lymphocytes from all mothers, varying in *SERT* genotype and degree of stress exposure, were assessed for miRNA expression. Total RNAs were isolated using the mirVana kit (Ambion, Foster City, CA, USA) to capture small RNAs. miRNA profiling was conducted using a previously used service provider (LC Sciences, Houston, TX, USA), which applies an in-house-developed μParaflo® technology platform. Each region of chips used consists of miRNA probes, which detect miRNA transcripts listed in Sanger miRBase Release 21 (http://www.mirbase.org/). Multiple control probes were included on each chip for quality controls of chip production, sample labeling, and assay conditions. Image digitization was done using "Array-Pro Analyzer" (Media Cybernetics, Rockville, MD, USA). Normalization was carried out using LOWESS (locally weighted scatterplot smoothing), a pair-wise regression method, on background-subtracted data. The purpose of the experiment was to identify miRNAs that differ in expression levels (DE miRNAs) among the comparison groups. ANOVA was applied to detect DE miRNAs ($p < 0.05$), considering the variability of the expression levels across all samples (within and among the groups). miRDB was used to identify miRNA predicted target genes (82). Pathway analysis was conducted using the DAVID tool (83).

Exome Sequencing

To explore other effects of maternal genotype, the same service provider was used to run whole-exome sequencing (WES). The Illumina HiSeq 2500/4000 platform was used with 50× coverage depth. Data were analyzed using GATK (Genome Analysis Toolkit) and applying an analytical pipeline that included removing low-complexity sequences, aligning of sequence against reference human genome, excluding duplicate reads, sorting nucleotide sequence alignment, and base quality score and variant calling [INDEL and single-nucleotide variants (SNVs)]. Annotation and filtering of variants included minor allele frequency filtration (MAF < 0.05 using databases such as dbSNP, 1000 Genome, TopMed, and ExAC). False-positive variants commonly seen in WES were sorted out and removed, using recommendation by Fuentes Fajardo et al. (84). The functional effect of variants was assessed using prediction programs (SIFT and PolyPhen2), for retained variants with MAF < 5% and residing within the coding regions or 10 bases upstream/downstream from splicing junctions. Variants were divided into three categories based on their predicted effect on the protein function, with the following description: high-impact effects (splice site acceptor, splice site donor, start lost, exon deleted, frame shift, stop gained, stop lost), moderate-impact effects (non-synonymous coding, codon change/insertion/deletion, UTR 5′/UTR 3′ deletion), and low-impact effects (synonymous/non-synonymous start/stop, start gained, synonymous coding). We focused on high-impact variants.

TABLE 2 | Eight DE microRNAs candidate for stress × SERT interactions (stress upregulated = red, downregulated = blue[c]).

		SERT genotypes				
		High stress			Low stress	
microRNA	*P*-value	SS	LS	LL	SS	LL
hsa-miR-6125	0.000009	3,952	7,249	5,973	1,854	2,854
hsa-miR-4787-5p	0.00002	3,086	5,952	6,561	1,601	2,325
hsa-miR-663a	0.00002	100	242	177	38	70
hsa-miR-7704	0.0001	6,175	11,183	7,739	2,281	4,289
hsa-miR-1224-5p[a]	0.001	1,491	1,814	1,663	309	485
hsa-miR-664b-5p	0.002	308	548	787	115	80
hsa-miR-331-3p[a]	0.002	363	457	398	976	1,018
hsa-miR-145-5p[b]	0.01	209	387	471	675	660

*See **Table 1** for grouping and sample sizes[c].*
[a]Exhibited a stress-dependent expression pattern in rodent brain samples from embryos exposed to prenatal stress (78).
[b]Has been reported in association with maternal stress (71, 85).
[c]Numbers represent normalized average expression level (signal intensity on the array).

TABLE 3 | Functional annotation of the predicted target genes for eight DE microRNAs listed in **Table 2**.

Term	Count	*P*-value	Fold enrichment	Bonferroni	Benjamini	FDR
Dopaminergic synapse	22	5.46E-06	3.1	0.001	0.001	0.007
Amphetamine addiction	14	3.82E-05	3.9	0.010	0.003	0.050
Cocaine addiction	11	3.32E-04	4.0	0.081	0.012	0.432
Glutamatergic synapse	16	1.22E-03	2.6	0.267	0.026	1.582
Circadian entrainment	14	2.04E-03	2.7	0.404	0.036	2.619
Cholinergic synapse	15	3.40E-03	2.4	0.579	0.053	4.337
Alcoholism	16	6.76E-03	2.2	0.821	0.069	8.455
Serotonergic synapse	14	7.52E-03	2.3	0.853	0.071	9.368
GABAergic synapse	12	7.94E-03	2.5	0.868	0.072	9.861

Using miRDB a total of 1,074 target genes were predicted for the DE microRNAs and DAVID was used for functional annotation of these genes. Count refers to the number of genes from the input list (i.e., predicted targets for the eight DE miRNAs) annotated with a given term.

RESULTS

MicroRNA expression in blood samples ($n = 34$) from mothers of children with ASD, with known pregnancy stress history, was profiled. Comparisons were conducted based on *SERT* genotypes (LL, LS, and SS) and prenatal stress level (high vs. low). Among the 2,500 mature miRNAs examined in all five groups, 119 miRNAs were found to be differentially expressed (DE). Ninety of the DE miRNAs (76%) showed a different pattern of expression in high vs. low stress-exposed groups (suggestive of being stress-dependent miRNAs); 77 (86%) of them were upregulated by stress and 13 (14%) were downregulated in the high- vs. low-stress groups, as shown in **Supplementary Table 1A**. Out of these DE miRNAs, the following three have been previously reported in association with stress: miR-1224-5p and miR-331-3p were found to be stress-dependent in offspring mouse brains by our group (78), and miR-145-5p has been reported in association with maternal stress (71, 85). Our previous work had suggested that prenatal stress exposure interacts with maternal stress susceptibility associated with the *SERT* genotype. To further explore this interaction, miRNA profiles were also assessed in the three groups exposed to a high level of prenatal stress, stratified by *SERT* genotypes. This analysis detected a smaller number of DE miRNAs ($n = 20$), as shown in **Supplementary Table 1B**. Moreover, five out of 20 (miR-663a, miR-664b-5p, miR-4787-5p, miR-6125, and miR-7704) were shared with the stress-dependent miRNAs, making them potential candidates for the *SERT*/stress mechanism.

For target predictions, we prioritized those DE miRNAs that are most likely to be associated with this G × E interaction model. To do so, we compiled a list of eight DE miRNAs (**Table 2**), including the five candidates for stress × *SERT* genotype interactions and the three that have been previously associated with stress, discussed above. A total of 1,074 genes were predicted for these eight DE miRNAs. Two hundred thirteen of them were annotated in the OMIM database. The leading functional target for these genes was the dopaminergic synapse, congruent with our effects on dopamine in the striatum described below in the gene × stress model (86), in addition to pathways associated with addiction and other neurotransmitter systems including

TABLE 4 | High-impact recurrent SNVs identified in each group.

Prenatal stress level	Gene	Variant	Chromosome	Group	Subject ID	Annotation	HGVS.c	HGVS.p	SERT genotype
High	AMY1C	rs140363602	chr1	G1a	10M, 51M	stop_gained	c.1054C>T	p.Arg352*	SS
	CPA4	rs145012020	chr7	G1a	10M, 25M	stop_gained	c.777G>A	p.Trp259*	
	GC	rs76781122	chr4	G1a	10M, 51M	start_lost	c.3G>T	p.Met1?	
	NOTCH2NL	rs140871032	chr1	G1a	10M, 44M	stop_gained	c.220C>T	p.Arg74*	
	NOTCH2NL	rs374113588	chr1	G1a	10M	stop_gained	c.154C>T	p.Arg52*	
	KIAA1919	rs117505745	chr6	G1b	32M, 53M	stop_gained	c.545T>A	p.Leu182*	LS
	LILRA1	rs150508449	chr19	G1b	23M, 31M	stop_gained	c.781G>T	p.Gly261*	
	LRRC9	rs368587449	chr14	G1b	32M, 48M	stop_gained	c.3781C>T	p.Arg1261*	
	LRRC9	rs35427175	chr14	G1b	45M	stop_gained	c.3113G>A	p.Trp1038*	
	NOMO2	rs200294351	chr16	G1b	28M, 31M, 53M	stop_gained	c.2122G>T	p.Glu708*	
	NOTCH2NL	rs140871032	chr1	G1b	22M, 46M	stop_gained	c.220C>T	p.Arg74*	
	OLFM4	rs34067666	chr13	G1b	12M, 22M	stop_gained	c.640C>T	p.Arg214*	
	RHBDD3	rs138870856	chr22	G1b	23M, 48M	stop_gained	c.867G>A	p.Trp289*	
	SULT1C3	rs112050262	chr2	G1b	46M, 53M	stop_gained	c.108G>A	p.Trp36*	
	VCX3B	rs5978242	chrX	G1b	23M, 46M	splice_donor_variant	c.387+1G>C	NA	
	AMY1C	rs140363602	chr1	G2a	27M, 43M	stop_gained	c.1054C>T	p.Arg352*	LL
	PRSS1	rs147366981	chr7	G2a	13M, 27M	stop_gained	c.166C>T	p.Gln56*	
	SULT1C3	rs112050262	chr2	G2a	27M, 43M	stop_gained	c.108G>A	p.Trp36*	
Low	NIT1	rs76502631	chr1	G2b	04M, 21M	splice_donor_variant	n.96+1G>A	NA	SS
	CBWD1	rs199631831	chr9	G3	29M	splice_acceptor_variant	c.2961C>G	p.Tyr987*	LL
	CBWD1	rs199901774	chr9	G3	15M	splice_donor_variant	c.816+1G>T	NA	

Functional effect of variants was assessed using SIFT and PolyPhen2 programs.

glutamate, GABA, the serotonergic system, and the cholinergic system (**Table 3**). Additionally, *DYRK1A* was a predicted target for three of these miRNAs (miR-1224-5p, miR-145-5p, and miR-663a).

Furthermore, we found that high-stress groups exhibited a notably elevated level of high-impact SNVs compared with the low-stress groups, as shown in **Supplementary Table 2**. When comparing groups in the two extreme ends (G1a vs. G3), total number of SNVs per subjects were 4.9 times more in high-stress-exposure mothers with the SS genotype than those with low-stress-exposure and the LL genotype. SNVs seen in more than one subject in each group are listed in **Table 4**. In this list, we prioritized SNVs identified in G1a, the high-risk genotype (SS) group. Recurrent variants in four genes, *AMY1C*, *CPA4*, *GC*, and *NOTCH2NL*, were observed in this group. The *NOTCH2NL* and *AMY1C* variants were also detected in the LS and LL genotype groups, respectively.

DISCUSSION

Our previous work supported a specific gene and stress interaction in the development of ASD (57). The present study provides evidence for epigenetic alterations in relation to a promising G × E model (prenatal maternal stress × *SERT* gene) in ASD detected in maternal blood samples. These findings are remarkable as these changes were detected in samples from mothers several years after stress-exposed pregnancies. Previous work has demonstrated an altered miRNA expression in the brains of offspring mice exposed to maternal stress and altered maternal serotonin transporter genotype (heterozygous KO) (78), with some of the same miRNA differentially expressed in the present study with human maternal blood. Additionally, DAVID revealed that the leading functional target of the miRNA differentially expressed by G × E in the maternal clinical samples was the dopaminergic synapse. This is of particular interest with the growing interest in the role of dopamine in social behavior (87), and the potential role for this as a treatment target in this subset of cases with ASD. The effects on dopaminergic synapse targets are also of particular interest given our recent finding that prenatal stress exposure in *SERT*-het mice resulted in increased striatal DA in offspring brains (86). Furthermore, these dopaminergic changes were reversed with DHA (86). The effects on glutamatergic and GABAergic targets are of particular interest in autism, given the importance of the excitatory/inhibitory imbalance in ASD (88), and the effects on serotonergic targets would be anticipated given the inclusion of maternal *SERT* genotype in the G × E interaction.

Persistent miRNA changes have been observed previously in other conditions, such as after cessation of smoking (89). Thus, the maternal miRNA changes observed after prenatal stress exposure associated with ASD appear to be long-lasting. This is an important step forward in our understanding of ASD, whose incidence appears to continue to rise, by identifying potential markers for an etiological subtype. Future work should explore these markers in children as well. Regulation of miRNA stability is currently an underappreciated area of research, but better understanding of its mechanism is likely to contribute to a broader range of regulation that can impact gene expression. New areas of investigation into how gene expression can be controlled by miRNA stability may provide novel advancements in therapeutic applications related to the fluctuations of gene expression associated with human disorders (90).

The finding that *DYRK1A* was targeted by three different DE miRNAs is of interest. *DYRK1A* was identified as a strong risk candidate gene for ASD based on a combination of recurrent *de novo* likely gene-disruptive mutations in affected individuals and their absence/very low frequency in controls (91). *De novo* dominant mutations in *DYRK1A* substantially reduce kinase function and account for ≈0.5% of severe developmental disorders (92). *DYRK1A* was found to be downregulated in samples from women exposed to Superstorm Sandy during pregnancy, across all trimesters (93). Overexpression of *Dyrk1a* causes a major deficit in the level of serotonin in the brain, as well as deficit of dopamine and adrenaline neurotransmitters in a transgenic mouse model for Down syndrome (94).

The exploratory exome sequencing revealed some findings of potential interest. First, the finding of *NOTCH2NL* variants in four individuals with high stress exposure is of particular interest due to its critical role in cortical development and radial glial stem cell proliferation, as well its association with the 1q21.1 distal deletion/duplication syndrome, where duplications are associated with autism (95). Additionally, the finding of *AMY1C* variants in four individuals with high stress exposure is of interest since salivary amylase is significantly correlated with repetitive behaviors in individuals with developmental disorders (96). Among the other SNVs, one variant, rs76781122 in *GC*, detected in two subjects from the high-stress-exposure group, in particular, caught our attention. This variant alters the start codon and creates a stop codon. The *GC* gene encodes a vitamin D-binding protein (VDBP), the major plasma carrier of vitamin D metabolites. A non-synonymous variant, R21L, in this gene has been reported in association with migraine, a condition often triggered by stress factors (97). Several SNPs in *GC* have been shown to effect vitamin D levels (98). The VDBP has also been associated with inflammatory-mediated conditions (99). Notably, vitamin D receptors are highly abundant in brain and involved in several key biological processes, including the serotonin-mediated pathways (100). One possible implication is that pregnant mothers, carriers of rs76781122, may have had decreased levels of VDBP, which further places them at risk for a compromised immune system in a stressful environment. When comparing the results from miRNAs profiling and WES, it was noted that six out of 1,047 predicted target genes (*MADD, EPS15, PCDHGA10, WWOX, PGPEP1L*, and *NDUFA10*) for the DE miRNAs (miR-1224-5p, miR-145-5p, miR-7704, miR-663a, miR-664b-5p, and miR-6125, respectively), also harbor SNVs in the high-stress groups. Further investigations are warranted to assess potential functional relation between these genes and the miRNAs.

Stress precipitates a systemic physiological response that involves inflammatory, cellular, and metabolic processes and their epigenetic regulation. Epidemiological studies have found increased susceptibility to schizophrenia, ASD, or ADHD to be associated with prenatal stress exposure. While exact pathophysiological mechanisms are still unknown, maternal immune activation, alteration of the hypothalamic–pituitary–adrenal axis, and epigenetic modifications regulating gene expression were proposed as potential causes of neuronal proliferation and migration disturbances in the developing fetus, which may lead to the increased susceptibility to these disorders (101, 102). Multiple studies using human whole blood reported that stress may cause shifts in the concentration levels of specific miRNAs and suggest their potential use as biomarkers in human whole blood (103–105).

While maternal exposure to stress during pregnancy is an elusive risk factor contributing to a wide range of ASD-like traits in offspring, a recent study involving children with ASD revealed that exposure to gestational stress can be used as a strong predictor of severity of ASD symptoms ($p = 0.048$) and communication abilities ($p = 0.004$), even after controlling for other variables. Moreover, significant increases in symptom severity were seen with multiple (two or more) prenatal stressful life events (29).

Recent research evidence suggests that miRNAs are both responsive and susceptible to significant environmental insults such as gestational stress and may increase the offspring vulnerability to stress-related psychopathological conditions (85).

The exact route through which maternal stress affects gene expression in the offspring's brain are not yet known, but involvement of epigenetic changes may be one such mechanism (106). Epigenetic processes such as microRNAs are among gene regulatory mechanisms that are influenced by environmental factors such as stress, and identifying their potential mis-regulation will contribute to narrowing the existing gap in our understanding of the mechanism of maternal stress and ASD.

The findings from the exome sequencing, while exploratory in nature, deserve further investigation. It will be critical to determine whether aspects of vitamin D metabolism, known to have a range of effects on offspring during development (107), might also contribute to developmental susceptibility to the effects of prenatal stress, or if the other SNVs might have critical salience, such as the potential for altered stress reactivity among mothers with *AMY1C* variants (96).

To our knowledge, this study is the first to examine a potential mechanism for this specific interaction between genetics and stressors during a specific prenatal period. These findings may serve as evidence of a biomarker for this mechanism, or possibly a common biomarker for several etiologies, which warrants further investigation. As numerous genes as well as *SERT* can affect stress reactivity, exploring epigenetic pathways by which this occurs more broadly will help to identify pathways of action regardless of the specific gene associated with stress exposure. Additionally, it will be critical for subsequent studies to examine the impact of other *SERT* variants, including the L(A) allele (108, 109), as well as others (110, 111). It will also be critical to better understand the time course of development of these markers, from at the time of initial stress exposure through birth, to determine other such markers that might be present earlier in the course. While the apparent persistence of these stress-associated miRNA changes is remarkable, and there is precedent for persistent miRNA changes in other settings (89), one cannot be certain as to the relationship here, and future studies will need to reevaluate these findings at an earlier time point, and eventually during pregnancy in a longitudinal study. However, this would not appear to be due to changes simply resulting from raising a child with ASD, since all samples were collected from mothers of children with ASD, and the miRNA findings herein are due to the effects of the G × E interaction within the population of mothers of children with ASD. We cannot exclude, though, the possibility that other maternal factors, may have contributed such as those identified in the exome sequencing, or it might be due to stress resulting from phenotypical differences in the G × E-associated cases, since previous work suggests that prenatal stress-associated autism can be more severe (29). Thus, future work will be needed to determine whether these miRNA findings might serve as a biomarker that could be targeted in approaches to mitigate the effects of prenatal stress.

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: NCBI Gene Expression Omnibus, accession no: GSE179222.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

DQB and ZT conceptualized and designed the study, coordinated and supervised data management, drafted the initial manuscript, and reviewed and revised the manuscript. ZT led the epigenetics analysis. DQB led the sample and clinical aspects. AS implemented the epigenetics analysis with ZT. AJ assisted with WES data analysis and literature review. DB assisted MT and PH with the genotyping. JN-M assisted with statistical data analysis and interpretation. PH obtained the clinical samples, stress surveys, and with DQB. MT oversaw the genetics for the samples. PH and MT also reviewed and revised the manuscript.

BF coordinated all aspects of the between-site collaboration and reviewed and revised the manuscript. All authors approved of the final manuscript as submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.668577/full#supplementary-material

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CD-1 Outbred Mice Produce Less Variable Ultrasonic Vocalizations Than FVB Inbred Mice, While Displaying a Similar Developmental Trajectory

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The production of ultrasonic vocalizations (USVs) in neonatal mice is a critical means of communication that is used to elicit maternal care. Alterations in neonatal USV production is also an indicator of neurological deficits. However, USVs have been predominately assessed in inbred animals and are significantly understudied in outbred mice, even though outbred animals better represent the genetic diversity of humans and are used in several neurological disorder models. To determine the reproducibility of USVs across models, we compared male and female CD-1 (outbred) and FVB (inbred) mice on postnatal days (PD) 4, 8, 12, 16, and 20. We found that CD-1 and FVB mice displayed a similar developmental trajectory of USVs. However, CD1 mice emitted more USVs on PD 12 than FVB mice. In addition, FVB mice emitted a longer duration of calls on PD 4 and 8 and a higher overall maximum and minimum frequency of USVs than CD-1 mice. No differences in mean amplitude were found between groups. We also detected numerous significant differences between outbred and inbred mice when comparing each group's call composition. We next assessed the relative variability of mouse vocalizations between groups, finding that outbred mice were less variable than inbred mice. For the spectral and temporal characteristics of the USVs, variability was similar between groups. Altogether, we found that CD-1 outbred mice display a similar, if not lower, degree of variability than FVB inbred mice when assessing neonatal USVs.

Keywords: USV, behavior reproducibility, neurodevelopmental disorders, methods, communication, neonatal vocalization

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INTRODUCTION

Vocal communication is a constituent of various species including mice, songbirds, dogs, dolphins, and humans (1–4). Producing vocalizations serves many purposes as they are used in neonates to elicit maternal care and are also used in adults to mark the presence of food, predators, or territory, and to assert social status and reproductive interest (5–9). However, vocalizations are also a reliable indicator of an animal's overall health, with disruptions in vocalizations characterizing numerous neurological diseases. Specifically, altered vocalizations have been found in neurodegenerative disorders such as Alzheimer's disease and frontotemporal dementia, as well as in neurodevelopmental conditions such as autism spectrum disorder,

tuberous sclerosis complex, epilepsy, and Tourette's syndrome (10–15). Importantly, these altered vocalizations in disease states are highly conserved across species, as they are observed in both clinical settings and in preclinical models, further reinforcing their pertinence to, and utility in, neurological conditions (3, 11, 13, 16–20). Therefore, vocal communication not only encompasses an expansive and vital behavior, but one that is highly relevant to human health.

The unique implications of vocal communication have been studied *via* the use of murine models. Vocal communication in mice refers to the production of ultrasonic vocalizations (USVs), which are whistle-like calls emitted between 30 and 90 kHz (21). USVs can be emitted across the lifespan, with pups emitting vocalizations in order to elicit maternal retrieval and adults vocalizing during mating behaviors however, USVs have been most comprehensively and commonly assessed in neonates, due to the consistency of neonatal USVs and the relative ease of their paradigm (22, 23). To date, the majority of both neonatal and adult USV research has used inbred mice due to their genetic stability and limited mouse to mouse variation (24). However, preferentially assessing inbred mouse vocalizations is potentially problematic. Outbred mice, by definition, are genetically complex, which in turn better resembles the complexity of the human genome and therefore may yield results that have a greater generalizability (25, 26). Furthermore, Tuttle et al. (27) assessed trait stability in inbred and outbred mice using 26 measures and found compelling evidence indicating that in most cases (20 out of 26) outbred mice are as variable, if not less variable, than inbred mice, counteracting a perceived strength of the inbred model. Moreover, there is evidence to suggest that more genetically diverse animals are less sensitive to changes in environmental and experimental conditions than their inbred counterparts, indicating that results may be more consistent, and thus more reproducible, across studies utilizing outbred mice (27, 28). However, despite the apparent advantages of outbred mice, few studies have investigated outbred mouse USVs and no study has comprehensively characterized the developmental trajectory of vocalizations in outbred mice and directly compared the USV profiles of neonatal inbred and outbred mice (29, 30).

Due to the importance of communicative behaviors and their pertinence to disease states and implications for human health, it is essential to know if inbred vocalizations, which constitute the majority of the literature, resemble the vocalizations of the more generalizable outbred mice. Furthermore, characterizing the developmental trajectory of outbred vocalizations would not only provide additional context for existing USV studies, but would also contribute to a foundation that other outbred studies could build upon. Therefore, to address these needs, our study assessed the vocalizations of CD-1 outbred mice throughout the neonatal period and compared them to FVB inbred mice from the same background strain.

MATERIALS AND METHODS

Animals and Housing

CD-1 outbred mice and FVB inbred mice (both originally derived from Swiss mice) were purchased from Charles River. The strain of a mouse has been shown to significantly affect the quantity of USVs produced, with some strains innately producing more vocalizations than others (3, 31). In order to minimalize strain dependent variance (to the extent that it is possible when comparing outbred and inbred animals), we used mice that were derived from the same background (Swiss). A total of 60 mice were tested in this study coming from 16 different litters: 15 male CD-1, 15 female CD-1, 15 male FVB, and 15 female FVB mice. This sample size was determined by an a priori power analysis. Mice were toe clipped on PD 4 after USV assessment which allowed specific mice to be identified throughout the course of the study. Mice were weighed following behavioral assessment at each timepoint. All animals were tested during the light cycle between 9 a.m. and 2 p.m. The mice were kept in a climate-controlled colony room on a 12-h light/dark diurnal cycle and given *ad libitum* access to food and water. All test procedures were carried out in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by Yale University's Institutional Animal Care and Use Committee.

Neonatal Ultrasonic Vocalizations

In order to garner a thorough understanding of the USV profile of outbred mice relative to inbred mice, we assessed vocalizations at 5 timepoints: PD 4, 8, 12, 16, and 20. These timepoints were chosen to be in accordance with other (inbred) vocalization development characterization studies (3, 31). Furthermore, while studies have shown that the strain of the mouse may affect the quantity of USVs produced, it has been well-established that virtually all neonatal inbred vocalizations follow a similar trajectory, with USVs increasing after birth, typically reaching a peak around days 7–9 and decreasing significantly at PD 14 (21, 32). Therefore, our selected timepoints encompass the typical trajectory of neonatal vocalizations, allowing for a comprehensive comparison.

USVs were elicited via the maternal separation paradigm which has been previously described (3, 33). Briefly, pups were habituated to a 22 °C testing room for 30 min prior to the testing period. The pups were then separated from their dam and placed into a clean housing cage preheated to an ambient nesting temperature (∼35 °C). Next the pups were individually removed from the housing cage and placed into a clean test cage contained within a 30 × 30 × 20 cm sound attenuating acrylic chamber. Ultrasonic vocalizations were recorded for a 2-minute duration using a broad-spectrum condenser microphone with a range spanning 1–125 kHz (CM16/CMPA, Avisoft Bioacoustics, Glienicke, Germany part #40011) and a recording interface (UltraSoundGate 116Hb, Avisoft Bioacoustics part # 41161/41162), in accordance with prior studies (18, 33). The microphone was suspended above the center of the cage, making the distance between each pup and the microphone approximately 7 inches. The pups were not restrained and were thus allowed to freely move for the duration of the trial. Upon completion of the trial, the test mouse was removed from the test chamber. Once testing concluded for all mice, they were returned to their home cage.

DeepSqueak Analysis

DeepSqueak analysis was conducted as previously described (34). Specifically, DeepSqueak was downloaded from Github and accessed via Matlab 2018a software. The.wav USV files were imported into DeepSqueak and the total analysis length was set to 0, the analysis chunk length to 6, the frame overlap to 0.0001 s, the frequency low cut off to 30 kHz, the frequency high cut off to 120 kHz, and the score threshold to 0. The detection parameters were set to "high recall" to ensure that all of the USVs present were detected. The files were then manually processed, with the tonality threshold being adjusted to optimize the signal-to-noise ratio for each file and the automatic detection boxes being redrawn as needed in order to accurately and consistently detect the spectral and temporal characteristics of the vocalizations. The call type composition per each strain was assessed by manually going through each file and labeling the detected calls. Specifically, the Scattoni call type taxonomy (2011) was used to identify the vocalizations and sort them into 1 of 10 discrete categories based off of internal pitch changes, call length, and call shape (3, 35).

Statistical Analysis

All data was analyzed using IBM SPSS Statistics 21.0 (IBM, USA) or GraphPad Prism 7 software (La Jolla, CA). The differences in the quantity of vocalizations between outbred and inbred mice across timepoints were analyzed with a repeated-measures ANOVA, with group and sex as between-subjects factors and the USVs emitted on PD 4, 8, 12, 16, and 20 as the within subjects variables. A similar analysis was run to analyze the differences in the average duration, minimum frequency, maximum frequency, and the mean amplitude (loudness) of the calls. The above parameters were chosen since each has been shown to be of particular relevance to communicative behaviors as a whole, as well as to atypical communicative behaviors in neurological conditions (3, 11, 13, 36–38). All interactions were clarified using the Tukey HSD post hoc analysis. The call type composition of each group was analyzed with a Pearson Chi-Square, along with individual z-tests, to compare significant call type proportions between groups. Call type composition was assessed on PD 8 to be in alignment with other studies and to maximize our study's points of comparison (3, 39). The relative dispersion of the USVs emitted per each timepoint between inbred and outbred mice were assessed via calculating the mean and standard deviation of the groups, as well as the coefficient of variability (CV), as this has previously been used to compare the behavioral variance between outbred and inbred animals (27). Furthermore, the weights of the mice in both groups were compared via a repeated measures ANOVA. A value of $p < 0.05$, was considered significant for each statistical test, with figures depicting the mean ± standard error of the mean (SEM).

RESULTS

Ultrasonic Vocalization Developmental Trajectory

Vocalization production was assessed over time between two groups, CD-1 outbred and FVB inbred mice, using a repeated measures ANOVA. Thirty mice (15 male and 15 female) were examined per group. When comparing male and female USV production, there was no main effect present for sex, nor any group by sex interaction, and no day by sex by group interaction (data not shown, F and p-values in **Supplementary Table 1**). As a result, data from male and female mice were pooled. We then found that there was a main effect of group, a day by group interaction, and a main effect for the within subjects variable of day ($F_{1,56} = 6.24$, $p = 0.02$, $F_{4,224} = 3.19$, $p = 0.01$, and $F_{4,224} = 46.20$, $p < 0.001$, respectively). *Post hoc* tests found that, for CD-1 outbred mice, there was no difference in the quantity of USVs produced on PD 4 vs. PD 8. However, on PD 12, CD-1 mice emitted significantly more USVs than at any other timepoint. CD-1 mice also emitted significantly fewer USVs on PD 16 than the preceding timepoints, with no USVs being emitted on PD 20. For FVB mice, there was no significant difference in USVs emitted for PD's 4, 8, and 12. However, on PD's 16 and 20 FVB mice emitted significantly fewer USVs, with no USVs again being emitted on PD 20. When comparing across groups, CD-1 mice emitted significantly more USVs than FVB mice on PD 12 ($p < 0.05$), however, there were no significant differences between groups for PD 4, 8, 16, and 20 (**Figure 1A**).

Trajectory of USVs' Spectral and Temporal Characteristics

We next assessed USV characteristics, including duration, minimum frequency, maximum frequency, and amplitude. For all USV characteristics, there was no effect of sex, no day by sex, or group by sex interactions, and no day by sex by group interaction (**Supplementary Table 1**). Female and male data were then pooled. For duration, we observed a main effect of group, an interaction between group and day, and a main effect for the within subjects variable of day ($F_{1,39} = 10.02$, $p < 0.01$, $F_{3,117} = 4.39$, $p < 0.01$, and $F_{3,117} = 28.01$, $p < 0.001$, respectively). *Post hoc* analysis revealed that CD-1 mice emitted shorter mean USVs on PD 4 ($p < 0.05$), with USVs increasing and plateauing on PD 8 and 12 ($p > 0.05$) before significantly decreasing on PD 16 ($p < 0.05$). Meanwhile, FVB mice emitted a shorter duration of USVs on PD 4 ($p > 0.05$), with USV duration increasing on PD 8 ($p > 0.05$) then decreasing on PD 12 ($p > 0.05$), with USVs being at their shortest on PD 16 ($p < 0.05$). When comparing CD-1 to FVB mice, we found that CD-1 mice emitted USVs of a significantly shorter duration on PD's 4 and 8, with no differences between groups on PD's 12 and 16 ($p < 0.05$) (**Figure 1B**).

When assessing the minimum frequency, there was a main effect of group ($F_{1,39} = 35.99$, $p < 0.001$), with CD-1 mice emitting a lower minimum frequency than FVB mice (**Figure 1C**). There was no day by group interaction and no main effect for the within subjects variable of day ($F_{3,117} = 2.03$, $p = 0.11$ and $F_{3,117} = 2.22$, $p = 0.09$, respectively).

When assessing the maximum frequency of USVs, there was a main effect of group ($F_{1,39} = 14.10$, $p = 0.001$), with CD-1 mice emitting an overall lower maximum frequency than FVB mice (**Figure 1D**). There was no day by group interaction ($F_{3,117} = 0.86$, $p = 0.46$). There was a main effect for the within subjects variable of day ($F_{3,117} = 12.65$, $p < 0.001$). Corresponding

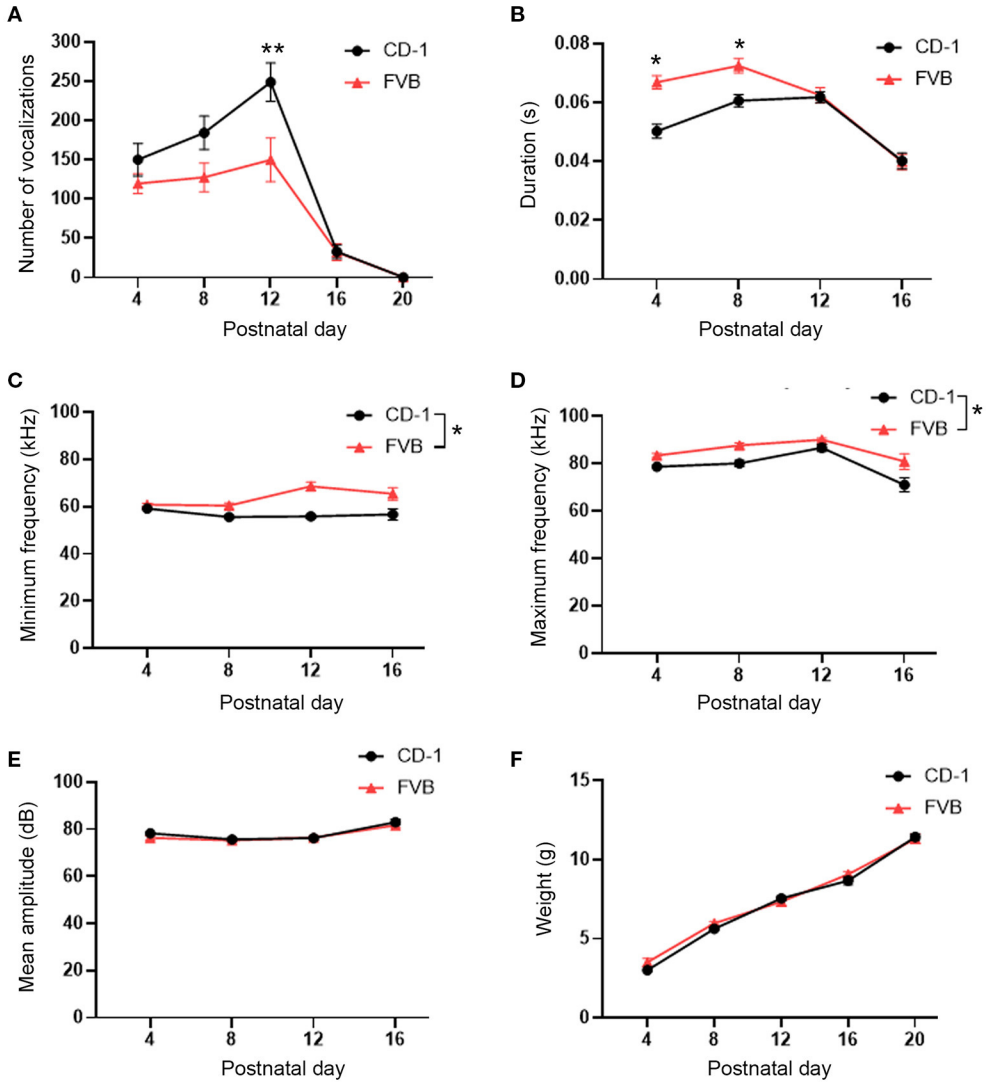

FIGURE 1 | USV developmental trajectory and comparison in CD-1 outbred and FVB inbred mice. **(A)** CD-1 and FVB mice display a similar USV development, however, CD-1 mice emitted significantly more USVs on PD 12 than FVB mice emitted on PD 12, with no other differences present between groups. **(B)** FVB mice emitted USVs of a shorter duration than CD-1 mice on PD 4 and 8, with no other differences present. **(C)** FVB mice emitted USVs of a higher overall minimum frequency than CD-1 mice. **(D)** FVB mice emitted USVs of an overall higher maximum frequency than CD-1 mice. **(E)** There were no differences in mean amplitude between CD-1 and FVB mice. **(F)** Animal's weights increased linearly overtime, with no differences between groups present. A total of 60 mice were assessed, 15 males and females per each group. The data points represent the mean and the error bars represent the standard error of the mean. $^{*}p < 0.05$, $^{**}p < 0.01$.

assessments revealed that the highest maximum frequencies were on PD 12 ($p < 0.05$), with the lowest maximum frequencies being emitted on PD 4 ($p < 0.05$) and PD 16 ($p < 0.05$).

For USV amplitude, there were no main effects of group and no day by group interaction ($F_{1,39} = 0.18$, $p = 0.67$ and $F_{3,117} = .61$, $p = 0.61$). There was a main effect for the repeated measures variable of day ($F_{3,117} = 15.83$, $p < 0.001$). Further tests revealed that vocalizations had the highest amplitude on PD 16 ($p < 0.05$) (**Figure 1E**). Therefore, while there was only a difference at one timepoint between outbred and inbred mice for USV production, there are numerous differences across many timepoints between outbred and inbred mice when assessing the spectral and temporal characteristics of USVs.

Call Type Composition Analysis

In addition to assessing the quantitative characteristics of vocalizations, we also assessed qualitative features of the calls. When examining the call types, a Pearson Chi-Square analysis revealed significant group differences between the composition of calls for CD-1 and FVB mice ($X^2_{8,N=7707} = 203.69$, $p < 0.001$). Proportional differences detected with z-tests found that CD-1 animals emitted a significantly greater quantity of complex, frequency steps, and composite call types, than FVB mice. CD-1 mice also emitted significantly fewer downward, two-component, and chevron call types, with no differences between groups for short, upward, and flat call types (**Figure 2**).

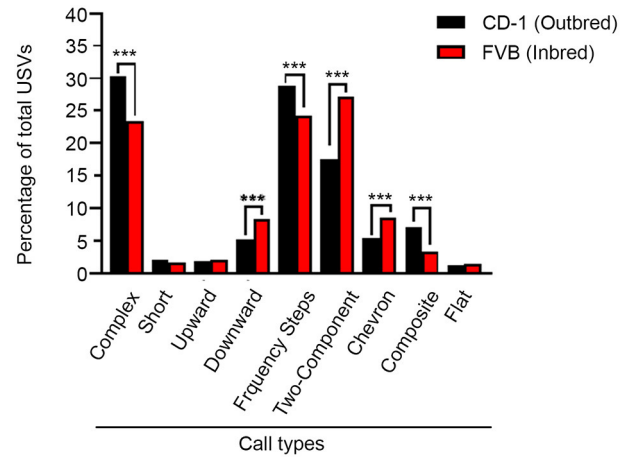

FIGURE 2 | Call type composition for CD-1 and FVB mice. PD 8 CD-1 mice emit more complex, frequency steps, and composite call types than FVB mice but less downward, two-component, and chevron call types. No differences between groups were found for short, upward, and flat call types. ***$p < 0.001$.

TABLE 1 | Variability in USV production per day and in total between groups.

	Ultrasonic vocalization quantity					
	CD-1			FVB		
	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD
PD 4	75.4	150.1	113.1	**57.3***	119.5	68.5
PD 8	**77.9***	127.6	99.4	80.4	126.0	101.2
PD 12	**53.9***	248.8	134.1	101.8	149.8	152.5
PD 16	**146.7***	32.8	48.2	182.1	32.1	58.5
Total	**91.6***	139.8	128.1	103.2	110.2	110.3

**The values with lower variability between groups are bolded. CV, coefficient of variability; $\bar{x}$, mean; SD, standard deviation.*

Weight Assessment Across Timepoints

The weight of a pup may be a factor that differentially affects USV production (40). To rule out this potential confound, the weights of CD-1 and FVB mice were directly compared across each timepoint. A repeated measures ANOVA was run and there was no main effect of group and no day by group interactions ($F_{1,56} = 0.64$, $p = 0.43$ and $F_{4,224} = 3.48$, $p = 0.07$, respectively) There was also no effect of sex for any parameters (**Supplementary Table 1**). However, there was a main effect of day ($F_{4,224} = 518.96$, $p < 0.001$). Tukey's test was preformed to further assess the data and found that the weight of the mice was lowest on PD 4. It also found that the weights of the mice on PD's 8, 12, 16, and 20 were all significantly higher than the preceding timepoints ($p < 0.05$) (**Figure 1F**). Therefore, the lack of difference in weight between CD-1 and FVB mice indicates that the size of the mice did not differentially affect vocal production.

Variability Assessment for the Quantity of Vocalizations

We next assessed the inherent variability of inbred and outbred USVs. The mean, standard deviation, and coefficient of variability were calculated per each timepoint and group and are listed in **Table 1** (the lower CV value between groups is bolded). We found that on PD 4, FVB mice were less variable in their vocal production than CD-1 mice. However, on PD 8, 12, and 16, CD- 1 mice exhibited less variability than FVB mice. We also combined the data and assessed the total variability between groups. We found that CD-1 mice were significantly less variable than FVB mice (>10% difference of CV between groups). Therefore, CD-1 mice emitted a less variable quantity of vocalizations than FVB mice on both a per day basis and overall.

Variability Assessment of the Spectral and Temporal Characteristics of Vocalizations

The mean, standard deviation, and coefficient of variability for the spectral and temporal characteristics of USVs are depicted in **Table 2**. For duration, FVB mice were slightly less variable than CD-1 mice on PD 4 and PD 16. However, CD-1 mice were slightly less variable than FVB mice on PD 12. On PD 8 there was a similar degree of variability between groups. When the variability of the total duration was compared, we found that FVB mice were slightly less variable than CD-1 mice. Therefore, there was minimal variability per day (<6% difference of CV) and overall (<1% difference of CV) between groups when assessing duration (**Table 2**).

When assessing the minimum frequency of the USVs elicited, we found that FVB mice were slightly less variable than CD-1 mice on PD 4, 12, and 16, however, CD-1 mice exhibited less variability than FVB mice on PD 8. When assessing the total variability between groups, FVB mice were slightly less variable than CD-1 mice. Thus, there was minimal variability per day (1–8% difference of CV) and overall (2% difference of CV) between groups (**Table 2**).

For maximum frequency, FVB mice were slightly less variable than CD-1 mice on each day. The total variability for maximum frequency between groups was also similar, with FVB mice being slightly less variable than CD-1 mice. Thus, there was limited variability for maximum frequency between groups per day (<6% difference of CV) and overall (<5% difference of CV) (**Table 2**).

Lastly, for mean amplitude, CD-1 mice were slightly less variable than FVB mice on PD 4. However, FVB mice were less variable than CD-1 mice on PD 12 and 16, and both groups exhibited the same variability at PD 8. When assessing the overall mean amplitude, we found similar variability between groups. Therefore, for mean amplitude, there was minimal variation between CD-1 and FVB mice per day (<3% difference of CV) and overall (1% difference of CV). Thus, across all spectral and temporal parameters, CD-1 and FVB mice displayed similar variability.

DISCUSSION

The present study characterized the developmental trajectory of CD-1 outbred mouse vocalizations and compared them to FVB inbred mice in order to elucidate the role genetic complexity may have on neonatal communicative behaviors

TABLE 2 | Variability in USV characteristics per day and in total between groups.

	Duration						Minimum frequency					
	CD-1			FVB			CD-1			FVB		
	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD
PD 4	25.6	0.050	0.013	**18.9***	0.067	0.013	13.3	59.2	7.9	**5.9***	83.5	5.0
PD 8	18.8	0.061	0.011	**18.4***	0.073	0.013	**7.9***	55.7	4.4	10.9	60.4	6.6
PD 12	**16.5***	0.063	0.010	20.2	0.061	0.012	12.8	56.9	7.3	**11.7***	68.2	8.0
PD 16	34.1	0.040	0.014	**28.4***	0.040	0.011	20.8	56.7	11.8	**16.8***	80.9	13.6
Total	27.4	0.054	0.015	**27.1***	0.062	0.017	14.1	57.1	8.1	**12.4***	63.3	7.8
	Maximum frequency						**Mean amplitude**					
	CD-1			FVB			CD-1			FVB		
	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD	CV	$\bar{x}$	SD
PD 4	10.8	78.7	8.5	**5.6***	60.9	3.4	**5.0***	78.3	3.9	6.1	76.4	4.7
PD 8	9.5	80.1	7.6	**6.7***	87.7	5.9	**5.2***	75.7	3.9	**5.2***	75.7	3.9
PD 12	9.9	86.6	8.6	**3.7***	90.4	3.3	6.4	75.9	4.9	**4.0***	77.0	3.1
PD 16	20.8	71.1	14.8	**16.6***	65.3	10.8	7.5	83.1	6.2	**4.2***	82.2	3.5
Total	14.2	79.5	11.3	**9.3***	85.9	8.0	7.0	78.0	5.5	**6.0***	77.2	4.6

**The values with lower variability between groups are bolded. CV, coefficient of variability; $\bar{x}$, mean; SD, standard deviation.*

and data reproducibility. We found that there was a similar trajectory of USV changes between inbred and outbred mice, with both models displaying an increase in USV production after PD 4 that peaked on PD 12, followed by a significant reduction in USVs occurring on PD 16, with USVs ceasing on PD 20. At 4 of the 5 timepoints, the groups were statistically indistinguishable from one another, providing compelling evidence that results obtained in inbred mice are largely reproducible in outbred animals when the animals are of a similar background strain. However, on PD 12, the quantity of vocalizations emitted was significantly different between groups, with CD-1 mice producing significantly more USVs than FVB mice. This difference in USV production is best explained by the increased genetic complexity of outbred mice relative to inbred mice. Indeed, although CD-1 and FVB mice come from the same background strain, there is still a large degree of genetic variability between them, resulting in a congruent but not identical phenotype between models, accounting for the observed discrepancy. Overall, we found that there are minimal differences between inbred and outbred mice for the quantity of vocalizations produced, indicating that results should be reproducible between models.

When we compared the spectral and temporal characteristics of calls, we found numerous differences, with FVB mice vocalizing for a significantly longer duration on PD 4 and 8 and emitting calls of an overall higher pitch (minimum and maximum frequencies) than CD-1 mice, with no differences present between models for the loudness (mean amplitude) of the USVs. The differences in pitch and duration between models is notable, as alterations in both of these parameters are important constituents of numerous neurodevelopmental disease states. Specifically, murine models of conditions such as autism, epilepsy, or Cowden syndrome have reported fluctuations in call duration and pitch (3, 18, 38). Furthermore, when neonatal cries have been assessed in autistic infants, changes in duration and pitch have also been observed, indicating that these parameters are not only conserved across species, but may be significant indicators of neurodevelopment (13, 37). Additionally, when assessing the call type composition of USVs emitted from CD-1 or FVB mice, we similarly observed numerous differences between groups. Therefore, our data suggests that the spectral, temporal, and qualitative characteristics of early life vocalizations are more susceptible to the inherent differences in outbred and inbred mice than the production of USVs are.

We next assessed the inherent behavioral variability of both models, per day and overall. We found that CD-1 mice were less variable than FVB mice in the quantity of USVs emitted at 3 of the 4 timepoints. Moreover, when we assessed the total variability of each model, we found that CD-1 mice were more than 10% less variable than FVB mice. With respect to the spectral and temporal aspects of the vocalizations, we found that inbred and outbred mice were similarly variable, with there being no more than a 2% difference between groups for the total variability of the duration, maximum frequency, and minimum frequency of the calls, and only a 6% difference for the amplitude. Altogether, this indicates that outbred mice are as, if not less, variable than inbred mice across numerous measures. Importantly, our data also indicates that in measures that have more inherent variability (USV production), outbred mice are significantly less variable than their inbred counterparts, whereas in measures that are inherently less variable (call characteristics), inbred mice are only slightly less variable than outbred mice. Thus, CD-1 mice display an overall more favorable variability index than FVB mice, directly counteracting the common perception that outbred mice are more variable than inbred mice.

Interestingly, similar behavioral variability in outbred mice relative to inbred mice has been previously reported (27). Tuttle et al. (27) assessed adult mice and found that in 20 of 26 behavioral measurements outbred mice were as variable as their inbred counterparts, and perhaps even less variable. Therefore, in both neonates (our study) and adults (27), outbred animals have not been found to be any more variable than inbred animals. This is significant, since outbred mice better resemble the complexity of the human genome and thus display increased external validity relative to inbred mice. Additionally, outbred animals may also be more resistant to minute changes in experimental and environmental conditions than their inbred counterparts (27). Therefore, outbred mice have select advantages over inbred mice and, as our study suggests, fewer disadvantages than previously thought. Collectively, our study supports the growing body of research challenging the perception that outbred mice are significantly more variable than inbred mice (27, 41).

Future studies could expand upon the present work by assessing the variability of other outbred mouse strains relative to inbred strains. This would help to determine if all outbred animals are less variable and therefore more optimal for USV assessment than inbred mice or if CD-1 mice are particularly well-suited to assess communicative behaviors. Additionally, while our study assessed 5 timepoints at 4-day intervals, other studies could assess different timepoints (such as PD 2, 6, 10, 14) to garner a more comprehensive understanding of the subtle nuances between outbred and inbred mouse communicative behaviors. Studies could also assess and compare USV production in adult inbred and outbred mice to determine if increased genetic complexity has a more pronounced effect in mature animals. Altogether, numerous studies have assessed vocalizations and USV development in mice, however, more work needs to be performed if the potential of vocalizations is to be maximized (3, 21, 35, 42).

While the generation of USVs is a vital behavior in mice, there is also compelling evidence indicating that neonatal communicative behaviors are equally important in clinical populations. Specifically, Esposito et al. (43) assessed the crying behaviors of infants and observed that infants with autism are less likely to cry when the parent leaves the immediate environment, and will cry without a known cause. Infants with autism have also been reported to cry at a higher pitch, display shorter crying bouts, and to have an irregular loudness of their cries relative to neurotypical infants (11, 37, 44). While vocalizing behaviors have been mostly studied in clinical ASD populations, altered vocalizations have also been observed in other neurodevelopmental conditions such as tuberous sclerosis complex, epilepsy, and Tourette's syndrome, as well as in neurodegenerative conditions such as Alzheimer's disease and frontotemporal dementia (10–15). Therefore, compelling research is emerging which indicates that vocalizing behaviors, particularly during the neonatal period, have significant ramifications for human health and may constitute an early life behavioral biomarker for various disorders. Thus, the identification of optimal murine models that present with minimal behavioral variability will help to further elucidate vocalizing behaviors, constituting a necessary endeavor with clear applications to human health.

CONCLUSION

Overall, our study assessed the consistency of USVs between inbred and outbred animals and was the first to assess the developmental trajectory of outbred mouse USVs relative to inbred mice, establishing a baseline of comparison for future studies. We found that both inbred and outbred animals from a Swiss background had the same USV developmental pattern and emitted approximately the same quantity of USVs on PD 4, 8, 16, and 20, indicating that USV results obtained in inbred mice should be similar to those obtained in outbred mice. However, there were numerous differences between outbred and inbred mice for the duration, minimum frequency, and maximum frequency of the USVs, as well as for each group's call type composition, suggesting that while USV production may be consistent between groups, the same is not necessarily true for other USV parameters. Importantly, we found that overall CD-1 mice displayed a more favorable variability index than FVB mice. Therefore, our study indicates that although inbred mouse models are valuable, their preferential use may not always be warranted nor necessarily ideal in all cases. Our study also indicates that additional studies need to be conducted that comprehensively examine the relative strengths and weaknesses of both inbred and outbred mice, as we found that common perceptions of models are not always accurate.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Yale University's Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

AB: research design and writing. HS: data analysis. MB: research design, experiment, data analysis, and writing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.687060/full#supplementary-material

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REVIEW
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Signaling Pathways and Sex Differential Processes in Autism Spectrum Disorder

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Autism spectrum disorders (ASDs) are a group of neurodevelopmental disorders associated with deficits in social communication and restrictive, repetitive patterns of behavior, that affect up to 1 in 54 children. ASDs clearly demonstrate a male bias, occurring ∼4 times more frequently in males than females, though the basis for this male predominance is not well-understood. In recent years, ASD risk gene discovery has accelerated, with many whole-exome sequencing studies identifying genes that converge on common pathways, such as neuronal communication and regulation of gene expression. ASD genetics studies have suggested that there may be a "female protective effect," such that females may have a higher threshold for ASD risk, yet its etiology is not well-understood. Here, we review common biological pathways implicated by ASD genetics studies as well as recent analyses of sex differential processes in ASD using imaging genomics, transcriptomics, and animal models. Additionally, we discuss recent investigations of ASD risk genes that have suggested a potential role for estrogens as modulators of biological pathways in ASD, and highlight relevant molecular and cellular pathways downstream of estrogen signaling as potential avenues for further investigation.

Keywords: autism spectrum disorder, female protective effect, estrogens, imaging genomics, animal models, genetics

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INTRODUCTION

Autism spectrum disorders (ASDs) are a group of neurodevelopmental disorders characterized by persistent deficits in social interaction and communication and by the presence of restricted, repetitive patterns of behavior, interests, or activities (1). While the clinical presentation of ASDs may be highly heterogeneous, individuals with ASD often experience difficulties with social-emotional reciprocity and non-verbal communication, stereotyped movements, rigid adherence to routines, and hyper- or hypo-sensitivity to sensory stimuli (1). At present, there are no approved pharmacological treatments that target these core deficits, which is due in part to our limited understanding of their underlying pathophysiology. ASDs have been increasing in prevalence in recent years, with estimates indicating that 1 in 54 children in the United States is affected (2), underscoring the need for improved insights into ASD biology. Recent human gene discovery efforts have been instrumental in this regard, with large-scale whole-exome sequencing studies leading to the identification of at least 102 genes that are strongly associated with risk (3–6). Interestingly, while ASD risk genes encode proteins involved in seemingly divergent functions, such as ion channels, synaptic cell adhesion molecules, and chromatin modifiers, there is growing evidence that these genes converge on common neurodevelopmental pathways, such as the

regulation of gene expression and neuronal communication (6), which are likely to play a central role in ASD biology.

Despite this progress in gene discovery, gaps in our understanding of the biology of ASD remain. ASDs show a clear male bias, occurring at least four times more often in males than females, yet the basis for this male predominance is not well-understood (2). While ascertainment bias favoring the recognition and diagnosis of ASD in males, as well as "camouflaging" of social deficits by females, might contribute, ASD is likely more common in males even after accounting for these factors (7). Interestingly, there is growing evidence from human genetics analyses supporting a "*female protective effect*" in ASD. The female protective effect theory assumes that the risk for ASD is quantitative and follows a normal distribution in the general population, but that females are protected against this risk and thus have a higher liability threshold for ASD diagnosis (5, 7). While the etiology of the female protective effect in ASD is unknown, one hypothesis is that differential exposure to sex steroid hormones may be a contributing factor (7). Interestingly, recent studies analyzing the function of ASD risk genes in animal models, including one from our group, independently identified estrogens as suppressors of ASD gene-associated cellular and behavioral phenotypes, implicating estrogens as potential modulators of biological pathways relevant to ASD (8, 9). However, studies directly assessing the role of estrogens in ASDs, both in clinical and preclinical models, are limited.

Here, we provide an overview of signaling pathways and mechanisms implicated by ASD genetics studies, as well as sex differential biological processes that may contribute to the female protective effect. In addition, we review clinical and preclinical studies suggesting a role for estrogens in ASD and discuss potential mechanisms by which estrogens might interact with relevant signaling pathways and neural cell types. While the effect of environmental exposure to estrogens on brain development has been an area of interest, this is beyond the scope of this review and has been reviewed elsewhere (10, 11). In this review, we focus on signaling pathways that have been implicated by studies of ASD risk genes and consider potential interactions between these pathways and established mechanisms of endogenous estrogen signaling in the brain. Taken together, investigations into the etiology of the increased male to female ratio in ASD—a central feature of ASD—are likely to inform our understanding of ASD pathophysiology more broadly and may provide novel avenues for pharmacological intervention.

ASD RISK GENE DISCOVERY

ASD risk gene discovery has been accelerating at a rapid pace in recent years, made possible by advances in genomic sequencing technologies. Large-scale, whole-exome and whole-genome sequencing studies have led to the identification of a growing list of "high confidence" genes that are strongly associated with ASD and are beginning to reveal common biological pathways. ASDs are known to be highly heritable, with greater concordance rates among monozygotic (50–90%) compared to dizygotic (up to 30%) twins (12–18). Early efforts at gene discovery in ASD led to the identification of genes associated with rare monogenic disorders, including Fragile X syndrome (*FMR1*), tuberous sclerosis complex (*TSC1, TSC2*), Rett syndrome (*MECP2*), and *PTEN* hamartoma tumor syndrome (*PTEN*) [reviewed in (19)]. Additional early studies using molecular cytogenetics and sequencing approaches led to the discovery of some of the first ASD-associated genes, including *NLGN4X* and *NRXN1,* which encode synaptic cell adhesion molecules (20–24), and *SHANK3,* encoding a synaptic scaffolding protein (25, 26).

While common variants likely account for the majority of the inherited risk of ASD (27–29), most progress in gene discovery in recent years, and all of the "high confidence" risk genes, were discovered through the identification of rare variants of large effect (occurring in <0.1% of the general population) (3–6). These studies involved whole-exome sequencing of individuals from multiple consortia, including the Autism Sequencing Consortium, the iPSYCH-Broad Consortium, the Simons Simplex Collection (SSC), and others. In particular, the Simons Simplex Collection is a cohort of simplex families consisting of an affected child, unaffected parents, and in most cases, an unaffected sibling, thus increasing the likelihood of identifying rare, *de novo* (not inherited) variants. Importantly, these studies found that rare *de novo*, likely gene-disrupting single-nucleotide variants (SNVs) occur more frequently in individuals with ASD than in unaffected siblings, and that the genes affected by such variants when recurrent in unrelated individuals represent bona fide risk genes (30).

This finding was consistent with the earlier discovery that *de novo* copy number variants (CNVs), i.e., microdeletions and microduplications, also occur significantly more often in individuals with ASD, particularly from simplex families, than unaffected individuals (31). The increased rate of rare and *de novo* CNVs in ASD was confirmed in subsequent investigations (5, 32, 33), which led to the discovery of additional ASD-associated genes and genomic regions, such as 15q11.2-13, 16p11.2, and 22q11.2 (5, 22, 24, 32–36). It is estimated that *de novo* CNVs and SNVs collectively account for ∼30% of simplex cases (5). Together, these groundbreaking studies led to the identification of the first "high confidence" ASD risk genes and shaped our understanding of the genetic architecture of ASDs, establishing a clear contribution of rare, *de novo* variants to ASD risk and a path forward for gene discovery (3–6, 30, 37, 38). SFARI Gene (https://gene.sfari.org/), an excellent resource established by the Simons Foundation for Autism Research Initiative (SFARI), is a database that ranks ASD risk genes based the strength of the evidence supporting their association with ASD on a 1–3 scale, where genes with score 1 have the strongest evidence for association (39).

Abbreviations: ASD, autism spectrum disorder; ER, estrogen receptor; LoF, loss of function; MAPK/ERK, mitogen activated protein kinase/extracellular signal-regulated kinase; PI3K/Akt/mTOR, phosphatidylinositol 3-kinase/protein kinase B/mechanistic target of rapamycin; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element-binding protein; GABA, gamma aminobutyric acid; NPC, neural progenitor cell.

BIOLOGICAL PATHWAYS IMPLICATED BY ASD RISK GENES

With expanding DNA-sequencing efforts, the number of ASD-associated genes has continued to climb, enabling the identification of related biological pathways involving these genes. In the largest exome-sequencing study of ASD to date, involving almost 12,000 individuals from family-based and case-control samples, Satterstrom et al. (6) identified 102 ASD risk genes (false discovery rate < 0.1) and examined biological pathways involving these genes. Specifically, they found that the 102 risk genes are expressed early in the developing brain and fall broadly into the following functional categories: gene expression regulation, neuronal communication, cytoskeleton, and other functions (6). "Gene expression regulation" includes chromatin modifiers and transcription factors, while "neuronal communication" encompasses a range of functions, including ion channels, synaptic cell adhesion molecules, intracellular signaling molecules, and axon guidance molecules (6). These findings are consistent with earlier studies implicating synaptic function as a central mechanism in ASD, based on the identification of risk genes encoding synaptic cell adhesion molecules and scaffolding proteins, including neurexins, neuroligins, and SHANK proteins (20–26). Additionally, the first ASD risk genes associated with monogenic disorders, e.g., *TSC1, TSC2, FMR1, NF1,* and *PTEN*, are directly or indirectly involved in mechanistic target of rapamycin (mTOR) signaling, a central pathway controlling protein translation, cellular proliferation, and survival, suggesting that mTOR may represent another common pathway in ASD (19).

Recent studies have also shown that ASD risk genes encode proteins involved in transcriptional regulation, chromatin remodeling, and synaptic formation (3, 4). A protein-protein interaction network of high confidence ASD risk genes and predicted risk genes also identified cell-cell communication, synaptic transmission, and transcriptional regulation as relevant clusters (3). Interestingly, Iossifov et al. (4) observed that the targets of *de novo*, likely gene disrupting mutations overlapped significantly in females and males with a lower intelligence quotient (IQ), but not males with a higher IQ. They found that this overlapping group of genes is enriched for embryonically expressed genes (mainly in females), suggesting an early developmental effect, as well as chromatin modifiers and targets of FMRP (encoded by *FMR1*), the Fragile X syndrome RNA-binding protein. Likewise, Pinto et al. (32) found that females with ASD are more likely than males to carry highly penetrant CNVs and CNVs disrupting FMRP targets. This suggests that females are more likely to carry mutations of greater impact and that less deleterious mutations show decreased penetrance in females, consistent with the female protective effect (discussed in the next section) (4, 7, 32).

Integrative genomics approaches aimed at analyzing expression patterns of ASD risk genes have also played a critical role in identifying relevant neural cell types and developmental time points. For example, Willsey et al. (40) utilized human brain transcriptome data from the BrainSpan project (41) encompassing early embryonic to late adult stages and multiple brain regions to construct spatio-temporal co-expression networks around nine high confidence ASD genes, and identified mid-fetal glutamatergic deep layer projection neurons as a point of convergence (40). Another study analyzed the same transcriptome dataset using weighted gene co-expression network analysis (WGCNA) (42), and remarkably, also found that ASD risk genes are enriched in glutamatergic projection neurons, though in superficial, not deep, layers (43). Further, an imbalance in excitatory-inhibitory signaling in the brain has previously been proposed as a central mechanism underlying ASD (44). By highlighting excitatory glutamatergic neurons as a point of convergence, these studies provide further support for this theory. The recent exome-sequencing study by Satterstrom et al. (6) also provides evidence for excitatory-inhibitory imbalance; using a single-cell RNA sequencing dataset from prenatal human forebrain (45), this study found that ASD risk genes are enriched in maturing and mature excitatory and inhibitory neurons, with the strongest enrichment in early excitatory neurons and striatal interneurons (6). Taken together, these studies highlight neuronal communication, synaptic transmission, and regulation of gene expression as convergent biological processes involving ASD risk genes and implicate excitatory and inhibitory neurons as neural cell types with relevance to ASD pathophysiology.

THE FEMALE PROTECTIVE EFFECT IN ASD

As discussed above, large-scale genetic studies have been productive in identifying genes strongly associated with ASD. They have also yielded the intriguing observation that females with ASD carry, on average, more deleterious mutations compared to males with ASD (46). That is, females with ASD are more likely to have an increased mutation "burden." For example, Sanders et al. (5) found that *de novo* CNVs occur at a greater rate in females with ASD. In addition, Satterstrom et al. (6) observed that *de novo*, protein-truncating variants in the most strongly associated ASD risk genes show a two-fold enrichment in females compared to males, consistent with findings from earlier exome-sequencing studies (3, 4). The apparent implication that females require a larger etiological "hit" to develop ASD supports the "female protective effect" model, which posits that there are innate factors that cause females to be relatively resilient to the disorder, leading to the overrepresentation of males in ASD. Still, the sex differential in diagnosis could be due to factors that cause males to be especially vulnerable to developing ASD as well as factors that cause females to be resilient to developing the condition; they are not mutually exclusive.

While there is highly suggestive evidence from genetic analyses supporting a female protective effect in ASD, the biological mechanisms responsible for female protection and/or male risk remain unclear. It has been proposed that multiple "hits," involving some combination of sex, genetics, and environmental triggers, lead to the imbalance of males and females in ASD (47). Interestingly, differences in sex steroid hormone levels have been proposed as a possible mechanism

contributing to resilience in females and/or increased risk in males [reviewed in (7)]. One of the most notable examples is the "extreme male brain" theory (discussed below). In addition, studies investigating the function of ASD risk genes in preclinical systems, including one from our group, identified estrogens as potential modulators of ASD gene-associated cellular and behavioral phenotypes (8, 9, 48). However, genes that directly affect sex steroid hormone signaling, e.g., estrogen or androgen receptors, have not been identified among the strongest ASD-associated genes (e.g., *ESR1*, which encodes Estrogen Receptor 1, is not listed in SFARI Gene; *AR*, encoding Androgen Receptor, has a SFARI gene score of 3), suggesting that estrogens and/or androgens may be acting indirectly to modulate signaling pathway deficits resulting from ASD risk gene mutations. Below we review investigations that have provided insights into sex differential processes and potential female protective factors in ASDs, including: genetic and transcriptomic analyses examining the sex chromosomes and sex-differential gene expression; imaging genomics studies integrating functional imaging with genetic findings; as well as clinical and preclinical studies investigating potential contributions of sex steroid hormones, with a particular focus on estrogens. Further elucidating these mechanisms will be important for identifying potential targets for treatment.

SEX DIFFERENTIAL PROCESSES IN ASD

Genetic Studies

One potential explanation for the female protective effect is that it may be driven by genetic factors, i.e., specific genes that confer risk and/or protection in males or females. Although it is reasonable to presume that the male bias could be due to ASD as an X-linked disorder, chromosome X gene mutations do not account for a substantial proportion of cases and most ASD-associated genes identified to date are autosomal [(6); reviewed in (7)]. Approximately 92% of all genes in SFARI Gene are autosomal, including ∼90% of those with the strongest evidence for association with ASD (SFARI gene score 1) (https://gene.sfari.org) (39). Moreover, large-scale genetic studies provide evidence that the same genes and genetic mechanisms likely contribute to ASD risk in males and females, suggesting that female protection and/or male risk may be driven by non-genetic factors. Specifically, by analyzing 10,220 individuals from 2,591 families from the SSC, Sanders et al. (5) found that *de novo*, damaging variants in high confidence ASD risk genes are randomly distributed in males and females, suggesting that a common set of genes contribute to risk in both sexes [(5); reviewed in (7)]. In addition, in the largest exome sequencing study of ASD to date with 35,584 individuals, including 11,986 with ASD, Satterstrom et al. (6) found that there is no significant difference in the types of genetic variants (i.e., protein-truncating, missense) that contribute to ASD risk in males vs. females, even though females are more likely to harbor damaging mutations in the strongest risk genes. However, there is evidence that one gene, *DDX3X*, located on the X chromosome, shows locus-specific enrichment in females (49). Interestingly, another study by Gockley et al. (50) investigated whether there is a single genetic locus that might confer resilience in females. By comparing single nucleotide polymorphisms (SNPs) on the X chromosome and genome-wide in females with and without ASD, they did not find evidence supporting a single protective genetic locus in females, though it is possible that several genetic loci may be involved (50). Taken together, large genetic studies provide strong support for the female protective model, though to date there is no clear evidence that a single gene or genetic mechanism in females or males accounts for resilience and/or risk.

Transcriptomic Studies

To investigate how differences in gene expression might contribute to the female protective effect in ASD, Werling et al. (51) performed the first integrative genomics analysis of sex-differential gene expression with relevance to ASD. Specifically, they compared sex-differential gene expression patterns in post-mortem adult and prenatal cortex from neurotypical individuals from the BrainSpan project (adult dataset: 5 subjects of each sex, 29 samples/subject; prenatal dataset: 4 subjects of each sex, 43 samples/subject) (41) with gene expression modules from a post-mortem cortical dataset from individuals with ASD [4/16 female ASD cases, 1/16 female controls in (52); 8/32 female ASD cases and 9/41 female controls in (53)]. This study led to several key findings. First, there was no evidence for enrichment of known ASD risk genes among genes showing sex-differential expression patterns in either adult or prenatal cortex, suggesting that risk genes themselves do not show sex-differential expression (51). Second, the authors found that genes that are upregulated in the post-mortem cortex of individuals with ASD (ASD-upregulated) were significantly enriched for male differentially expressed genes, i.e., genes showing higher expression in neurotypical males. Intriguingly, the ASD-upregulated gene module is associated with astrocyte and microglial markers, indicators of immune system function, and astrocyte and microglial markers were also found to be enriched among male differentially expressed genes. In contrast, ASD-downregulated gene modules were more highly enriched for female differentially expressed genes and overlapped with synaptic and neuronal markers. Of note, this association was not likely to be due to the male predominance of the cases and controls in the post-mortem studies, given that they were sex balanced, and the potential confounds of sex were accounted for in the analyses of ASD-associated gene expression (51–53). Third, these findings were recapitulated in a prenatal neocortical dataset, including the lack of enrichment of ASD risk genes among sex-differentially expressed genes and the overlap of male differentially expressed genes and ASD-upregulated genes. This study indicates that genes whose expression is dysregulated in post-mortem brains of individuals with ASD, but not ASD risk genes themselves, show sex-differential expression patterns (51). This implies that it is the pathways downstream of ASD risk genes that might interact with sexually dimorphic biological processes to influence the sex bias in ASD (51). Furthermore, the enrichment of astrocyte

and microglial markers in the male-differentially expressed and ASD-upregulated gene modules suggests that typical sex differences in immune system function may be relevant to ASD biology and will be important to investigate as a potential contributor to the sex bias in ASD in future studies (7, 51).

Imaging Genomics

Imaging genomics is a cutting-edge approach to integrate neuroimaging and genomic data from individuals with ASD to gain a greater understanding of interactions between neural circuit and genetic mechanisms in ASD. Our group conducted an imaging-genomics analysis to characterize the female neural profile in ASD, which, in turn, could provide insights into potential female protective factors (54). To accomplish this, we integrated task-based functional MRI (fMRI) data obtained in response to point-light displays of whole-body human motion, a well-established measure of social perception (55), and genetic data regarding median size of rare, genic CNVs (i.e., those containing one or more genes) as a measure of mutation severity, in a sex-balanced cohort of ASD and typically-developing youth (8–17 years-old; total imaging $n = 184$, males $= 94$, females $= 90$). Differences in brain response between our sex-balanced cohorts could be due to environmental factors, such as the cumulative effects of life experiences, and/or to biological factors, such as the effects of genetic mutations. The integration of genetic data would help determine to what degree genetically-mediated processes contribute to a possible etiological mechanism. Interestingly, previous research identified hypoactivation of the posterior superior temporal sulcus during biological motion perception as a "neural signature" of ASD (55). However, as with much of the ASD literature, this study was conducted with a male-predominant sample. In contrast, we found that ASD females, compared to typically-developing females, showed significant hypoactivation to biological motion in primarily sensorimotor, striatal, and frontal brain regions (**Figure 1A**). Furthermore, ASD females compared to ASD males had larger median size of rare, genic CNVs containing gene(s) expressed in early development of these brain regions, especially the striatum (**Figure 1B**), which was replicated in an independent cohort (SSC) of ASD females and males. The striatum is a subcortical structure involved in motor planning as well as cognitive processes, such as social and language functions, and may link motivation to voluntary behavior. Not only do these larger CNVs support the female protective effect model, but they also suggest that impacts to the striatum may particularly contribute to female risk for autism. Therefore, caution should be applied to making inferences about female autism based on male-predominant studies.

We proposed that the brain regions underlying the female protective effect may consist of those that not only differentiate typically-developing females from ASD females but also differentiate typically-developing females from typically-developing males, reflecting what is unique about the female perception of social stimuli. Comparing the brain response of these cohorts to biological motion, we found the female protective effect to be potentially represented by frontal and parietal regions of the salience and central executive brain networks, involved in processes such as monitoring salient stimuli, attention, working memory, and cognitive control. Although a lack of transcriptome data for important brain regions comprising these networks precluded the integration of genetic data as had been done for the female autism neural profile, important insights were still gained into the regions relating to female resilience in social perception. However, further studies are needed to elucidate the genetic and/or environmental factors affecting the differential development of these networks.

Studies of Prenatal Sex Steroids

One of the most well-known theories for the sex bias in ASD is the "extreme male brain theory" proposed by Simon Baron-Cohen, which holds that ASD presents as an extreme version of the male brain, where individuals with ASD exhibit greater abilities in male "systemizing" as opposed to female "empathizing" characteristics (56). Consistent with this idea, Baron-Cohen proposed that fetal exposure to sex steroids, specifically fetal androgens, might contribute to the sex bias in ASD (57). A number of clinical studies, many of which are from Baron-Cohen's group, have directly investigated associations between prenatal exposure to sex steroid hormones, including androgens and estrogens, and ASD (reviewed in **Table 1**) (58–64). In support of this theory, Baron-Cohen's research group identified a positive correlation between fetal testosterone levels measured in amniotic fluid by amniocentesis and the development of ASD traits in typically developing male ($n = 118$) and female ($n = 117$) children at ages 6–10 years in a longitudinal cohort study (58). This significant positive relationship between fetal testosterone levels and ASD traits was also observed in typically developing toddlers aged 18–24 months (59). In another study, Baron-Cohen et al. (60) compared levels of cortisol and the sex steroids progesterone, 17α-hydroxy-progesterone, androstenedione, and testosterone, from amniotic fluid samples of males who were later diagnosed with ASD ($n = 128$) and typically developing males ($n = 217$) from the Danish Historic Birth Cohort, and found that the levels of all hormones were positively correlated with each other and that all hormones were elevated in the ASD group on "a latent generalized steroidogenic factor" ascertained by principal components analysis (60). This study provided evidence that increased steroidogenic activity may be associated with ASD.

By analyzing the same cohort from the Danish Historic Birth Cohort of males with ($n = 98$) and without ASD ($n = 177$), Baron-Cohen et al. (61) found that elevated levels of prenatal amniotic estradiol, estriol, and estrone were significantly associated with an ASD diagnosis in univariate logistic regression analysis (61). Of the steroidogenic hormones analyzed in the previous study (60), only progesterone levels were found to be significantly associated with an ASD diagnosis (61). Interestingly, this study suggests that increased levels of estrogens are associated with an ASD diagnosis in males, though due to the limited number of females with ASD in this cohort, additional studies are needed to investigate whether this association exists for females with ASD (61). Baron-Cohen et al. (61) viewed the

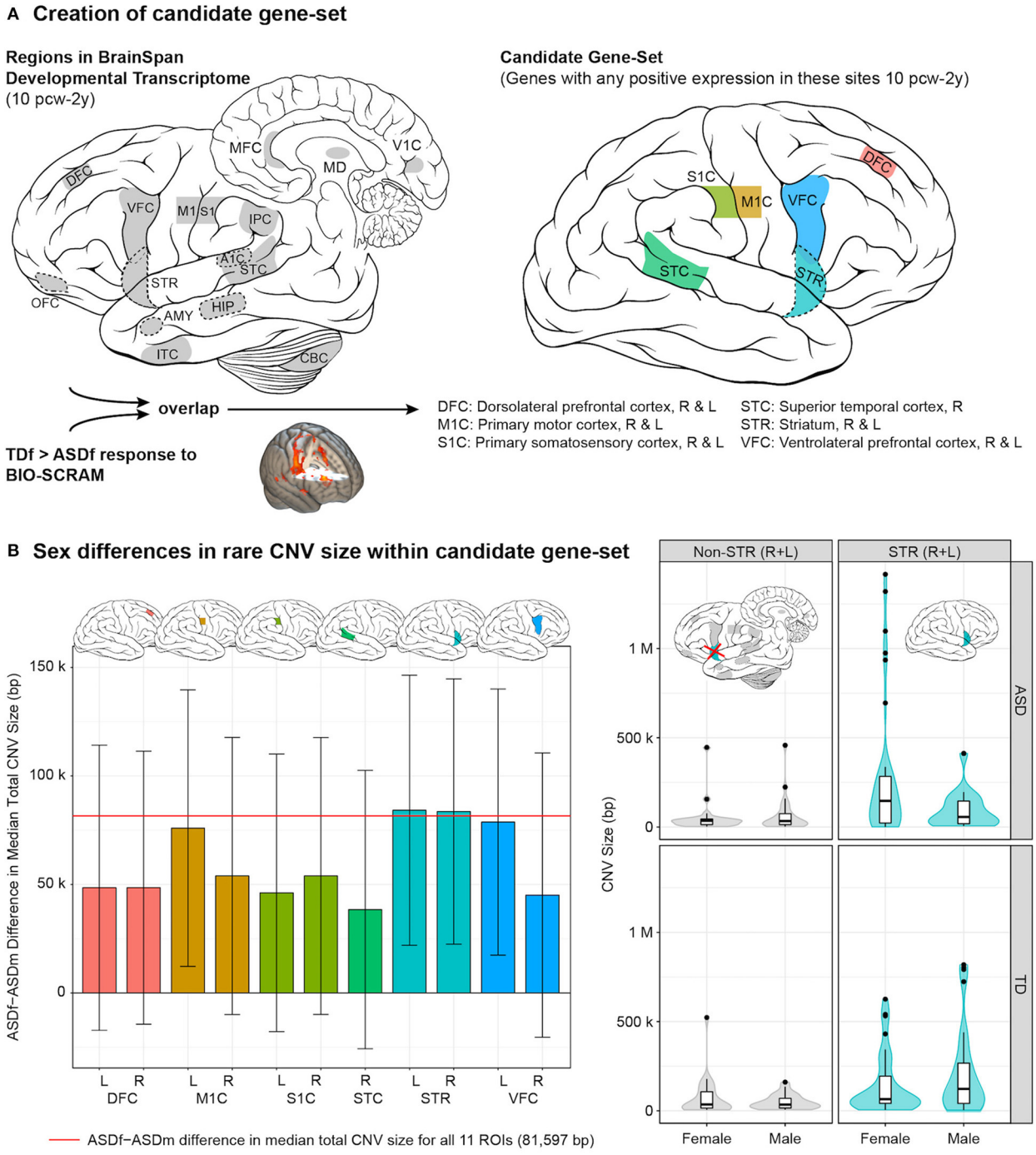

FIGURE 1 | Female autism neurogenetic profile. **(A)** Creation of candidate gene-set involved identifying 11 regions of interest (ROIs) that had both been characterized in the BrainSpan Developmental Transcriptome (left) and showed a significant typically-developing female > ASD female (TDf > ASDf) response to biological motion (BIO-SCRAM); genes were then identified that showed positive expression in these overlapping regions (right) between 10 post-conceptual weeks (pcw) through 2 years (y). **(B)** (Left) bar plot with standard errors demonstrating the sex difference (ASDf - ASDm) in median total CNV size, in base pairs (bp), within the candidate gene-set. Plots of the CNV size difference are provided for each of the 11 ROIs. (Right) distribution of size of rare CNVs containing gene(s) expressed in right (R) and left (L) striatum (STR) from 10 pcw to 2 y (right panels, cyan) by group and sex. For comparison, "non-STR (R + L)" includes rare CNVs containing gene(s) characterized in BrainSpan that were not positively expressed in R/L-STR from 10 pcw to 2 y (left panels, gray). Of note, rare CNVs often contained gene(s) that were expressed in multiple brain regions (e.g., STR plus additional ROI[s]). Violin plots depict a Gaussian kernel density estimate, and are overlaid with Tukey-style boxplots. Brain art adapted from illustrations created by Patrick Lynch and C. Carl Jaffe, commons.wikipedia.org/wiki/File:Brain_human_lateral_view.svg and Brain_human_sagittal_section.svg, licensed under a Creative Commons Attribution 2.5 Generic License, 2006. Figure constructed by Allison Jack PhD and reprinted with permission from Jack et al. (54).

TABLE 1 | Clinical and postmortem studies of sex steroid hormones in autism spectrum disorder.

Prenatal studies	Results	Sample type	Age at assessment	Samples size/Sex	Diagnosis
Auyeung et al. (58)	• Positive association of fetal testosterone levels and higher scores on the Childhood Autism Spectrum Test (CAST) and the Child Autism Spectrum Quotient (AQ-Child) in typically developing children	Amniotic fluid	6–10 years	235 M = 118, F = 117	Typically developing children
Auyeung et al. (59)	• Positive association between fetal testosterone levels and scores on the Quantitative Checklist for Autism in Toddlers (Q-CHAT) in typically developing children. • Males had higher Q-CHAT scores indicating more "autistic traits" compared to females	Amniotic fluid	18–24 months	129 M = 66, F = 63	Typically developing children
Baron-Cohen et al. (60)	• Positive association of levels of progesterone, 17α-hydroxy-progesterone, androstenedione, testosterone, and cortisol with each other in amniotic fluid • Elevated levels of all hormones in the ASD group on a generalized latent steroidogenic factor that accounted for much of the variation in the data by principal component analysis	Amniotic fluid	Individuals born between the years 1993 and 1999 in the Danish Historic Birth Cohort	Control (n = 217); ASD (n = 128)/Males only	ICD-10 diagnosis of any ASD code
Baron-Cohen et al. (61)	• Increased levels of prenatal amniotic estradiol, estriol, and estrone were predictive of an ASD diagnosis • Elevated prenatal amniotic estradiol was the most significant predictor of ASD diagnosis in univariate logistic regression model	Amniotic fluid	Individuals born between the years 1993 and 1999 in the Danish Historic Birth Cohort	Control (n = 177); ASD (n = 98)/Males only	ICD-10 diagnosis of any ASD code
Bilder et al. (62)	• Higher estradiol and lower sex hormone binding globulin levels were associated with ASD risk in a cohort enriched for prenatal metabolic syndrome	Prenatal maternal serum (early second trimester)	Individuals born to mothers participating in the FASTER study from 1999 to 2002	Control (n = 19); ASD (n = 53)/Samples were matched by sex and birth year	ASD diagnostic billing codes; special education autism exceptionality status
Clinical studies	**Results**	**Sample type**	**Age at assessment**	**Samples size/sex**	**Diagnosis**
Sharpe et al. (63)	• B-lymphocytes from individuals with ASD showed less growth depression and mitochondrial upregulation in response to estradiol and dihydrotestosterone compared to unaffected siblings and unrelated controls	B-lymphocytes	Samples from the AGRE tissue bank; controls were age- and sex-matched	Unrelated controls (n = 11), M = 10, F = 1; Unaffected fraternal twins (n = 10), M = 4, F = 6; unaffected siblings (n = 22), M = 11, F = 11; ASD (n = 11), M = 10, F = 1	ASD diagnosis from AGRE
Altun et al. (64)	• Decreased serum GPER levels in the ASD group	Blood	Control = 6.12 ± 2.55 years; ASD = 5.33 ± 2.61 years	Control (n = 40), M = 32, F = 8; ASD (n = 45), M = 40, F = 5	DSM-V diagnosis of ASD
Postmortem studies	**Results**	**Brain regions**	**Age at tissue collection**	**Samples size/sex**	**Sample source**
Sarachana et al. (65)	• Decreased aromatase and RORA proteins in frontal cortex of ASD subjects relative to sex- and age- matched controls • Aromatase protein strongly correlates with protein levels of RORA, which transcriptionally regulates aromatase	Frontal cortex	Age- and sex-matched controls	Control (n = 22); ASD (n = 12)	Autism Tissue Program
Crider et al. (66)	• Decreased ERβ and CYP19A1 (aromatase) mRNA in ASD subjects; no change in ERα • Decreased mRNA levels of the ER co-activators: SRC-1, CBP and P/CAF in ASD subjects • Decreased ERβ and CYP19A1 protein levels in ASD subjects	Middle frontal gyrus	Control: 11.70 ± 1.584; ASD: 11.80 ± 1.609	Control (n = 13), M = 12, F = 1; ASD (n = 13), M = 13, F = 0	NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland

AGRE, Autism Genetic Resource Exchange; ASD, Autism spectrum disorder; CBP, CREB-binding protein; ER, Estrogen receptor; ERα, Estrogen receptor Alpha; ERβ, Estrogen receptor Beta; F, female; FASTER, First and Second Trimester Evaluation of Risk; GPER, G protein-coupled estrogen receptor; ICD-10, International Statistical Classification of Diseases and Related Health Problems, 10th revision; M, male; NICHD, National Institute of Child Health and Human Development; P/CAF, p300/CREB-binding protein-associated protein; RORA, Retinoic acid-related orphan receptor-alpha; SRC-1, steroid receptor co-activator-1.

results of this and their previous study as complementary, in that both suggest that increased prenatal steroidogenic activity may be associated with ASD.

In line with this theory, one study found that females with congenital adrenal hyperplasia ($n = 34$), a condition caused by an enzyme deficiency that leads to an increase in fetal androgen levels, have higher scores on the Autism Spectrum Quotient compared to unaffected female relatives ($n = 24$) (67). In addition, a matched case-control study of children ages 4–17 born in Sweden from 1984 to 2007 ($n = 23{,}748$ ASD cases and $n = 208{,}796$ controls) found that there is a 59% increased risk of ASD in children of women with polycystic ovary syndrome, which leads to increased androgen levels, and a greater risk if women had both PCOS and obesity (68). Further, males with aneuploidies affecting the Y chromosome (e.g., 47, XYY; 48, XXYY) were found to be 20 times more likely to have an ASD diagnosis than males in the general population (69). In a study of 860 individuals with Klinefelter syndrome in Sweden with 86,000 population controls, Klinefelter syndrome was found to be associated with a six times higher risk of ASD as well as an increased risk of schizophrenia and ADHD (70). Another study found that 21% of individuals with Turner syndrome (45, X) ($n = 98$) met the criteria for a diagnosis of ASD, which was higher than the national rates of ASD in females in the United Kingdom (0.3%) (71). Collectively, these studies suggest that altered exposure to sex steroids may be associated with ASD, though additional research will be important to elucidate potential mechanisms.

Post-mortem Studies of Sex Steroids

A limited number of post-mortem studies have examined the expression of genes related to sex steroid signaling in individuals with ASD (**Table 1**). For example, Crider et al. (66) found that levels of estrogen receptor-beta (ERβ) mRNA and protein were significantly reduced in the middle frontal gyrus of ASD subjects ($n = 13$) compared to controls ($n = 13$) (66). In addition, expression levels of coactivators of the estrogen receptor, including steroid receptor co-activator 1, CREB-binding protein, and p300/CREB-binding protein-associated protein, were lower in the middle frontal gyrus of ASD compared to control subjects (66). Another post-mortem study found that protein levels of retinoic acid-related orphan receptor-alpha (RORA), whose expression is differentially regulated by estradiol and dihydrotestosterone, as well as aromatase, the enzyme that converts testosterone into estrogen and whose expression is regulated by RORA, were significantly reduced in the frontal cortex of individuals with ASD ($n = 12$) compared to controls ($n = 22$) (65). However, additional post-mortem studies with larger samples sizes are needed.

FUNCTIONAL STUDIES OF ASD RISK GENES IDENTIFY ESTROGENS AS POTENTIAL MODULATORS

Interestingly, recent preclinical studies investigating the function of ASD risk genes in distinct model systems, including one from our group, found that estrogens might play a modulatory role in signaling pathways related to ASD and other neurodevelopmental disorders (**Table 2**) (8, 9, 48, 72). First, our group investigated the ASD and epilepsy risk gene, *Contactin Associated Protein-like 2* (*CNTNAP2*), and identified estrogenic compounds in an unbiased screen as a suppressor of a behavioral phenotype in zebrafish lacking the function of this gene (8). *CNTNAP2*, which encodes a cell adhesion molecule in the neurexin family, was first identified as a risk gene by a linkage study in consanguineous families from the Old Order Amish population with cortical dysplasia focal epilepsy syndrome (73). Our group utilized zebrafish to analyze the function of this gene, because they are highly tractable and amenable to large-scale small molecule screens (74, 75). We found that zebrafish mutants of *cntnap2* display selective deficits in GABAergic neurons, particularly in the forebrain, consistent with findings in mouse *Cntnap2* knockouts (76). Using a large-scale assay to quantify rest-wake activity (77), we found that zebrafish *cntnap2* mutants have a specific behavioral phenotype of nighttime hyperactivity (**Figures 2A,B**). To identify pharmacological compounds that suppress the *cntnap2* mutant behavioral "fingerprint," we screened small molecules by comparing the mutant behavioral phenotype to an existing dataset of the behavioral profiles of wild-type fish exposed to 550 psychoactive compounds across multiple parameters (77) (**Figure 2C**). Interestingly, we found that estrogenic compounds were significantly enriched in the top ranks of small molecules that anti-correlate with, or generate the opposite profile of, the *cntnap2* behavioral "fingerprint" (**Figure 2D**). Further, in a screen of 14 psychoactive compounds, including those with estrogenic activity, we found that the plant-derived estrogen, biochanin A, most selectively reversed the mutant behavioral phenotype by decreasing nighttime activity with little effect on other measures of rest and activity and with greater specificity than risperidone, which is FDA-approved to treat aggression and irritability in ASD (8) (**Figures 2E–G**). While the mechanisms mediating the rescue by estrogens are not clear, it is possible that estrogens might act by modulating deficits in GABAergic and glutamatergic signaling in mutants. While early exposure to biochanin A did not reverse the structural GABAergic deficits (8), additional studies are needed to determine how estrogens might affect excitatory and inhibitory circuits in these mutants.

Remarkably, another recent study also found that estrogens might act as modulators of cell proliferation phenotypes across multiple ASD risk genes (9). Willsey et al. (9) performed an *in vivo* CRISPR screen in *Xenopus tropicalis* targeting 10 high confidence ASD risk genes. By capitalizing on a unique feature of *Xenopus,* in which it is possible to generate unilateral mutants by introducing CRISPRs into one cell at the two-cell stage and compare phenotypes on either side of the midline at later stages, the authors found that disruption of high confidence ASD risk genes resulted in altered telencephalon size as well as an increased ratio of neural progenitor cells to neurons in this region (9). Disruption of ASD risk genes in human induced pluripotent stem cell (iPSC)-derived neural progenitor cells (NPCs) using CRISPR interference (CRISPRi) also led to an increase in the proportion of proliferative cells, which together with the

TABLE 2 | Preclinical studies of estrogens in genetic models of ASD and other neurodevelopmental disorders.

Study	Gene(s)	Model system	Developmental stage	Brain region(s)	Results
Olivetti et al. (48)	*ARX*	Mouse	Early postnatal and Adult	Cortex	• Early postnatal exposure to estradiol prevented spasms in infancy and seizures in adult mutants • Estradiol reversed deficits in neuropeptide Y-positive cortical interneuron and striatal cholinergic interneuron populations
Hoffman et al. (8)	*CNTNAP2*	Zebrafish	Larval (4–6 days post fertilization)	N/A	• Estrogens, including the plant-derived estrogen, biochanin A, and β-estradiol-17-cypionate, identified in a drug screen, suppressed a behavioral phenotype of nighttime hyperactivity in zebrafish *cntnap2* mutants
Erli et al. (72)	*DISC1*	Rat	Embryonic	Cortical neuronal culture	• Acute 17-β-estradiol rescued dendritic spine deficits and reduced DISC1 aggregation in neurons overexpressing wild-type or mutant DISC1 • Acute 17-β-estradiol rescued dendritic spine deficits due to DISC1 knockdown • Chronic 17-β-estradiol rescued dendritic spine deficits and reduced DISC1 aggregation in neurons expressing mutant DISC1 • Chronic 17-β-estradiol increased the number of spines positive for pre- and post-synaptic markers in control and mutant DISC1-expressing neurons
Willsey et al. (9)	*ADNP; ANK2; ARID1B; CHD2; CHD8; DYRK1A; NRXN1; SCN2A; SYNGAP1; POGZ*	Frog (*Xenopus tropicalis*)	Tadpole	Telencephalon	• An estrogenic agonist, estramustine, identified in a drug screen reversed the phenotype of increased proliferation in the *Xenopus* telencephalon induced by chemical disruption of the ASD risk gene, *DYRK1A* • Disruption of estrogenic signaling led to a reduction in telencephalon size in *Xenopus* • 17-β-estradiol reversed increased proliferation in NPCs derived from human CRISPRi cell lines disrupting the ASD risk genes *DYRK1A, NRXN1,* or *ADNP*

ASD, Autism spectrum disorder; ARX, Aristaless-Related Homeobox; ADNP, Activity-dependent neuroprotective protein; ANK2, Ankyrin 2; ARID1B, AT-Rich Interaction Domain 1B; CHD2, Chromodomain Helicase DNA Binding Protein 2; CHD8, Chromodomain-helicase-DNA-binding protein 8; CNTNAP2, Contactin-associated protein-like 2; CRISPRi, clustered regularly interspaced short palindromic repeats interference; DISC1, Disrupted-in-schizophrenia 1; DYRK1A, Dual specificity tyrosine phosphorylation regulated kinase 1A; NPCs, neural progenitor cells; NRXN1, Neurexin 1; POGZ, Pogo Transposable Element Derived with ZNF Domain; SCN2A, Sodium Voltage-Gated Channel Alpha Subunit 2; SYNGAP1, Synaptic Ras GTPase-activating protein 1.

findings in *Xenopus*, suggests that dysregulated neurogenesis may represent a convergent pathway linking these genes (9). Intriguingly, in a screen of 133 FDA-approved oncology drugs to identify compounds that reverse altered proliferation in the *Xenopus* telencephalon (induced by chemical disruption of the ASD risk gene, *DYRK1A*), 3 of the top 17 compounds identified as modulating this phenotype affect estrogen signaling. These include estramustine, an estrogenic agonist, which rescued the phenotype by decreasing the number of neural progenitor cells relative to neurons, along with raloxifene and fulvestrant, which are estrogenic inhibitors that led to an increase in the neural progenitor cell to neuron ratio. Estradiol also had similar effects in rescuing an altered proportion of proliferative cells in human NPCs following disruption of ASD risk genes. This study found that estrogen signaling is involved in regulating neurogenesis in the telencephalon, and identified Sonic hedgehog (SHH) signaling as a potential pathway by which estrogens may affect neurogenesis. Importantly, this study provides support for the idea that estrogens may play a modulatory role in the developing brain by conferring resilience against the negative effects of ASD risk gene disruption, possibly through its effects on neurogenesis (9).

In addition, there is evidence that exposure to estradiol during the early postnatal period reduced seizures in a mouse model of the X-linked epilepsy gene, *ARX* (48), and that estradiol exposure reversed the reduction in dendritic spine density associated with overexpression or knockdown of the schizophrenia-associated gene, *DISC1*, in rat cortical neuronal cultures (72) (**Table 2**). While the study in mice was performed at doses comparable to physiological levels of estradiol in fetal plasma (48), translating the concentrations of estradiol and other estrogenic compounds from zebrafish, *Xenopus*, and *in vitro* studies to physiological levels in mammals represents a challenge, and additional research is needed to establish dose equivalency. Nonetheless, these studies suggest that estrogens may function as modulators of signaling pathways downstream of risk genes associated with ASD and other neurodevelopmental disorders.

ESTROGEN SIGNALING PATHWAYS WITH RELEVANCE TO ASD

These preclinical studies raise important questions regarding how estrogens affect basic mechanisms of brain development, though the precise mechanisms by which they might influence signaling pathways relevant to ASD have yet to be elucidated. In the following sections, we consider known cellular and molecular effects of estrogens in the brain that might overlap with

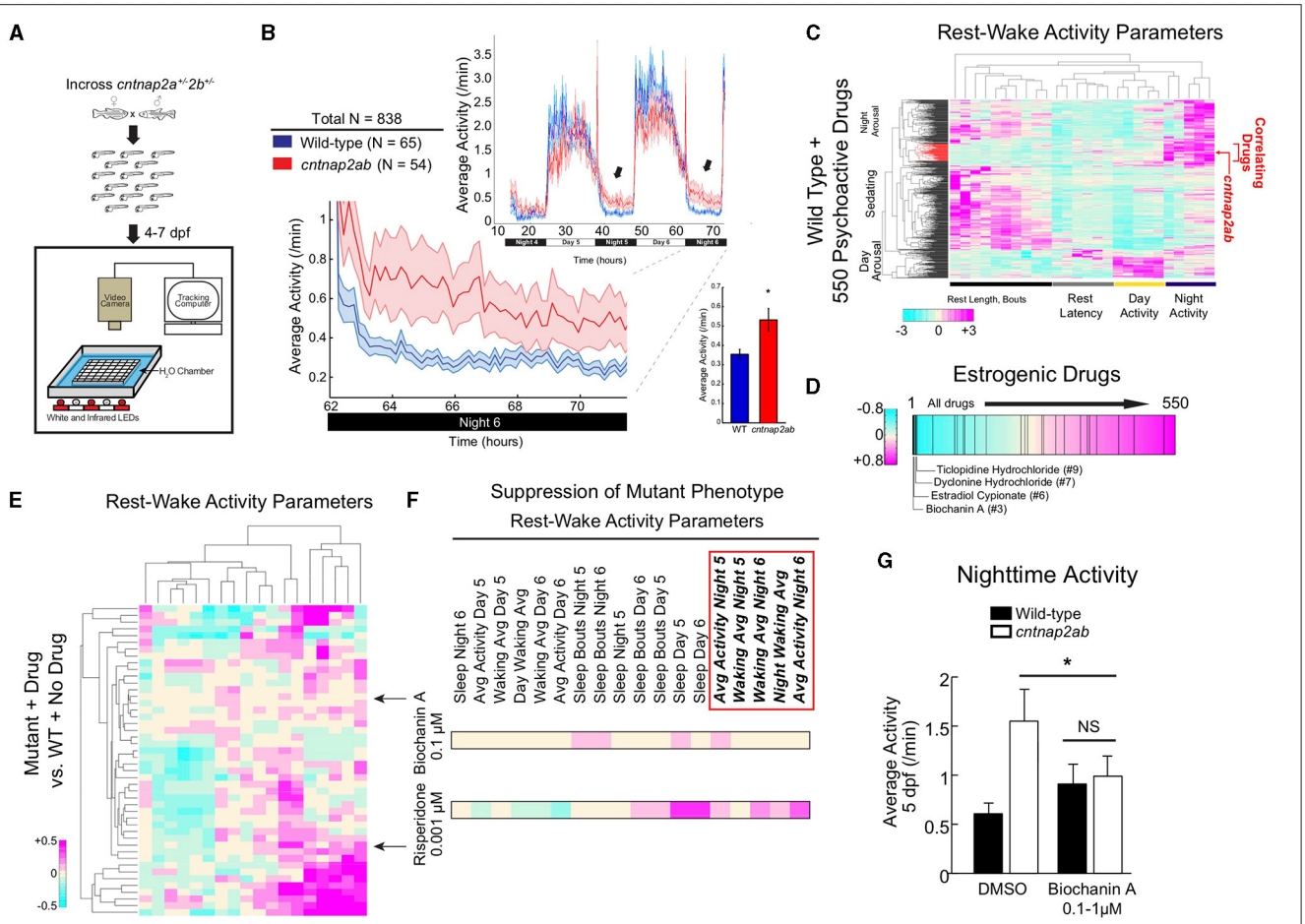

FIGURE 2 | The plant-derived estrogen, biochanin A, reverses nighttime hyperactivity in zebrafish mutants of the ASD risk gene, *CNTNAP2*. **(A)** Experimental set-up. Locomotor activity of zebrafish larvae is tracked on a rest-wake cycle from 4 to 6 days post fertilization (dpf) using an automated assay (77, 78). **(B)** Locomotor activity of *cntnap2a*$^{\Delta 25/\Delta 25}$*cntnap2b*$^{\Delta 7/\Delta 7}$ (*cntnap2ab*, red) and wild-type (WT; blue) sibling-matched larvae is shown over 72 h. Hyperactivity in mutants worsens on successive nights (arrows). The magnified activity profile on night 6 is shown. Inset graph shows average locomotor activity of *cntnap2ab* vs. wild-type. *$p = 0.00012$ (one-way ANOVA, comparing all genotypes on all nights); $p = 0.0193$, 0.0236, and 0.0073, nights 4, 5, and 6, respectively. **(C)** Hierarchical clustering of the *cntnap2ab* behavioral fingerprint (red arrow) compared with the fingerprints of wild-type larvae exposed to a panel of 550 psychoactive agents from 4 to 6 days post fertilization (dpf) (77). Each rectangle in the clustergram represents the Z score, or the average value in standard deviations relative to the behavioral profiles of wild-type exposed to DMSO alone (magenta, higher than DMSO; cyan, lower than DMSO). The *cntnap2ab* profile correlates with agents that induce nighttime arousal ("Correlating Drugs"). **(D)** Rank-sorting of the anti-correlating dataset with respect to estrogenic compounds shows significant enrichment of estrogenic agents in the top ranks ($p = 0.0003$ by random permutation). Black lines indicate drugs defined as having estrogenic activity (25 compounds in total). **(E)** Hierarchical clustering of the behavioral fingerprints of *cntnap2a*$^{\Delta 121/\Delta 121}$*cntnap2b*$^{31i/31i}$ larvae exposed to 14 psychoactive agents at three doses each relative to the wild-type + no drug fingerprint. Each rectangle in the clustergram represents the Z score of drug-exposed mutants relative to untreated wild-type (magenta, higher than wild-type; cyan, lower than wild-type). **(F)** Magnified sections of the clustergram show relative suppression of the mutant fingerprint by biochanin A (0.1 µM) compared with risperidone (0.001 µM). The red box highlights parameters that measure nighttime activity. **(G)** Effect of the blind addition of biochanin A (0.1–1 µM) or DMSO on nighttime activity in the progeny of incrosses of *cntnap2a*$^{\Delta 25/+}$*cntnap2b*$^{\Delta 7/+}$ fish at 5 dpf. *$p = 0.045$ (two-way ANOVA, gene x dose interaction). Figure adapted and reprinted with permission from Hoffman et al. (8).

signaling pathways and sexual differential processes implicated in ASD. These pathways might represent potential avenues for future investigation.

Sexual Differentiation of the Mammalian Brain

The gonadal sex steroid hormones, estradiol and testosterone, affect sexual differentiation of the mammalian brain. To place the preclinical studies discussed in the previous section in context, here we briefly review these mechanisms, though for a more extensive discussion, see excellent reviews by McCarthy (11) and Ferri et al. (47). During an *in utero* organizational period, fetal testosterone is produced by the testes in males, controlling masculinization of the body and brain, while an absence of hormones is associated with feminization [reviewed in (47)]. Specifically, the aromatization hypothesis, based on studies of perinatal rats, holds that testicularly derived fetal testosterone is aromatized locally to estradiol in the male brain. Estradiol then induces masculinization in males, while the lack

of exposure to androgens, and its aromatized product, estradiol, leads to female brain development [reviewed in (11)]. Estradiol produced by the placenta results in high maternal levels of estradiol, which are sequestered by α-fetoprotein, a binding globulin that prevents maternal estradiol from masculinizing the brain (11). α-fetoprotein further protects female fetuses from masculinization; therefore, the impact of estrogens is differential in males vs. females (11). However, in addition to being exposed to maternal and gonadally-derived estradiol, fetuses and newborns are exposed to estradiol synthesized locally in the brain, which can exert effects independent of sexual differentiation (11). Overall, the impact of estrogens is restricted to a specific developmental window (mid-to-late gestation in primates), and includes both processes of sexual differentiation and reproduction-independent processes (11).

Genomic and Non-genomic Effects of Estrogens

Estrogens exert their effects through genomic and non-genomic modes of action. In the classical genomic mechanism, estradiol, the bioactive form of estrogen, binds to estrogen receptor-alpha (ERα) and estrogen receptor-beta (ERβ) in the cytoplasm, causing the estrogen receptors to dimerize and translocate to the nucleus. The nuclear estradiol-estrogen receptor complex binds directly to estrogen response elements in the promoters of target genes and acts as a ligand-dependent transcription factor, regulating the expression of hundreds of genes (79). Estrogens also exert effects through non-genomic mechanisms, which involve the binding of estradiol to membrane-bound estrogen receptors, resulting in the rapid activation of intracellular signaling cascades, including mitogen activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), phosphatidylinositol 3-kinase/protein kinase B/mTOR (PI3K/Akt/mTOR), and cAMP response element-binding protein (CREB)/brain derived neurotrophic factor (BDNF) [reviewed in (80)]. Estrogen binding to G-protein-coupled estrogen receptor (GPER) has been shown to initiate the rapid actions of estrogens in *in vitro* studies (81–83), though the precise mechanisms by which estrogens induce rapid non-genomic effects *in vivo* are not well-understood and may involve ERα and ERβ acting in a "non-classical" manner (84, 85). Estrogen signaling via these pathways has been shown to function in synaptogenesis (86–88) and play a neuroprotective role (discussed below). For example, acute administration of 17-β-estradiol was found to activate ERK1/2 in cultured embryonic rat cortical neurons and lead to a transient increase in dendritic spines (88). 17-β-estradiol was also found to activate phosphorylation of ERK and Akt, as well as the N-methyl-D-aspartate receptor subtype 2B (NR2B) subunit of NMDA receptors, in rat cortical synaptoneurosomes, providing evidence that estradiol acts directly at cortical synapses and regulates intracellular signaling pathways (86). There is also evidence for "crosstalk" between the ERK/MAPK and PI3K/Akt pathways downstream of estrogen signaling. For example, treatment with PI3K/Akt inhibitors blocked estradiol-mediated phosphorylation of both ERK and Akt in rat cortical synaptoneurosomes (86). In addition, 17-β-estradiol was shown to rapidly induce both MAPK and PI3K signaling *in vitro* in embryonic rat cortical neurons (89). Interestingly, a recent study examined mice lacking aromatase in forebrain neurons, which have decreased regional estradiol, and found that they display decreased MAPK/ERK and PI3K/Akt signaling, as well as reduced CREB/BDNF in the cerebral cortex and hippocampus (90). These mice also exhibit reduced synaptic density in the forebrain, decreased long-term potentiation (LTP) amplitude in hippocampal slices, as well as significant deficits in hippocampal-dependent spatial reference memory, recognition memory, and contextual fear memory. Restoring 17-β-estradiol acutely rescued the LTP deficit, while treatment with an ERK inhibitor prevented the rescue, providing evidence that estrogen signaling via ERK is important for learning and memory (90).

Interestingly, there are several lines of evidence implicating these intracellular signaling pathways in ASD, independent of their role in estrogen signaling. For example, as discussed above, dysregulation of PI3K/Akt/mTOR signaling has been described in rare monogenic disorders associated with ASD, including tuberous sclerosis complex, Fragile X syndrome, and neurofibromatosis [reviewed in (19)]. In addition, alterations in CREB and cAMP signaling have been identified in Fragile X syndrome (91, 92), and an allosteric inhibitor of an enzyme that degrades cAMP was recently found to significantly improve cognition in adult males with Fragile X syndrome in a randomized, placebo-controlled phase 2 trial (93). Moreover, a recent preclinical study found that male, but not female, mice with the 16p11.2 hemideletion have deficits in reward learning and increased ERK1 activation in the striatum, suggesting that regional alterations in ERK signaling might modulate sex-differential behaviors in the context of an ASD-associated CNV (94). Studies aimed at elucidating the connection between these intracellular signaling pathways and ASD, as well as their possible modulation by estrogens, represent important avenues for further investigation.

Estrogen Effects on Excitatory and Inhibitory Signaling

Estrogens have been shown to play a role in GABAergic neuron development as well as protect against glutamate-induced neurotoxicity. Estradiol influences the shift from excitatory to inhibitory GABAergic signaling in the developing brain. That is, $GABA_A$ receptors, which are ligand-gated chloride channels, exhibit depolarizing effects during the neonatal period and classical hyperpolarizing signaling at later stages. This shift from excitation to inhibition is mediated by changes in chloride co-transporter expression and activity, which alter the reversal potential for chloride [see excellent review by McCarthy (11)]. Estradiol has been shown to enhance the depolarizing effects of GABA in developing hypothalamic and hippocampal neurons (11, 95, 96). However,

estradiol-promoted enhancement of the excitatory GABA effect may result in a lower threshold for excitotoxicity (11). Therefore, estradiol seems to have opposite effects on GABAergic and glutamatergic signaling in the developing brain, increasing GABA-induced excitotoxicity, while protecting against glutamate-induced excitotoxicity (11).

The neuroprotective effects of estrogen have been studied most extensively in the context of hypoxia and ischemia in the adult brain [see reviews by (97, 98)], yet less is known about its neuroprotective role in the developing brain (11). Interestingly, estrogen signaling via the intracellular pathways discussed above has been shown to mediate its neuroprotective effects. For example, estrogens were found to induce neuroprotective effects against glutamate-induced excitotoxicity in embryonic rat primary cortical neurons by activation of ERK/MAPK signaling (99). PI3K/Akt signaling has also been shown to mediate estrogen-induced neuroprotection. For example, pretreatment of cultured rat fetal cortical neurons with estradiol for 24 h prior to glutamate exposure significantly reduced glutamate-induced neurotoxicity, while exposure to a PI3K inhibitor significantly attenuated the neuroprotective effect of estradiol (100). In a follow-up study, estradiol was shown to induce phosphorylation of CREB and upregulate the anti-apoptotic protein, Bcl-2, in a PI3K/Akt-dependent manner, suggesting that estradiol exerts neuroprotective and anti-apoptotic effects via activation of the PI3K/Akt pathway (101).

Estrogens have also been shown to affect signaling and/or induce neuroprotective effects in other neural cell types that have been implicated in ASD, including oxytocin neurons (102, 103), dopaminergic neurons (104–106), as well as astrocytes and microglia (107–109), though a detailed discussion of these mechanisms is beyond the scope of this review. While estrogens have broad neurodevelopmental effects on multiple neural cell types, additional investigations are needed to determine which of these mechanisms might be most relevant to ASD pathophysiology.

Estrogen Effects on Synapse Formation and Function

As discussed above, estrogens have been shown to function in synaptogenesis (87, 88, 110). In particular, estradiol induces rapid effects at the synapse, including the activation of metabotropic glutamate receptors (mGluRs) mediated by ERα and ERβ, intracellular signaling cascades, actin polymerization, and local protein synthesis, which together may function in the "fine-tuning" of neural circuits (110). Interestingly, estrogens have been shown to play a critical role in synaptic plasticity and memory consolidation in the hippocampus [see excellent review by Frick (84)]. Studies have shown that infusion of 17-β-estradiol into the dorsal hippocampus or dorsal third ventricle enhances object recognition memory consolidation in young ovariectomized female mice, such that mice spend significantly more time with a novel vs. a familiar object following estradiol infusion [(111); reviewed in (84)]. Estradiol-induced memory consolidation requires ERK phosphorylation, which is activated by interactions between ERα and ERβ and metabotropic glutamate receptor 1 (mGluR1) (112, 113). Inhibitors of MAPK, PI3K, and mTOR (rapamycin) have been shown to block ERK phosphorylation and estradiol-induced hippocampal memory consolidation, indicating that the rapid activation of these signaling pathways is involved in this process (114–116). Estradiol-induced ERK activation also leads to phosphorylation of CREB, as well as increased histone H3 acetylation (117). Blocking histone acetylation also prevents estradiol-induced memory consolidation, demonstrating that epigenetic effects downstream of estrogen signaling play an important role in this process as well (118). All together, these studies highlight a central role for estrogens in shaping synaptic function and neural circuits.

DISCUSSION

In the past 10 years, there has been remarkable progress in ASD risk gene discovery, enabling the identification of common mechanistic pathways involving these genes, including the regulation of gene expression, synaptogenesis, and excitatory-inhibitory imbalance, leading to insights into basic biological processes that may be disrupted in ASD. It has long been recognized that ASD has a strong male bias, even when taking into account ascertainment bias, which may contribute to a decreased tendency to diagnose ASD in females, yet the basis for the male predominance is not well-understood. At a genetics level, there is evidence for a "female protective effect," such that females are more likely to carry mutations of greater impact, suggesting that females have a higher liability threshold for ASD diagnosis and/or that males have a lower liability threshold (7). While the etiology of the female protective effect is not well-understood, recent studies using genetics, transcriptomics, imaging genomics, as well as animal models are beginning to shed light on potential relevant mechanisms.

First, genetics studies to date have not identified a unifying genetic mechanism that accounts for resilience in females and/or risk in males (5, 7, 50), suggesting that the sex bias may not be driven by primarily genetic factors. Consistent with this finding, the first transcriptomic study of sex-differential processes in ASD (7) found that genes with dysregulated expression patterns in post-mortem brains of individuals with ASD, but not ASD risk genes themselves, show sex-differential expression patterns. This intriguing finding suggests that factors downstream of ASD risk genes likely interact with sex-differential processes to contribute to the sex bias in ASD. Second, a recent imaging genomics study from our group found that females with ASD show decreased activation of sensorimotor, striatal, and frontal brain regions in response to biological motion and have larger rare, genic CNVs containing genes expressed in these brain regions, particularly the striatum (54). In addition to finding a novel convergence in genetics and imaging data, this study further indicates that brain regions responsible for differences in the perception of social stimuli in typically developing females and males may also contribute to the female protective effect in ASD (54). Third, consistent with the "extreme male brain" theory, studies of

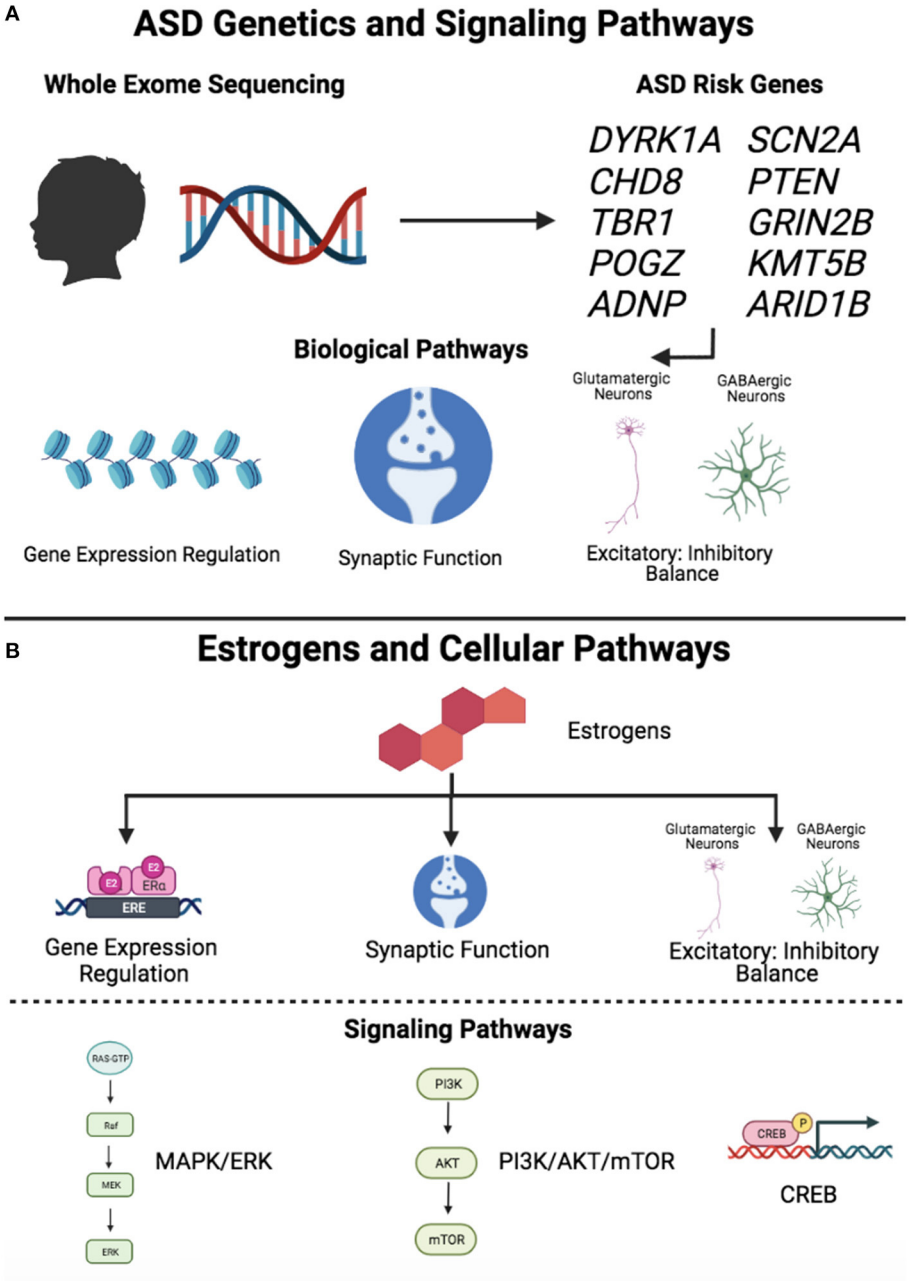

FIGURE 3 | Signaling pathways downstream of ASD risk genes and estrogens. **(A)** ASD genetics and signaling pathways. Whole exome sequencing studies have led to the identification of high confidence ASD risk genes, which converge on common biological pathways, including gene expression regulation, synaptic function, and excitatory-inhibitory balance. **(B)** Estrogens and cellular pathways. Estrogens have been shown to affect several biological pathways, including gene expression regulation, synaptic function, and excitatory-inhibitory balance. Estrogens exert effects through intracellular signaling pathways, including MAPK/ERK, PI3K/Akt/mTOR, and CREB. Figure created with BioRender.com. "Gene Expression Regulation" and "PI3K/AKT/mTOR" symbols (Estrogens and Cellular Pathways, lower panel) adapted from "Estrogen Receptor Signaling" and "KRAS Signaling Pathways," respectively, by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.

prenatal exposure to sex steroid hormones in humans indicate that increased steroidogenic activity might be associated with increased ASD risk (60, 61) [reviewed in (7, 47)]. However, the mechanisms by which differential exposure to androgens and/or estrogens might contribute to risk or resiliency are not well-understood. Additional clinical studies with larger sample sizes of males and females will be important for further clarifying this association.

Interestingly, recent preclinical studies from our group and others identified estrogenic compounds as potential suppressors of ASD gene-associated cellular or behavioral phenotypes in unbiased *in vivo* pharmacological screens in zebrafish and *Xenopus* (8, 9), and found that estrogens rescue seizures in a genetic mouse model of epilepsy (48) and cellular phenotypes associated with *in vitro* dysregulation of *DISC1* (72). While these investigations raise important questions regarding how estrogens might affect basic mechanisms of brain development, translating findings from animal and *in vitro* systems to humans presents various challenges. On the one hand, estrogens were found to suppress abnormal cellular and behavioral phenotypes associated with risk gene loss of function in preclinical studies (8, 9, 48, 72). At the same time, as discussed above, studies of prenatal exposure to sex steroids in humans found that increased steroidogenic activity is associated with ASD risk (60, 61). One possible interpretation is that the preclinical studies reflect the modulatory effects of estrogens on basic neurodevelopmental processes independent of their role in sexual differentiation. That is, estrogens may contribute to resilience during brain development by modulating signaling pathways, cellular phenotypes, and/or neural circuits that are dysregulated downstream of ASD risk genes. For example, our group hypothesized that estrogenic compounds may act by modulating GABAergic and glutamatergic signaling deficits in zebrafish *cntnap2* mutants (8). In addition, Willsey et al. (9) found that estrogens may affect neurogenesis by regulating the expression of genes in the SHH pathway. Moreover, identifying equivalent developmental time points across different systems further complicates directly translating findings from preclinical to clinical studies. Future analyses aimed at investigating the mechanisms underlying the effects of estrogens in preclinical studies, as well as the functions of estrogens and androgens in mammalian brain development and sexual differentiation will be essential for resolving these findings.

While additional investigations are needed to determine the extent to which estrogens might modulate brain development with relevance to the sex bias in ASD, it is interesting to observe that there are multiple pathways downstream of estrogen signaling that intersect with biological processes implicated by ASD risk genes (**Figure 3**). First, estrogens have been shown to affect synaptogenesis and induce neuroprotective effects via the PI3K/Akt/mTOR, cAMP/CREB, and ERK/MAPK intracellular signaling pathways (86, 88, 90, 99–101), which have been independently implicated in ASD. Studies examining the interactions between estrogens and these pathways in the context of ASD gene-associated preclinical models are likely to be highly informative. Second, estrogens affect GABAergic neuron development and protect against glutamate-induced toxicity (11), suggesting that they may contribute to excitatory-inhibitory balance, which has been proposed as a central mechanistic pathway in ASD and other neurodevelopmental disorders (44). Third, estrogens affect synaptogenesis in developing neurons (90, 110) and play a clear role in learning and memory by promoting synaptogenesis in the adult hippocampus (84). Given that synaptic function has been identified as a key pathway in ASD, investigating the molecular mechanisms by which estrogens affect synaptogenesis and circuitry in the developing brain is likely to be important for gaining a greater understanding of their potential role as modulators. Emerging research into the roles of sex steroids in the developing brain along with studies integrating genetics, transcriptomics, and neuroimaging are likely to inform our understanding of sex differential biology in ASD. In particular, interactions between pathways implicated by ASD risk genes and the neurodevelopmental effects of estrogens may represent important avenues for future evaluation.

SEARCH STRATEGY AND SELECTION CRITERIA

We searched PubMed for articles in all year ranges with multiple combinations of search terms including, "autism spectrum disorder," "estrogen," "estradiol," "brain," "genetics," "female protective effect," "sex differential processes," "neuroprotection," "MAPK," "ERK," "PI3K," "Akt," "mTOR," "CREB," "cAMP," "GABA," "glutamate," "synapse." Articles were selected based on relevance to topics covered in this review.

AUTHOR CONTRIBUTIONS

KE, AG, and EH contributed to the conceptualization, literature review, writing of this article, and approved it for publication. All authors contributed to the article and approved the submitted version.

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The Role of Ion Channel-Related Genes in Autism Spectrum Disorder: A Study Using Next-Generation Sequencing

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The clinical heterogeneity of autism spectrum disorder (ASD) is closely associated with the diversity of genes related to ASD pathogenesis. With their low effect size, it has been hard to define the role of common variants of genes in ASD phenotype. In this study, we reviewed genetic results and clinical scores widely used for ASD diagnosis to investigate the role of genes in ASD phenotype considering their functions in molecular pathways. Genetic data from next-generation sequencing (NGS) were collected from 94 participants with ASD. We analyzed enrichment of cellular processes and gene ontology using the Database for Annotation, Visualization, and Integrated Discovery (DAVID). We compared clinical characteristics according to genetic functional characteristics. We found 266 genes containing nonsense, frame shift, missense, and splice site mutations. Results from DAVID revealed significant enrichment for "ion channel" with an enrichment score of 8.84. Moreover, ASD participants carrying mutations in ion channel-related genes showed higher total IQ (p = 0.013) and lower repetitive, restricted behavior (RRB)-related scores (p = 0.003) and mannerism subscale of social responsiveness scale scores, compared to other participants. Individuals with variants in ion channel genes showed lower RRB scores, suggesting that ion channel genes might be relatively less associated with RRB pathogenesis. These results contribute to understanding of the role of common variants in ASD and could be important in the development of precision medicine of ASD.

Keywords: autism spectrum disorder, next-generation sequencing, ion channel genes, common variants, restricted repetitive behavior

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INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder whose essential features include persistent impairment in reciprocal social communication and restricted, repetitive behaviors and interests (American Psychiatric Association and DSM-5 Task Force., 2013). Although ASD is now widely known to the public, with a reported prevalence of 18.5 per 1,000 children aged 8 years in the United States (Maenner et al., 2020), proper diagnosis and treatment are still major challenges for clinicians because of the heterogeneity of the disorder. Regardless of severity, most patients with ASD require suitable therapy considering their individual symptoms (Lord et al., 2018). Among various

treatments applied for ASD patients, pharmacotherapy has proven effective in reducing behavioral problems associated with ASD (DeFilippis and Wagner, 2016). Previous studies have reported that over 30% of ASD patients used at least one antipsychotic drug and that the use of medication tends to be higher in ASD children (Rosenberg et al., 2010; Schubart et al., 2014). Pharmacologic treatment in ASD is usually focused on controlling restricted, repetitive behaviors (RRBs), irritability, and aggressive behaviors that are disruptive in behavioral therapy, in social situations, and in daily life (Williamson and Martin, 2012; Fung et al., 2016). Medications are more often prescribed if ASD patients are diagnosed of other comorbid psychiatric illnesses, and antipsychotics are the most frequently prescribed pharmacotherapy in ASD with intellectual disability (Houghton et al., 2017).

The diversity of genes related to ASD pathogenesis appears to be closely associated with the clinical heterogeneity of ASD (Persico and Napolioni, 2013). In addition to ASD-related syndromes or rare chromosomal abnormalities, additive effects from common genetic variants are also known to be related to ASD etiology (Lovato et al., 2019). Moreover, as rare gene variations with a high effect size account only for 10% of idiopathic autism (Persico and Napolioni, 2013), a cumulative effect for common genetic polymorphisms, such as single-nucleotide polymorphism (SNP), with a low effect size are thought to be important in explaining genetic components of ASD (Klei et al., 2012; Gaugler et al., 2014). Meanwhile, recent studies have indicated that common variants could be informative in identifying and diagnosing ASD (Wang and Avillach, 2021) and that cumulative dysfunction of genes by common variants could affect the severity of ASD manifestations (Toma, 2020). Additional research suggests that noncoding variants, as well as single-nucleotide variants and mosaic single-nucleotide variants, are implicated in autism susceptibility (Dias and Walsh, 2020).

However, defining the role of common variants in ASD still faces several obstacles. First, while common variants have been found to be related to ASD etiology in several studies, results have proven difficult to replicate, with an enormous number of genes suspected to be involved in ASD (Lovato et al., 2019). Also, it can be difficult to demonstrate the genetic contribution of a single common gene variant to ASD alone, because ASD shares genetic risks with other psychiatric illnesses, such as schizophrenia, and other neurodevelopmental disorders (Lee et al., 2013). Moreover, stochastic factors during gene expression and environmental factors can also affect the onset of ASD (Geschwind, 2011).

In recent years, next-generation sequencing (NGS), such as whole-genome sequencing, whole-exome sequencing, or clinical exome sequencing, has found use in identifying novel mutations in genes related to ASD (Jiang et al., 2013; Lovato et al., 2019). Most of the genes shown to be associated with ASD can be functionally classified into specific molecular pathways (Sahin and Sur, 2015; Parenti et al., 2020): the pathways include protein synthesis, transcriptional and epigenetic regulation, and synaptic signaling, affecting the functions of neurons and synapses important in neurodevelopment (De Rubeis et al., 2014; Sahin and Sur, 2015). Nevertheless, despite advancements in understanding of the molecular pathology of ASD, it is still unclear how molecular pathway alterations affect ASD phenotypes. For this reason, application of NGS in clinical settings remains limited. Understanding of the linkage between genotypes and ASD phenotypes, however, may help contribute to finally achieving proper diagnosis and predicting prognosis and individualized therapy.

In the present study, we investigated the role of genes in ASD phenotype in consideration of genetic functions in molecular pathways using NGS. We reviewed genetic results and clinical scores clinically used for ASD diagnosis. By excluding rare ASD-related syndromes and rare copy number variants, we only focused on common gene variants. To examine the relationship between ASD phenotype and genotype, we analyzed clinical scores for social function, RRB, and cognitive function in relation to the genetic results.

MATERIALS AND METHODS

Participants

In total, 197 children who underwent NGS for genetic evaluation were included in this study. All children were diagnosed with ASD by specialized child psychiatrists according to the diagnostic criteria suggested in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (American Psychiatric Association and DSM-5 Task Force., 2013). The diagnosis of ASD was confirmed with Autism Diagnostic Interview-Revised (Lord et al., 1994) and Autism Diagnostic Observation Schedule-2 scores (Lord et al., 2000). Only children who showed severe autistic symptoms, morphologic problems, or other comorbidities were recommended for genetic evaluation in our clinical setting. Data were collected by retrospectively reviewing medical records for the children. Information on demographics, clinical symptom scores, genetics, comorbidities, and medications was collected. Among 197 children with ASD, we excluded children from analysis if any of the clinical symptom scores described below were missing. Ninety-six children were excluded before analysis due to insufficient clinical data. Afterward, seven children who were diagnosed with ASD-related syndromes (tuberous sclerosis or Rett syndrome) were additionally excluded from this study. This study was approved by the applicable institutional review boards for research with human subjects at Severance Hospital, Yonsei University College of Medicine, where this study was performed. Written informed consent agreeing to donation of human biologic materials was acquired from the participant's legal guardian/next of kin.

Clinical Assessments

Autistic characteristics and intellectual function were assessed using the scales and tests listed below. For the assessment of intellectual function, the Korean-Wechsler Intelligence Scale for Children-IV (K-WISC-IV) (Gwak et al., 2011) or the Korean Wechsler Preschool and Primary Scales of Intelligence-IV (K-WPPSI-IV) (Park et al., 2016) was administered depending on the children's age and ability to perform the test. The Korean-Bayley-III scale was also used for participants who were unable to

perform intelligence tests. The Childhood Autism Rating Scale (CARS) was used to distinguish ASD from other developmental disorders and to assess the severity of ASD. A cutoff score of 30 points was applied, and the reliability and validity of the Korean version of CARS have been verified (Shin and Kim, 1998). The Social Communication Questionnaire (SCQ), which is based on the Autism Diagnostic Interview-Revised, was utilized to assess ASD symptoms (Anthony and Catherine, 2003). The Korean version of the SCQ was verified as a reliable and valid screening tool for autism in the Korean population (Kim et al., 2015).

The Social Responsiveness Scale (SRS) is a 65-item questionnaire of social interactions exhibited by children over the past 6 months (Constantino and Gruber, 2012). The test focuses on social impairments in naturalistic social settings and is measured by parents or teachers. It consists of five subscales (social awareness, social cognition, social communication, social motivation, and mannerisms). Each question is scored from 0 to 3 points, and the sum of scores for the social awareness, social cognition, social communication, and social motivation subscales is considered reflective of social communication, a core symptom of ASD in DSM-5. Similarly, the mannerisms subscale represents RRB symptoms, which is also a core symptom of ASD. We previously confirmed the clinical validity of the SRS in Korean children and suggested the relevance of SRS subscales to DSM-5 ASD diagnosis (Cheon et al., 2016). T-scores are used to resolve problems of differences in raw scores by sex or rater (parent or teacher). In this study, we used T-scores of each subscale, as well as total T-scores. T-scores over 75 indicate severe symptoms; T-scores between 60 and 75 are considered indicative of mid-to-moderate severity (Aldridge et al., 2012).

Next-Generation Sequencing

For exome sequencing, the xGen Inherited Diseases Panel (Integrated DNA Technologies, Coralville, IA, United States) including 4,503 candidate genes was used. The genes included in this panel are known to be related to ASD, intellectual disability, and other neurodevelopmental disorders.

The genomic DNA extracted from the children's blood was used for library preparation and target capture using a custom panel targeting candidate genes. The NextSeq 550Dx System (Illumina, San Diego, CA, United States) was used to perform massively parallel sequencing. With our custom analysis pipeline, quality control and sequence analysis were proceeded, and copy number analysis was performed (Kim et al., 2019). The GRCh37 (hg19) built as the reference sequence was applied for mapping and variant calling while using the Burrows–Wheeler alignment (BWA) tool (version 0.7.12). HaplotypeCaller and MuTect2 in the GATK package (3.8-0) and VarScan2 (2.4.0) were used to identify single-nucleotide variations (SNVs) and insertion and deletions (indels). Online databases including the Human Gene Mutation Database (HGMD), Online Mendelian Inheritance in Man (OMIM), Clinvar, dbSNP, 1000 Genomes, the Exome Aggregation Consortium (ExAC), the Exome Sequencing Project (ESP), and the Korean Reference Genome Database (KRGDB) were used for analyses and variant annotation.

Classification of variants was conducted using a scoring algorithm implemented in the DxSeq Analyzer (Dxome, Seoul, Korea), based on the standards and guidelines established by the American College of Medical Genetics (ACMG) (Richards et al., 2015). We excluded genetic variants classified as benign or likely benign based on ACMG guidelines in NGS clinical reports by physicians in laboratory medicine. Afterward, variants were lined in order of higher probability of pathogenicity according to ACMG guidelines. Among various variants, we selected five variants with the greatest likelihood of being pathogenetic from each patient.

Gene Ontology

Using the result of NGS, we analyzed enrichment of cellular processes and gene ontology using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009; Sherman and Lempicki, 2009). Children were then classified according to genetic characteristics.

Statistical Analysis

Independent t-tests were used to estimate group differences in demographics and clinical scores. The chi-squared test was used for comparing categorical variables. Logistic regression analysis was applied to evaluate the relative risk of a group to another. Statistical significance was defined at $p < 0.05$. All analyses were performed using the Statistical Package for the Social Sciences software (version 25.0; SPSS Inc., Chicago, IL, United States).

RESULTS

Applying the exclusion criteria, we excluded 94 children with ASD from final analysis (**Figure 1**). Children with ASD were about 6 years old on average (6.12 years, ranging from 3 years, 6 months to 15 years, 4 months). The male-to-female ratio was about 3:1, and 41 participants were using antipsychotics (aripiprazole or risperidone) because of excessive RRBs or irritability. The average IQ score was 51.87, ranging from 31 to 85. The T-score for total SRS was 85.53 on average, and all subscale T-scores in SRS exceeded 70 on average. CARS scores varied widely, from 21.00 to 51.50 (**Table 1**).

Excluding known benign variants, we found that children carried 0 to 34 variants, with 14.44 variants per child on average. We collected up to five SNVs in children with ASD that had the highest probability of being pathogenic according to ACMG guidelines. In total, we collected 266 genes containing nonsense, frame shift, missense, and splice site mutations. More than one-third of genes (91 genes) overlapped at least twice. Variants in *TSC2* (12 times), *RAI1* (9 times), *CHD7* (7 times), and *RELN* (7 times) were most frequently found among ASD children.

Results from DAVID highlighted significant enrichment for "ion channel" (UP_Keywords), with an enrichment score of 8.84 (corrected p = 1.9xe-13). In functional annotation clustering, 30 genes were involved in the ion channel cluster: *CACNG2*, *CACNA1A*, *CACNA1C*, *CACNA1D*, *CACNA1G*, and *CACNA1H* were associated with calcium voltage-gated channels; *SCN1A*, *SCN10A*, *SCN2A*, *SCN3A*, *SCN7A*, *SCN9A*, and *SCN1B* were involved in sodium voltage-gated channels; *KCNMA1*, *KCNT1*, *KCNH2*, *KCNQ2*, *KCNQ4*, *HCN1*, *HCN2*,

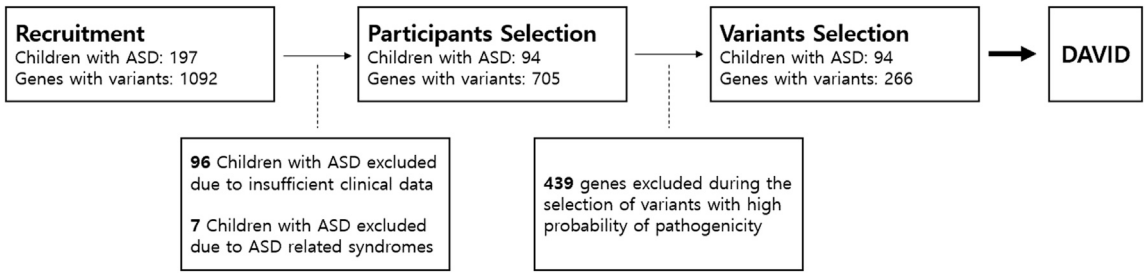

FIGURE 1 | Participants and gene selection. We first recruited 197 children with ASD who underwent next-generation sequencing analysis. In total, 1,092 genes were detected with variants in NGS. We only included participants whose clinical assessment was complete with no missing data. After participant selection, we only selected five variants that were most likely pathogenic in each patient based on ACMG guidelines. A total of 439 genes with variants were excluded. Only 266 genes remained from participant and variant selection. The 266 genes were included in enrichment analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID). ASD, autism spectrum disorder.

TABLE 1 | Demographic and clinical data for children with ASD and group comparisons.

	All (*n* = 94)	**Variants in ion channel genes (*n* = 37)**	**No variants in ion channel genes (*n* = 57)**	***p* value**
Male:female	63:31	26:11	37:20	
Age (years)	6y + 0.12m	5y + 6.81m	6y + 3.56m	0.141
IQ	51.87	55.22	49.70	0.013*
SRS_T	85.53	81.64	87.98	0.104
SCQ	16.42	15.92	16.76	0.563
CARS	32.09	31.55	32.45	0.404
Medication (*n*)	37	11	26	0.035*

Independent t *tests were performed to compare average values for age, IQ, SRS_T, SCQ, and CARS between two groups. The chi-square test was proceeded to analyze correlations between ion channel gene variants and medication use. ASD, autism spectrum disorder; IQ, Intelligence Quotient; SRS_T, Social responsiveness scale total score; SCQ, Social Communication Questionnaire; CARS, Childhood Autism Rating Scales. *p < 0.05.*

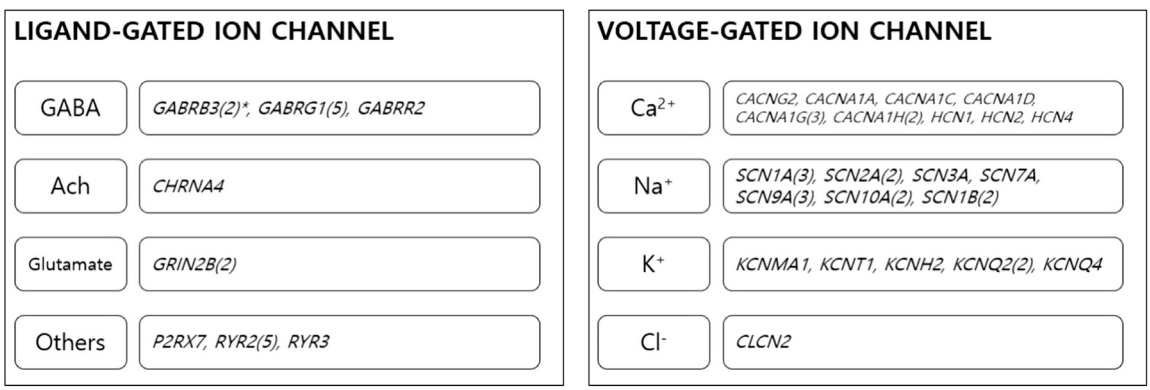

FIGURE 2 | Ion channel genes. Thirty genes were classified as ion channel-related genes. The genes were involved in the function of several ion channels. *Numbers in brackets stand for number of overlapping genes among children with ASD. GABA, gamma aminobutyric acid; Ach, acetylcholine.

and *HCN4* were related to potassium channels; and *CLCN2* and *CHRNA4* were involved in chloride channel function. *GABRB3*, *GABRG1*, *GABRR2*, *GRIN2B*, *P2RX7*, *RYR2*, and *RYR3* were also highlighted in ion channel functional cluster annotation (**Figure 2**). Details on variants of ion channel related genes are described in **Supplementary Material S1**.

In our data, 37 children with ASD had at least one variant in a gene involved in ion channel function. ASD children carrying variants in genes related to ion channels (ion channel group) showed significantly higher IQ (p = 0.013) and mannerism subscale scores in SRS (p = 0.003) than other children that did not. Other clinical scores were not significantly different between

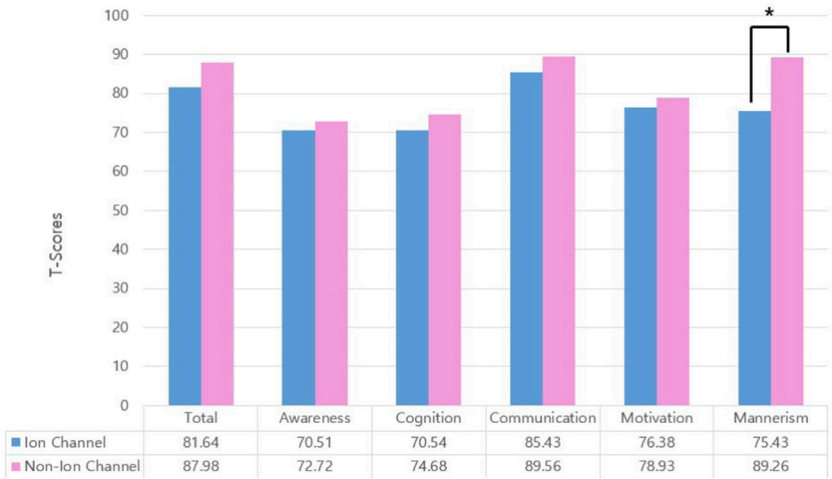

FIGURE 3 | Group comparison of Social Responsiveness Scale (SRS) total T-scores and subscale T-scores. Comparison of children with variants in ion channel-related genes versus others. Only mannerism subscale scores were significantly different (*$p = 0.003$). **Total:** SRS total T-score; **awareness:** social awareness subscale T-score; **cognition:** social cognition subscale T-score; **communication:** social communication subscale T-score; **motivation:** social motivation subscale T-score; **mannerism:** mannerism subscale T-score.

TABLE 2 | Differences in clinical scores between ASD children with and without antipsychotics use.

	Antipsychotics use (n = 41)	No antipsychotics use (n = 53)	p value
IQ	48.66	54.36	0.009*
SRS_T	88.02	83.56	0.244
SCQ	17.44	15.61	0.202
CARS	34.41	30.22	<0.001*

*ASD, autism spectrum disorder; IQ, Intelligence Quotient; SRS_T, Social responsiveness scale total score; SCQ, Social Communication Questionnaire; CARS, Childhood Autism Rating Scales. *p < 0.05.*

TABLE 3 | Logistic regression analysis for antipsychotics use in ASD.

Variables	Antipsychotics use		
	OR	95% CI	p value
Univariable logistic regression analysis			
IQ	1.058	1.012–1.105	0.012*
CARS	1.270	1.113–1.450	<0.001*
Ion Channel Group	0.381	0.159–0.914	0.031*
Multivariable logistic regression analysis			
CARS	1.244	1.085–1.426	0.002*

*ASD, autism spectrum disorder; OR, odds ratio; CI, confidence interval; IQ, Intellectual Quotient; CARS, Childhood Autism Rating Scales. p values are calculated using analysis of logistic regression. In multivariable logistic regression analysis, IQ, CARS, and ion channel group were adjusted for antipsychotics use. *p < 0.05.*

groups (**Table 1**; **Figure 3**). Chi-square analysis indicated that the ion channel group and medication use were significantly related ($p = 0.035$). On the other hand, children with ASD who were on pharmacotherapy showed significantly lower IQ ($p = 0.009$) and higher CARS scores ($p < 0.001$). There were no differences in SRS total/subscale scores between participants with and without pharmacotherapy (**Table 2**). Univariate logistic regression analysis revealed that the ion channel group was at a lower risk of undergoing pharmacotherapy, compared to other children (odds ratio = 0.381, $p = 0.031$). Logistic regression also revealed that ASD children with lower IQ scores were more prone to use medication (odds ratio = 1.058, $p = 0.012$) and that higher CARS score were related to medication use (odds ratio = 1.270, $p < 0.001$). Multivariable logistic regression analysis for medication use in ASD children showed that only a high CARS score was predictive of a greater likelihood of receiving pharmacotherapy in ASD (odds ratio = 1.244, $p = 0.002$) (**Table 3**).

DISCUSSION

In this study, we examined the role of common genetic variants in ASD phenotype by comparing clinical scores in ASD children with different genetic characteristics. Functional cluster annotation revealed significant enrichment for genes involved in ion channels. ASD children with ion channel-related genetic variants presented with significantly higher IQ and less severe RRBs, leading to less exposure to antipsychotics. Our findings suggested that different molecular pathways regulated by related genes are associated with the different aspect of ASD phenotype. Finding the linkage between the molecular pathway and ASD characteristics may contribute to predict prognosis and precision medicine in ASD at the clinical site.

As NGS was proceeded for comparatively severe ASD patients in clinic, the average of IQ was 51.87, and the average of SRS total T-score was within severe criteria. Considering the result of

genetic analysis that children with ASD possess more than 10 variants on average, additive genetic burdens of variants might have played a role in severe symptoms (Pizzo et al., 2019).

Impacting brain development in the prenatal period, defects in ion channels in the brain are critical not only in the pathogenesis of epilepsy but also in other neurodevelopmental disorders, including ASD (D'Adamo et al., 2020; Smith and Walsh, 2020). Mutations in ion channel-related genes seem to induce loss of function or gain of function of cell signaling (Imbrici et al., 2016), resulting in impairment of neuronal networks (Sanders et al., 2018). Although it would be difficult to discriminate the specific function of a particular ion channel in ASD pathogenesis, imbalances in excitation and inhibition have been emphasized in the development of neurodevelopmental disorders (Rubenstein and Merzenich, 2003). Additionally, both excitation and inhibition might play roles in complex neuronal circuits (Nelson and Valakh, 2015). In this study, genes associated with several ion channels were included in a functional cluster. Among 30 genes in the ion channel functional cluster, several genes appeared repeatedly in more than two children with ASD. *GABRG1*, one of the most frequently detected genes in our data, is a gamma-aminobutyric acid (GABA) receptor subunit gene. Although it is not yet clear whether mutated *GABRG1* directly affects ASD pathogenesis, studies have highlighted GABA receptor genes as potentially important in ASD (Ma et al., 2005): for example, GABA receptor density was found to be reduced in ASD (Blatt et al., 2001). Interestingly, a GABA gene cluster on human chromosome 4 was shown to be related to vulnerability to social context in youth (Villafuerte et al., 2014; Trucco et al., 2020), suggesting that these genes affect social functioning. Another frequently detected gene in our data was *RYR2*, which is involved in calcium channel activation in the outer membrane of the endoplasmic reticulum (George et al., 2003). The gene is usually associated with dysregulation of cardiac muscles, but when expressed in the brain, the gene may take part in social functioning and delayed development (Lu and Cantor, 2012). Mutations in calcium voltage-gated channel-related genes (*CACNA1A*, *CACNA1C*, *CACNA1D*, *CACNA1G*, and *CACNA1H*) and sodium voltage-gated channel-related genes (*SCN1A*, *SCN2A*, *SCN3A*, *SCN7A*, *SCN9A*, *SCN10A*, and *SCN1B*) were also detected by our clinical exome sequencing, in line with previous studies reporting genetic associations with ASD (Schmunk and Gargus, 2013). Although it is unclear how much these genes contribute to actual ASD pathogenesis, they do, at the very least, appear to enhance susceptibility to ASD (Schmunk and Gargus, 2013).

One particularly noteworthy finding in this study is that children with variants in ion channel-related genes showed significantly lower RRB scores, suggesting that channelopathy is unlikely to be associated with RRB pathophysiology. In this study, lower RRB scores were significantly related to less use of antipsychotics, an important issue in managing ASD children. Genes related to RRB etiology have been shown to be highly heritable (Ronald et al., 2006) and to be independent of genes affecting social functioning (Ronald et al., 2005). Genetic differences in ASD core symptoms are also supported by differences in RRB symptom severity by sex (Szatmari et al., 2012). Although it remains difficult to explain the genetic differences between social impairment and RRBs, neurobiological factors, such as cortical–basal ganglia pathways, might be closely related (Turner et al., 2006). Considering it is the phenotypic heterogeneity and complex pathophysiology of RRBs in ASD, various genes may be involved (Lewis and Kim, 2009). In addition, neurotransmitter genes, such as dopamine (Lewis and Bodfish, 1998), glutamate (Purcell et al., 2001), serotonin (Di Giovanni et al., 2006), and GABA (Shao et al., 2003) genes, have been shown to be associated with RRBs (Lewis and Kim, 2009). While the cumulative burden of common genetic variants likely affects ASD phenotype the most, we assume that ion channel-related genes may be less connected to RRBs. Although the ion channel group was significantly associated with medication use in our study, the overall severity of ASD represented by CARS scores was more strongly associated with pharmacotherapy of ASD than the RRB score. This might be because RRBs are not the only reason for pharmacotherapy in ASD: emotional problems can also account for antipsychotic use (Stepanova et al., 2017).

There are several limitations to this study. First, the sample size was relatively small, compared to other genetic studies. As this study reviewed medical records retrospectively, we made an effort to include only ASD children with sufficient clinical scores. Also, NGS was only conducted for severe ASD patients at our clinic. As such, the average IQ score was 51.87, and the average total T-score for SRS fell within severe criteria. This limits the generalizability of our results to individuals with less severe ASD. Second, we used both K-WISC-IV and K-WPPSI-IV for intelligence tests because of differences in age at examination, resulting in un-unified subscales of IQ. Third, as reports on genetic results of NGS in Korean individuals with ASD are scarce, we were unable to compare our results within this population. Also, as we only reviewed medical records, we could not compare our results with a healthy control group. Similar studies including healthy controls should be followed to avoid false-positive results. Fourth, considering the preschool age and the low intellectual function, we did not evaluate the comorbidity of attention-deficit/hyperactivity disorder (ADHD) because of the diagnostic instability (Bunte et al., 2014). As ADHD could also possess genetic variants related to the ion channel pathway (Thapar et al., 2016), longitudinal follow-up of comorbidities should be followed. Fifth, we analyzed common variants in genes that seemed to have a low-to-moderate effect size, rather than rare variants. Our selection thereof may put into question if the genetic variants truly affect ASD etiology. Also, considering that genetic analysis has indicated that children with ASD possess more than 10 variants on average, we suspect that additive genetic burden from variants might have played a role in the more severe symptoms seen in our patients (Pizzo et al., 2019). In spite of these weaknesses, we present one possible way in which to interpret the meaning of numerous common variants in ASD. Also, we attempted to discriminate relatively pathogenic variants using ACMG guidelines.

In conclusion, we found several ion channel-related genes to be involved in ASD etiology. Although mutations in ion channel genes are expected to present low-to-moderate

effect sizes, they might enhance susceptibility to ASD. Moreover, participants with variants in ion channel genes showed lower RRB scores, suggesting that ion channel genes might not be strongly associated with RRB pathogenesis. These results contribute to helping further understanding of the role of common variants in ASD and could prove to be important in the development of precision medicine for ASD.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because it includes the patient's genetic data for clinical purpose. Requests to access the datasets should be directed to kacheon@yuhs.ac.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Boards for research with human subjects at Severance Hospital, Yonsei University College of Medicine. Written informed consent from the participant's 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

Conceptualization, JL, SH, JA, and KC. Data curation, SL, JC, and KC. Formal analysis, SL and JC. Methodology, JL, SH, JA. Supervision, KC. Writing—original draft, LS. Writing—review and editing, JS, SH, JA, SL, JC, and KC.

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SUPPLEMENTARY MATERIAL

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SYSTEMATIC REVIEW
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Systematic Review and Bioinformatic Analysis of microRNA Expression in Autism Spectrum Disorder Identifies Pathways Associated With Cancer, Metabolism, Cell Signaling, and Cell Adhesion

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Background: Previous studies have identified differentially expressed microRNAs in autism spectrum disorder (ASD), however, results are discrepant. We aimed to systematically review this topic and perform bioinformatic analysis to identify genes and pathways associated with ASD miRNAs.

Methods: Following the Preferred Reporting Items for Systematic reviews and Meta-Analyses, we searched the Web of Science, PubMed, Embase, Scopus, and OVID databases to identify all studies comparing microRNA expressions between ASD persons and non-ASD controls on May 11, 2020. We obtained ASD miRNA targets validated by experimental assays from miRTarBase and performed pathway enrichment analysis using Metascape and DIANA-miRPath v3. 0.

Results: Thirty-four studies were included in the systematic review. Among 285 altered miRNAs reported in these studies, 15 were consistently upregulated, 14 were consistently downregulated, and 39 were inconsistently dysregulated. The most frequently altered miRNAs including miR-23a-3p, miR-106b-5p, miR-146a-5p, miR-7-5p, miR-27a-3p, miR-181b-5p, miR-486-3p, and miR-451a. Subgroup analysis of tissues showed that miR-146a-5p, miR-155-5p, miR-1277-3p, miR-21-3p, miR-106b-5p, and miR-451a were consistently upregulated in brain tissues, while miR-4742-3p was consistently downregulated; miR-23b-3p, miR-483-5p, and miR-23a-3p were consistently upregulated in blood samples, while miR-15a-5p, miR-193a-5p, miR-20a-5p, miR-574-3p, miR-92a-3p, miR-3135a, and miR-103a-3p were consistently downregulated; miR-7-5p was consistently upregulated in saliva, miR-23a-3p and miR-32-5p were consistently downregulated. The altered ASD miRNAs identified in at least two independent studies were validated to target many autism risk genes. *TNRC6B, PTEN, AGO1, SKI*, and *SMAD4* were the most frequent targets, and miR-92a-3p had the most target autism risk genes. Pathway enrichment analysis showed that ASD miRNAs are significantly involved in pathways associated with cancer, metabolism (notably Steroid biosynthesis, Fatty acid metabolism, Fatty acid biosynthesis, Lysine degradation, Biotin metabolism), cell cycle, cell signaling (especially Hippo, FoxO, TGF-beta, p53,

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Thyroid hormone, and Estrogen signaling pathway), adherens junction, extracellular matrix-receptor interaction, and Prion diseases.

Conclusions: Altered miRNAs in ASD target autism risk genes and are involved in various ASD-related pathways, some of which are understudied and require further investigation.

Keywords: systematic review, microRNA, autism spectrum disorder, gene, signaling pathway, bioinformatic analysis

INTRODUCTION

Autism spectrum disorder (ASD) is a category of clinically and genetically heterogeneous neurodevelopmental disorder characterized by impaired social function and repetitive, restricted behaviors (1). Genetics plays a significant role in the cause of ASD. Hundreds of different genetic loci, including non-coding mutations (2), single-nucleotide variants, chromosome abnormalities, and copy number variations have been associated with ASD (3, 4). However, many different variants share common biological pathways (5). Thus, identifying converging biological pathways and molecular mechanisms responsible for this disorder has much translational and clinical value (5).

MicroRNAs (miRNAs) are a class of small, non-coding RNAs with the main functions of regulating mRNA destabilization and modifying protein levels (6). One miRNA can target up to hundreds of different mRNAs, and a single mRNA may be regulated by many miRNAs. Thus, miRNAs–mRNAs form complicated gene regulatory networks and participate in various biological functions (7), including brain development and function (8). By regulating local gene expression, miRNAs can control cell fate determination, neurogenesis, cell migration, neuronal polarization, synapse development, and synaptic plasticity (9). In neurodevelopmental disorders, miRNAs are often dysregulated, indicating that miRNAs play an important part in the etiology and/or maintenance of neurological disorders (9).

Since the first study performed by Abu-Elneel and his colleagues in 2008 (10), miRNA expression profiling in ASD has been performed in brain tissue samples (10–18), serum (19–23), blood (24–30), peripheral blood monocytes (31–33), lymphoblast cell lines (34–37), saliva (38–40), reprogrammed induced pluripotent stem cell-derived neurons (41), olfactory mucosal stem cells, and skin fibroblasts (42) in studies comparing ASD persons and controls. There were a significant number of miRNAs in ASD samples and control with different expressions; however, many discrepancies among studies exist. The discrepancies may be due to differences in tissue types, genetic and environmental origin of the tissue sources, RNA extraction methods, miRNA detection, and validation methods (such as microarray, miRNA-seq, and RT-qPCR), data normalization methods, identification of new miRNAs, and miRNAs' annotation changes in the miRbase database, which stores information about individual microRNAs since 2002 (43). The biological function of these microRNAs in ASD pathogenesis remains little known, and there remains a question as to which miRNAs may be significant specific signatures as personalized biomarkers or therapeutic targets.

Until now, the literature reviews of ASD miRNAs were primarily narrative, and conclusive results were not available for comprehensive functional analysis (44). Therefore, an updated systematic review is needed. The objective of this research was to systematically review the literature to assess which microRNAs were altered in children and adults with ASD when compared to non-ASD controls from case-control studies and standardize them to miRBase version 22.1 (43). Furthermore, validated gene

Abbreviations: ADCY3, adenylate cyclase 3; ADI-R, Autism Diagnostic Interview-Revised; ADOS, Autism Diagnostic Observation Schedule; AGO1, argonaute RISC component 1; Akt, also known as protein kinase B; AMPK, 5′ adenosine monophosphate-activated protein kinase; ASD, Autism spectrum disorder; BTRC, beta-transducin repeat containing E3 ubiquitin protein ligase; CARS, Childhood Autism Rating Scale; CARS-II, Childhood Autism Rating Scale, Second edition; CDH5, cadherin 5; CDH8, cadherin 8; CDH9, cadherin 9; CHD8, chromodomain helicase DNA binding protein 8; CSNK1E, casein kinase 1 epsilon; Ct, cycles-to-threshold; CTNNB1, catenin beta 1; CUL3, cullin 3; DSM-5, Diagnostic and Statistical Manual of Mental Disorders, 5th Edition; DSM-IV, Diagnostic and Statistical Manual of Mental Disorders, 4th Edition; DSM-IV-TR, Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision; ECM, extracellular matrix; FC, fold change; FDR, false discovery rate correction; FoxO, forkhead box O; GNAI1, G protein subunit alpha i1; IGF1, insulin like growth factor 1; IL6, interleukin 6; ITPR1, Inositol 1,4,5-trisphosphate receptor type 1; KDM3B, Lysine Demethylase 3B; KDM6B, lysine demethylase 6B; KEGG, Kyoto Encyclopedia of Genes and Genomes; KMT2A, lysine methyltransferase 2A; KMT2C, lysine methyltransferase 2C; KMT5B, lysine methyltransferase 5B; LAMB1, laminin subunit beta 1; MAPK, mitogen-activated protein kinase; MECP2, Methyl CpG binding protein 2; MED13, mediator complex subunit 13; mTOR, mammalian (mechanistic) target of rapamycin; MTOR, mechanistic target of rapamycin kinase; N/A, not available; NF-kappa B, Nuclear factor-kappa B; NPAS2, neuronal PAS domain protein 2; NR1D1, nuclear receptor subfamily 1 group D member 1; NR4A2, nuclear receptor subfamily 4 group A member 2; NSD1, nuclear receptor binding SET domain protein 1; PAX6, paired box 6; PBMC, peripheral blood monocytes; PCDH10, protocadherin 10; PER1, period circadian regulator 1; PER2, period circadian regulator 2; PI3K, phosphatidylinositol 3′ -kinase; PIK3R2, phosphoinositide-3-kinase regulatory subunit 2; PPP2R1B, protein phosphatase 2 scaffold subunit Abeta; PRISMA, Preferred Reporting Items for Systematic reviews and Meta-Analyses; PTEN, phosphatase and tensin homolog; qRT-PCR, quantitative reverse-transcription/real time polymerase chain reaction; RELN, reelin; RHEB, Ras homolog, mTORC1binding; RNA-Seq, ribonucleic acid sequencing; RORA, RAR related orphan receptor A; RORB, RAR related orphan receptor B; SETD1B, SET domain containing 1B; SHANK3, SH3 and multiple ankyrin repeat domains 3; SKI, Sloan–Kettering Institute proto-oncogene; SMAD, mothers against decapentaplegic homolog; TBL1XR1, transducin beta like 1 X-linked receptor 1; TCF4, transcription factor 4; TGF, transforming growth factor; THBS1, thrombospondin 1; THRA, thyroid hormone receptor alpha; TNRC6B, trinucleotide repeat containing adaptor 6B; TPO, thyroid peroxidase; TRIP12, thyroid hormone receptor interactor 12; TSC1, tuberous sclerosis complex 1; TSC2, tuberous sclerosis complex 2; Wnt, Wingless-type/Int-1.

targets of these miRNAs were obtained and pathway enrichment analysis was used to assess the physiological impact of miRNA dysregulation in ASD pathology. Our study may clarify the ambiguities and contradictions in this research field and promote future further studies to better understand microRNAs' function in ASD. This may also contribute to the use of microRNAs as potential personalized biomarkers and therapeutic targets.

MATERIALS AND METHODS

Criteria for Considering Studies for This Review

Types of Participants

We included children and adults with ASD diagnosed by an established classification system or clinical assessment, including individuals with autistic disorder, Asperger's disorder, and pervasive developmental disorder–not otherwise specified (PDD-NOS). Participants with comorbidities were not excluded.

Types of Exposures

The exposure was ASD diagnosis.

Types of Control

The control participants were non-ASD individuals.

Types of Outcome Measures

The outcome measure was microRNA expression level.

Types of Studies

Original research case-control studies written in English were eligible for this review. Literature reviews, non-human studies, comments, opinion articles, expert opinions, letters, news reports, hypotheses, conference summaries, book sections, patent descriptions, same study reports, non-ASD or non-microRNA expression studies, and studies that are not case-control were excluded.

Database Search Strategies for Identification of Studies

We conducted a systematic literature search in Web of Science, PubMed, Embase, Scopus, and OVID databases without initial date restriction up to and including May 11, 2020. The search criteria in five databases are provided in **Supplementary Material 1**. No language restriction was applied. The search yielded 2,718 references.

Selection of Studies

All references were managed in the EndNote X9 software (Thomson Reuters, New York, NY, USA). Initially, duplicate references were removed. Two reviewers (ZXH and GFC) independently screened titles and abstracts based on the inclusion and exclusion criteria. Lists were compared, and a consensus was reached through discussion or with a third reviewer in case of disagreement. This systematic review followed the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statements (**Figure 1**; **Supplementary Material 2**), with some modifications (45).

Data Collection

Two reviewers (ZXH and HRG) independently collected the following data items from the full text and supplementary data of each included study: first author, year of publication, country of study, tissue types, diagnostic measure, sample sizes, the age of cases and controls, the gender of cases and controls, miRNA expression assay type, lists of upregulated and downregulated miRNAs with statistically significant expression changes, and differential expression criteria. Lists were compared, and a consensus was reached through discussion or with a third reviewer (GFC) in case of disagreement. All miRNA names from different studies were standardized to miRBase version 22.1 (43). Pre-miRNAs, viral miRNAs, and non-miRNAs were not used in the bioinformatic analysis.

Enrichment for ASD Risk Genes Among Validated Targets for Each Overlapping miRNAs in ASD

To explore the experimentally validated target genes of each microRNAs that were dysregulated in at least two independent studies (ASD-miRNAs), we obtained ASD-miRNAs targets that had been experimentally verified using reporter assays, western blots, microarrays, or next-generation sequencing studies, among other methods, from the miRTarBase database (**Supplementary Material 3**) (46). We did not perform further filtering based on strength of evidence. Next, we systematically evaluated and compared whether targets of the differentially expressed miRNAs are enriched for ASD risk genes (**Supplementary Material 4**) from the Simons Foundation Autism Research Initiative (SFARI, https://gene.sfari.org/database/human-gene/) (47) and AutDB database (http://autism.mindspec.org/autdb/HG_Home.do) (48), which are based on candidate gene studies, common variant association, genetic syndromes, and copy number variation.

Subgroup Analysis of Tissues and Pathway Enrichment Analysis of Validated Targets for Each ASD-miRNAs

To further explore the associated biological pathways of ASD-miRNAs in specific tissues, firstly the web-based software portal Metascape was used to conduct Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathways analysis of validated targets from miRTarbase for each of ASD-miRNAs (49), with a background set of genes that are expressed in the brain, salivary glands, blood, and immune cells (**Supplementary Material 5**, data available from v20.1.proteinatlas.org) (50) for miRNA detected in these tissues. There are 16,227 genes in the brain (data available from https://www.proteinatlas.org/humanproteome/brain/human+brain), 15,218 in the salivary glands (data available from https://www.proteinatlas.org/humanproteome/tissue/salivary+gland), and 14,812 in "blood and immune cells" (data available from https://www.proteinatlas.org/humanproteome/celltype/blood+26+immune+cells). Studies using brain tissue, reprogrammed induced pluripotent stem cell-derived neurons, or olfactory mucosal stem cells were classified as "brain" tissue. Studies using serum, blood, lymphoblast cell lines, or

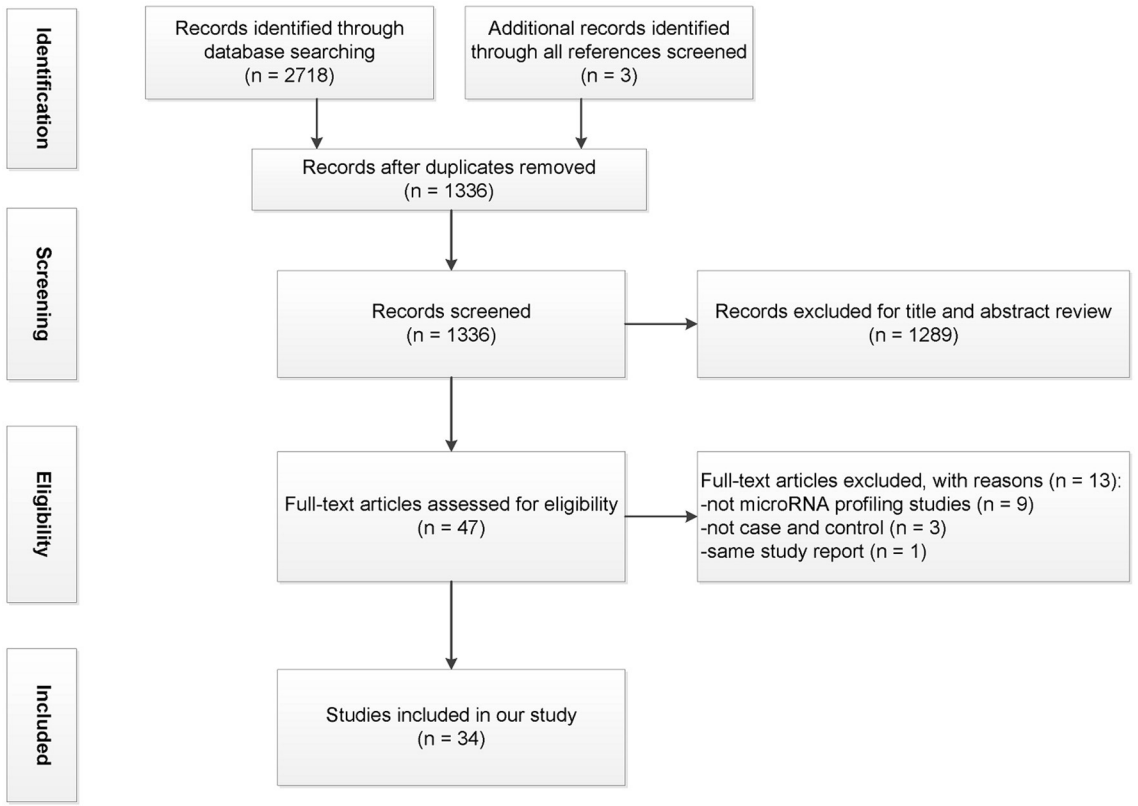

FIGURE 1 | Flowchart illustrating the process of study selection.

peripheral blood mononuclear cells were classified as "blood or immune cell" tissue. Studies using saliva were classified as "salivary gland." Terms with p-value <0.01, a minimum count of 3, and enrichment factor of >1.5 were considered as significant (using hypergeometric test and Benjamini–Hochberg p-value correction).

Explore KEGG Pathways That Are Commonly Targeted by Multiple miRNAs

Moreover, to investigate KEGG pathways that are simultaneously controlled by multiple ASD-miRNAs, dysregulated ASD-miRNAs in the brain, or consistently upregulated and downregulated ASD-miRNAs in blood or saliva, were inputted into the online software DIANA-miRPath v3.0 (51), respectively. The KEGG analysis was used, and the human species was selected. In the optional gene filter menu, genes that are expressed in the brain, salivary glands, blood, and immune cells (data available from v20.1.proteinatlas.org, http://www.proteinatlas.org) (50) were uploaded respectively. The interactions dataset selected for all microRNAs was TarBase v7.0, based on previous positive and negative experiments. In the advanced statistics options, the Fisher's Exact Test (Hypergeometric Distribution), Benjamini–Hochberg's False Discovery Rate (FDR) correction, and more conservative statistics (DAVID's EASE score) with a p-value threshold of 0.05 were selected. After inputting miRNAs into the software, "Pathways union" was selected to identify pathways containing more than one associated miRNA, which can give meta-analysis statistics for the assessment of combined miRNA action.

Quality Assessment of Selected Studies

We assessed the methodological quality of the included studies using the Newcastle-Ottawa Scale (NOS) for case-control studies (52). The scale includes three categories: selection, comparability, and exposure. A higher total quality score indicates better study quality, and the highest quality score is 10 (**Supplementary Material 6**).

RESULTS

Selection and Characteristics of Included Independent Studies

Using Web of Science, PubMed, Embase, Scopus, and OVID databases, a total of 2,718 studies were identified; another three articles were added from the review citation. Among them, 1,385 were duplicates; a total of 1,336 articles were included in the title and abstract screening after which 48 articles remained. After evaluating the full text, 14 studies were excluded. **Figure 1** displays the PRISMA flowchart for the study selection process.

Details of the characteristics of the selected studies are shown in **Supplementary Material 6**. The year of publication was from 2008 to 2020. In total, this review pooled results from over 1,000 subjects with ASD and almost 1,000 controls. Five studies included only male participants (10, 18, 29, 35, 41). One study included only female participants (53). In all, there are more males than females with ASD (773:192). Participants' ages ranged from 2 to 81 years. In all 34 included studies, the participants had ASD diagnoses. The Autism Diagnostic Interview-Revised (ADI-R) was the most used diagnostic instrument among the studies, followed by the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5), Autism Diagnostic Interview-Revised (ADOS), Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision (DSM-IV-TR), and Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV). Six studies did not provide the ASD diagnostic criteria but did report that the participants had ASD, including autistic disorder, Asperger's disorder, and pervasive developmental disorder–not otherwise specified (PDD-NOS) (10, 14, 18, 33, 35, 53); thus, they are included in this study. Ten of these studies involved brain tissue (10–18, 53), 15 involved peripheral blood (19–33), four employed lymphoblastoid cell lines (34–37), three examined saliva (38–40), 1 examined reprogrammed induced pluripotent stem cell-derived neurons (41), and 1 examined olfactory mucosal stem cells and skin fibroblasts (42). Variation in these studies is due to differences in the patients investigated, the tissue examined, microRNA profiling and analysis methods, the number of samples, and the statistical analysis approach used.

Differentially Expressed miRNAs

In the 34 selected miRNA expression profiling studies, 285 differentially expressed mature miRNAs were reported that compared over 1,000 subjects with ASD and almost 1,000 controls (**Supplementary Material 6**). Of the 68 differentially expressed miRNAs identified in at least two studies (ASD-miRNAs), 29 miRNAs had a consistent direction, 15 upregulated and 14 downregulated (**Table 1**), and 39 inconsistently dysregulated (**Table 2**). Among them, miR-23a-3p, miR-106b-5p, miR-146a-5p, miR-7-5p, miR-27a-3p, miR-451a, miR-181b-5p, and miR-486-3p are the most frequently reported (**Tables 1**, **2**). Certain microRNAs changed in different ways depending on gender; for example, miR-148b-3p was shown to be downregulated solely in male ASD participants (**Tables 1**, **2**; **Supplementary Material 6**).

Subgroup analysis of tissues showed that in brain samples, six miRNAs (miR-146a-5p, miR-155-5p, miR-1277-3p, miR-21-3p, miR-106b-5p, and miR-451a) were consistently upregulated, 1 miRNA (miR-4742-3p) was consistently downregulated, and seven miRNAs were inconsistently dysregulated (**Supplementary Material 6**). In blood and immune cell samples, four miRNAs (miR-146a-5p, miR-23b-3p, miR-483-5p, and miR-23a-3p) were consistently upregulated, seven miRNAs (miR-15a-5p, miR-193a-5p, miR-20a-5p, miR-574-3p, miR-92a-3p, miR-3135a, and miR-103a-3p) were consistently downregulated, and 19 miRNAs were inconsistently dysregulated (**Supplementary Material 6**). In saliva samples, one miRNA (miR-7-5p) was consistently upregulated, two miRNAs (miR-23a-3p and miR-32-5p) were consistently downregulated, and two miRNAs were inconsistently dysregulated (**Supplementary Material 6**).

Validated Gene Targets of Each Dysregulated microRNAs Overlap With ASD Risk Genes

Compared to experimentally validated microRNA-targets based on miRTarBase, each ASD-miRNA targets many genes. miR-92a-3p, miR-15b-5p, miR-93-5p, and miR-155-5p are among microRNAs that have the greatest number of validated ASD risk gene targets (**Table 1**, **Supplementary Materials 7**–**9**). The most frequently targeted ASD candidate genes were *TNRC6B*, *PTEN*, *AGO1*, *AGO2*, *SKI*, and *SMAD4* (**Supplementary Materials 7**–**9**).

Subgroup Analysis of Tissues and ASD-miRNAs Associated KEGG and Reactome Pathways

Using the Metascape, a diverse range of KEGG and Reactome pathways were significantly enriched for validated gene targets of each dysregulated microRNAs (**Supplementary Materials 7**–**9**). Furthermore, using the software DIANA-mirPath v.3, bioinformatics analysis revealed that among the 7, 11, and 3 consistently dysregulated ASD-miRNAs in the brain, blood, and saliva respectively, there were various commonly targeted pathways (**Figures 2A–C**). Enriched KEGG pathways were most significantly associated with cancer, metabolism (notably steroid biosynthesis, fatty acid metabolism, fatty acid biosynthesis, lysine degradation, biotin metabolism), cell cycle, cell signaling (especially Hippo, FoxO, TGF (transforming growth factor)-beta, p53, thyroid hormone, and estrogen signaling pathway), adherens junction, extracellular matrix–receptor interaction, prion diseases, etc., (**Figures 2A–C**).

DISCUSSION

In this report, we systematically reviewed the ASD-related miRNAs from 34 independent profiling studies. Up until now, more than 285 mature microRNAs have been identified (**Supplementary Material 6**), of which 68 had altered levels in at least two independent studies comparing autistic persons with controls (**Tables 1**, **2**). The most frequently altered miRNAs in all studies include miR-23a-3p, miR-106b-5p, miR-146a-5p, miR-7-5p, and miR-27a-3p. MiRNAs that were dysregulated in at least two studies were validated to target a number of ASD risk genes. Among them, *TNRC6B*, *PTEN*, *AGO1*, *AGO2*, *SKI*, and *SMAD4* were the most frequent (**Supplementary Materials 7**–**9**), and miR-92a-3p has the most target ASD risk genes (**Table 1**, **Supplementary Material 8**). Bioinformatic analysis showed that validated targets of each microRNA were enriched in various KEGG and Reactome pathways. Moreover, tissue subgroup enrichment analysis by using mirPath v.3 showed that various commonly targeted pathways were identified for the 7, 11, and 3 consistently dysregulated ASD-miRNAs

TABLE 1 | Consistently dysregulated miRNAs reported in at least two expression profiling studies.

miRNA	Chromosome location	Studies/References	No. of ASD samples (male: female)	Male: female ratio of ASD samples	No. of autism risk genes targeted by the miRNA[a]
Upregulated					
miR-146a-5p	5	(11, 17, 34, 42, 54)	N/A	N/A	24
miR-155-5p	21	(11, 13, 18)	63:12	5–6: 1	77
miR-106a-5p	X	(10, 22)	27:2	13–14: 1	49
miR-10a-5p	17	(13, 36)	58:17	3–4: 1	43
miR-335-3p	7	(13, 38)	64:15	4–5: 1	35
miR-142-3p	17	(11, 28)	34:8	4–5: 1	34
miR-23b-3p	9	(34, 35)	14:3	4–5: 1	28
miR-107	10	(13, 35)	56:10	5–6: 1	27
miR-223-3p	X	(13, 37)	45:10	4–5: 1	11
miR-146b-5p	10	(10, 34)	16:3	5–6:1	13
miR-483-5p	11	(26, 32)	105:20	5–6:1	8
miR-21-3p	17	(11, 13)	55:12	4–5:1	6
miR-191-5p	3	(35, 38)	30:5	6:1	5
miR-1277-3p	X	(13, 54)	45:11	4–5:1	1
miR-494	14	(11, 26)	27:5	5–6:1	N/A
Downregulated					
miR-15a-5p	13	(10, 26, 28)	54:9	6:1	73
let-7a-5p	9, 11, 22	(11, 26, 39)	188:31	6–7:1	48
miR-193a-5p	17	(29, 32, 39)	256:43	5–6:1	3
miR-92a-3p	X, 13	(26, 34)	20:6	3–4:1	118
miR-15b-5p	3	(10, 26)	30:3	10:1	91
miR-20a-5p	13	(26, 32)	105:20	5–6:1	71
miR-148b-3p	12	(10, 35)	24:0	24:0	45
miR-211-5p	15	(11, 35)	21:2	10–11:1	44
miR-4742-3p	1	(12, 15)	10:10	1:1	11
miR-148a-5p	7	(32, 39)	249:43	5–6:1	10
miR-151a-3p	8	(19, 39)	209:33	6–7:1	6
miR-3135a	3	(23, 28)	48:12	4:1	5
miR-574-3p	4	(26, 32)	105:20	5–6:1	3
miR-3687	21, 22	(13, 28)	69:16	4–5:1	3

[a] *Based on validated target genes in miRTarbase, N/A: not available.*

in brain, blood, and saliva, respectively. Our study clarified miRNAs involved in ASD and explored their target genes and significantly associated pathways, which are the focus of our discussion next.

miR-23a-3p and miR-27a-3p belong to the same cluster and participate in several ASD-related pathways, such as lysine degradation and fatty acid metabolism (**Supplementary Materials 7–9**; **Figures 2A,B**). Our results showed that miR-23a-3p targets many ASD risk genes, including *PTEN*, *TSC1*, and *KDM3B* (**Supplementary Material 7**). miR-23a plays a crucial role in neurogenesis (9). For example, miR-23a-3p controls oligodendrocyte differentiation and myelin formation by targeting *PTEN* and manipulating PTEN/PI3K/Akt/mTOR pathway (55). It also directly targets *TSC1*, which is crucial in the mTOR signaling pathway associated with synaptic protein defects in autistic persons (54). miR-27a-3p targets many ASD risk genes, including *IGF1*, *KMT2A*, *KMT2C*, *KMT5B*, and *IL6* (**Supplementary Material 8**). For example, miR-27a-3p targets insulin-like growth factor-1 (*IGF-1*), which binds to the IGF-1 receptor and activates PI3K and MAPK pathways and various downstream signaling pathways, including mTOR, p53, and FoxO signaling pathway. Insulin-like growth factor-1 plays an important role in brain development (56); it has shown a potential therapeutic role for ASD *in vitro* (57) and in individuals with ASD (58) and Rett syndrome (59). *KMT2A*, *KMT2C*, *KMT5B*, and *NSD1* all participate in post-translational chromatin modification such as histone lysine methylation and are thus linked to transcriptional activation or repression (60). These genes are also involved in lysine degradation and play an important role in both normal human development and developmental disorders, including ASD (61). A recent study showed that miR-27a-3p has protective effects on the blood–brain barrier, brain injury, and hippocampal neuron injury (62). Therefore, its dysregulation may result in neurologic dysfunction. miR-23a-3p and miR-27a-3p are dysregulated not only in brain samples (10) but also in the

TABLE 2 | Inconsistently dysregulated miRNAs reported in at least two expression profiling studies.

miRNA	Genome location[a]	Studies/References		N ASD (male: female) Up	Male: female ratio of N ASD Up	N ASD (male: female) Down	Male: female ratio of N ASD Down
		Up	Down				
miR-23a-3p	19	(13, 34, 35, 54)	(10, 38, 40)	59:14	4–5:1	57:19	3:1
miR-106b-5p	7	(10, 19, 28, 35, 54)	(35)	96:14	6–7:1	11:0	11:0
miR-7-5p	9, 15, 19	(11, 38, 40)	(10, 32)	54:21	2–3:1	101:17	5–6:1
miR-181b-5p	1, 9	(33, 36)	(19, 33)	39:18	2–3:1	74:18	4–5:1
miR-27a-3p	19	(19)	(10, 29, 38)	48:7	6–7:1	39:5	7–8:1
miR-486-3p	8	(21, 36)	(24, 28)	31:9	3–4:1	48:12	4:1
miR-451a	17	(11, 16, 35)	(26)	29:3	9–10:1	17:3	5–6:1
miR-93-5p	7	(35)	(10, 32)	11:0	11:0	101:17	5–6:1
miR-19b-3p	13, X	(11, 19)	(26)	58:9	6–7:1	17:3	5–6:1
miR-195-5p	17	(19, 35)	(26)	59:7	8–9:1	17:3	5–6:1
miR-21-5p	17	(11)	(10, 54)	10:2	5:1	13:1	13:1
miR-103a-3p	5, 20	(54)	(26, 32)	0:1	0:1	105:20	5–6:1
miR-32-5p	9	(37)	(38, 40)	N/A	N/A	44:19	2–3:1
miR-145-5p	5	(29)	(28, 32)	7:0	7:0	112:23	4–5:1
miR-619-5p	12	(28, 54)	(13)	24:7	3–4:1	45:10	4–5:1
miR-132-3p	17	(34)	(10, 35)	3:3	1–1:1	24:0	24:0
miR-140-3p	16	(20, 38)	(40)	41:13	3–4:1	25:14	1–2:1
miR-199a-5p	1, 19	(29, 36)	(28)	20:7	2–3:1	24:6	4:1
miR-328-3p	16	(30)	(23, 28)	18:12	1–2:1	48:12	4:1
miR-484	16	(13)	(10)	45:10	4–5:1	13:0	13:0
miR-193b-3p	16	(10)	(28)	13:0	13:0	24:6	4:1
miR-320a	8	(10)	(19)	13:0	13:0	48:7	6–7:1
miR-186-5p	1	(35)	(37)	11:0	11:0	N/A	N/A
miR-424-5p	X	(13)	(28)	45:10	4–5:1	24:6	4:1
miR-221-3p	X	(13)	(42)	45:10	4–5:1	N/A	N/A
miR-144-3p	17	(11)	(30)	10:2	5:1	18:12	1–2:1
miR-940	16	(13)	(26)	45:10	4–5:1	17:3	5–6:1
miR-223-5p	X	(37)	(32)	N/A	N/A	88:17	5–6:1
miR-423-5p	17	(32)	(37)	88:17	5–6:1	N/A	N/A
miR-34c-5p	11	(29)	(37)	7:0	7:0	N/A	N/A
miR-363-3p	X	(13)	(34)	45:10	4–5:1	3:3	1:1
miR-663a	20	(34)	(19)	3:3	1:1	48:7	6–7:1
miR-338-5p	17	(11)	(15)	10:2	5:1	5:5	1:1
miR-199b-5p	9	(36)	(35)	13:7	1–2:1	11:0	11:0
miR-379-5p	14	(11)	(32)	10:2	5:1	88:17	5–6:1
miR-204-3p	9	(54)	(13)	0:1	0:1	45:10	4–5:1
miR-628-5p	15	(38)	(40)	19:5	3–4:1	25:14	1–2:1
miR-127-3p	14	(38)	(54)	19:5	3–4:1	0:1	0:1
miR-874-3p	5	(13)	(30)	45:10	4–5:1	18:12	1–2:1

[a]*Chromosome location, N/A: not available.*

saliva of autistic persons (38). Thus, they may be potential biomarkers of ASD.

hsa-miR-106b-5p and hsa-miR-93-5p belong to the microRNA-25-93-106b cluster; they are vital for "neural stem/progenitor cell proliferation and neuronal differentiation" and are involved in various psychiatric or neurologic disorders (63, 64). They both target many ASD risk genes including *MECP2*, *PTEN*, and *SMAD4*, and they are involved in various pathways, including adherens junction, circadian clock, long-term depression, mTOR, and estrogen signaling pathway (**Supplementary Materials 7**, **8**). Methyl CpG binding protein 2 (*MECP2*) is abundantly expressed in neurons and is essential for neuronal function and development (65). *MECP2* under-expression and overexpression have been associated with Rett syndrome (3, 66) and *MECP2* duplication syndrome, respectively, and many neurologic disorders including ASD

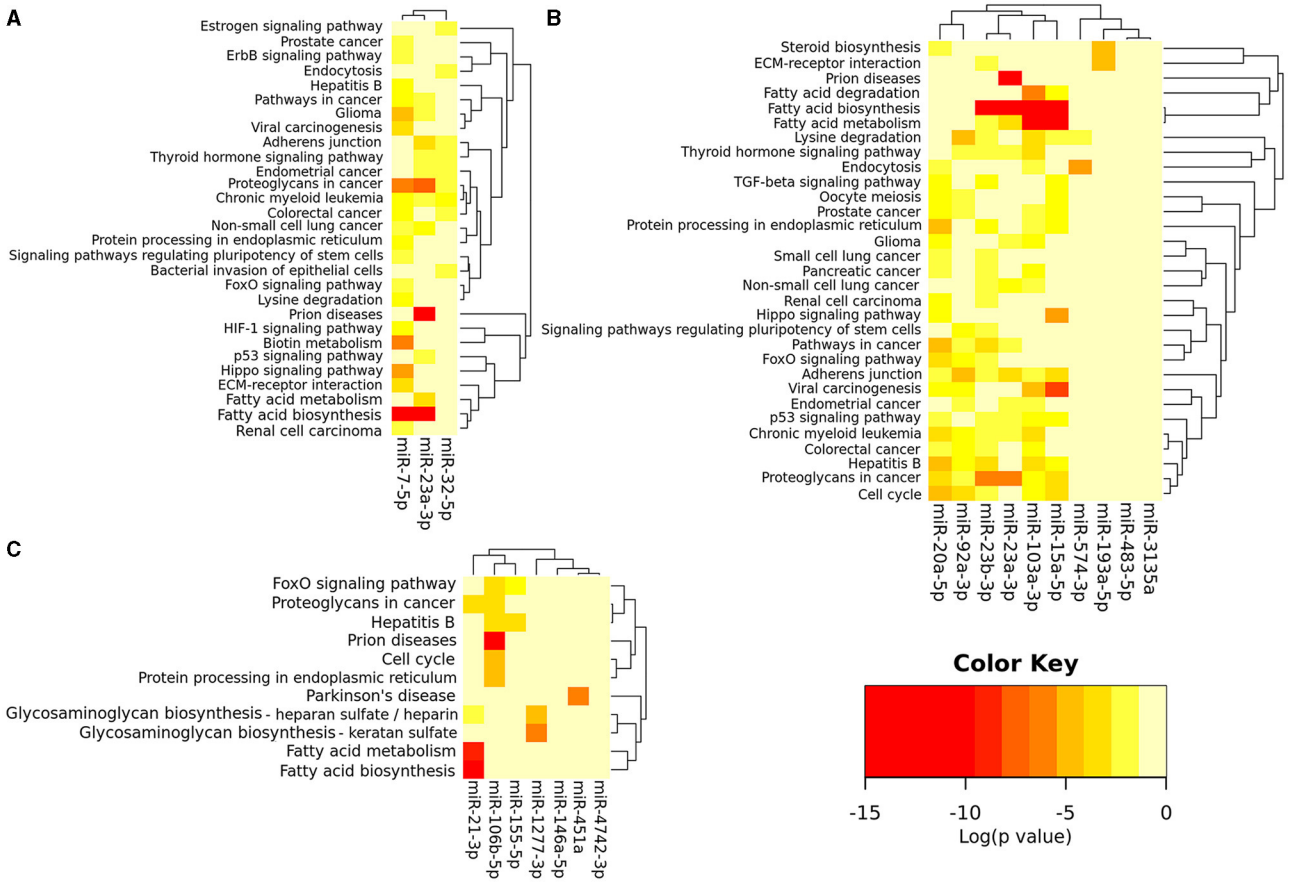

FIGURE 2 | miRNAs vs. pathways heatmap (Clustering based on significance levels) of the consistently dysregulated ASD-miRNAs in blood and immune cells **(A)**, saliva **(B)**, and brain tissue **(C)**. The heatmap was created from the DIANA mirPath v 3.0. Darker shades represent more significant. Dendrograms depicts hierarchical clustering results for miRNAs and pathways, respectively. The miRNA axis represents the clustered miRNAs with similar pathway targeting patterns. A similar clustering is shown on the pathway axis. Interactions dataset: TarBase v7.0; Analysis: pathways union; *p*-value threshold: 0.05; FDR Correction, Conservative Statistics.

(3, 67). Besides, many cases of Rett syndrome and *MECP2* duplication syndrome fit the diagnostic criteria for ASD (3, 68). As a multifunctional protein, MECP2 affects various metabolites (including glutamate and catecholamine catabolites), genes (including *BDNF*), and pathways (including AKT/mTOR and neurotrophin signaling pathway). Therefore, more research is needed to investigate the target genes and pathways of hsa-miR-106b-5p and hsa-miR-93-5p to better understand neurologic disorders including ASD and Rett syndrome.

Notably, miR-146a-5p (11, 17) and miR-155-5p (11, 13, 18) were both consistently upregulated in brain tissues of ASD persons **(Supplementary Material 6)**, and they play a critical role in regulating immune response (69) and neuroinflammation (70). In line with this, our results showed that they target immune/inflammation-associated genes (including *IL6* and *SMAD4*) and pathways (such as NF-kappa B, TGF-beta, and Toll-like receptor signaling pathway) (**Figure 2C**; **Supplementary Material 7**). miR-146a-5p has also been consistently upregulated in lymphoblastoid cell line (34), skin fibroblasts (42), and olfactory mucosal stem cell (42) samples from people diagnosed with ASD. This may be an anti-inflammatory compensatory reaction since miR-146a-5p primarily acts as a negative inflammation regulator (70). However, evidence showed that miR-146a-5p dysregulation can affect neuronal development and differentiation and may thus lead to ASD (17, 42, 71).

miR-7-5p is abundant in neurons and neuroendocrine organs and is a prototypical neuroendocrine miRNA. It regulates many genes in the brain (**Supplementary Material 7**) (72, 73). For example, miR-7-5p inhibits the expression of ASD risk gene *PAX6* (74), a crucial transcription factor for neural tissue development, and regulates dopaminergic neuron differentiation (75). miR-7-5p also regulated the expression of ASD risk gene *SHANK3* and affected the dendritic spines in hippocampal neurons (76). Moreover, miR-7 could regulate cerebral cortex development through the p53 pathway (77). Our bioinformatic analysis shows that its related pathways include fatty acid biosynthesis, biotin metabolism, lysine degradation, and the Hippo signaling pathway (**Supplementary Material 7**, **Figure 2B**). Therefore, it is reasonable to speculate that

miR-7 dysregulation may lead to neurologic disorders including ASD.

Each ASD-miRNA was validated to target several ASD risk genes and involves many signaling pathways (**Supplementary Materials 7–9**). *TNRC6B*, *PTEN*, *AGO1*, *AGO2*, *SKI*, and *SMAD4* are among the most commonly targeted ASD genes (**Supplementary Materials 7–9**). *TNRC6B*, *AGO1*, and *AGO2* participate in miRNA-mediated translational inhibition (78), which can affect a variety of cellular functions, given the various targets in this pathway. *PTEN* gene has been identified as a strong candidate gene for ASD (79). Defective *PTEN* protein interacts with tumor protein p53 to suppress energy production in neurons, causing detrimental changes in mitochondrial DNA and abnormal levels of energy production in the cerebellum and hippocampus, regions of the brain vital to social function and cognition, and Pten haploinsufficient mice exhibit autistic-like behavior with brain mitochondrial dysfunction and accumulation of mitochondrial DNA loss (80). Moreover, PTEN antagonizes PI3K-AKT signaling, which is essential for axon guidance and dendritic outgrowth (81). So *PTEN*-deficiency may affect neuronal connectivity, synaptic plasticity, and the development of autistic behavior (81). The SKI protein regulates the TGF-beta pathway by interacting with SMAD proteins, such as SMAD4, and plays a key role in tissue development (including the brain) during embryogenesis (82).

Tissue subgroup analysis showed that ASD-miRNAs in the brain, blood, and saliva participate in the FoxO signaling pathway (**Figures 2A–C**; **Supplementary Materials 7–9**), and several ASD risk genes are present in this pathway (**Supplementary Materials 7–9**). Recently, FoxO transcription factors have emerged as important regulators of cell development and function in the nervous system (83) and are implicated in neurological diseases such as Parkinson's disease and Huntington's disease (83, 84). A growing body of evidence demonstrates that miRNAs can directly regulate FoxO transcripts in various physiological and pathological conditions (85). However, there is still a lack of literature on the FoxO signaling pathway in ASD.

Notably, ASD-miRNAs were implicated in many cancer pathways (**Supplementary Materials 7–9**; **Figures 2A–C**). This supports evidence that ASD shares overlapping genes and pathways with cancers (86). For example, several ASD-miRNAs (miR-21-3p, miR-106a-5p, miR-155-5p, miR-92a-3p, miR-23a-3p, miR-106b-5p, and miR-19b-3p) have been shown to target *PTEN* (**Supplementary Materials 7–9**), a ubiquitously expressed tumor suppressor that is commonly inactivated in human cancers (87). Additionally, several ASD risk genes, including *PTEN*, are regulators of p53, which act as a tumor suppressor. Besides, the p53 tumor suppressor network cross-talks with the miRNA regulation system (88). For example, dysregulated microRNAs in ASD (miR-15a-5p, miR-19b-3p, and miR-92a-3p) target the p53 pathway (**Supplementary Material 8**; **Figure 2A**). In contrast, the p53 pathway could downregulate microRNAs such as let-7a and miR-17/92 cluster (miR-19b-1, miR-20a, and miR-92a) and upregulates microRNAs such as miR-107, miR-34c, miR-145, miR-15a, miR-23b, and miR-486 (89, 90). These miRNAs mediating p53 function in tumor suppression are also dysregulated in ASD. Evidence showed that the *p53* gene is more deficit in ASD children and their fathers (91). So dysregulated miRNAs and the p53 signaling pathway may impact each other mutually and contribute to ASD. Since ASD and cancer have mechanistic similarities, these have clinical importance, such as repurposing feasible cancer drugs for ASD-targeted therapies (86).

It should be highlighted that ASD-miRNAs are implicated in various metabolic pathways (**Supplementary Materials 7–9**; **Figures 2A–C**). This is consistent with the clinical finding that ASD persons have various metabolic problems (92–94). One explanation is mitochondria dysfunction since mitochondria are vital for bioenergetic and biosynthetic processes, including lipids, amino acids, and nucleotide metabolism (95). Many microRNAs including miR-23a-3p, miR-23b-3p, miR-107, and miR-103 are found in mitochondria (96); they may regulate various biological pathways, such as p53 signaling, neurotrophin signaling, TGF cycle, cell cycle, and ubiquitin-mediated proteolysis (97). For example, miR-23a-3p and miR-23b-3p bind to (glutaminase) GLS-mRNA and suppress its translation; thus, they are involved in the mitochondrial amino acid metabolism (96, 98). miR-107 and miR-103 regulate insulin signaling and glucose homeostasis (99). Cellular nutrient and energy-sensing by mTOR signaling regulates almost all aspects of metabolism and mitochondrial biogenesis and plays an important role in glucose homeostasis, lipid homeostasis, immune function, brain function, cancer, etc. (100, 101). Notably, several ASD risk genes are present in mTOR signaling, including *IGF1*, *MTOR*, *PIK3R2*, *PTEN*, *RHEB*, *TSC1*, and *TSC2*. Some of them (*PTEN* and *TSC2*) are direct target genes of p53, which regulates mTOR signaling and many metabolic activities (102). Taken together, microRNA, metabolism, and mitochondria are closely interconnected and associated with other cellular pathways, which may partially explain the etiology of mitochondrial dysfunction and diverse clinical manifestations of ASD.

It is well-known that immune dysregulation/inflammation participates in the pathogenesis of ASD. Reinforcing this perspective, our pathway enrichment analysis shows that ASD-miRNAs are involved in immune/inflammation-associated pathways, such as the NF-κB, Hippo, TGF-beta, and mTOR signaling pathways (**Figures 2A–C**; **Supplementary Materials 7–9**). The TGF-beta signaling pathway is a crucial regulator of the immune system and plays a critical role in the regulation of inflammation and embryo development (103). Evidence shows that TGF-beta1 regulates the PI3K/Akt/Wnt/beta-catenin signaling pathway and restores hippocampal synaptic plasticity and memory (104). TGF-beta1 also activates the MAPK signaling pathway and modulates neurogenesis (105). TGF-beta can also activate other pathways including mTOR signaling pathways and the NF-κB pathway (106). Moreover, TGF-beta signaling cross-talks with miRNAs and upregulates miRNAs such as the miR-181 family, the miR-17/92 cluster, miR-155, and the miR-23/24/27 cluster; downregulates the miR-200 family, miR-203, let-7, miR-34a, and miR-584 (107). On the other hand, miRNAs including the miR-106b/205 cluster (including miR-106b-5p and miR-93-5p) regulate TGF-beta signaling by convergently suppressing a range

of TGF-beta signaling components, such as *SMAD4* (107). This may partly explain the reason why microRNAs are dysregulated in ASD. However, the functions of TGF-beta in ASD are little understood (108). Further research is needed to investigate the role of microRNAs and TGF-beta signaling in immune dysregulation in ASD.

The cadherin genes (including *PCDH10*, *CDH5*, *CDH8*, *CDH9*, and *CDH15*) are ASD risk genes and function as essential cell adhesion molecules. Some of them may interact with beta-catenin, which is encoded by the *CTNNB1* gene (a validated target of miR-155-5p, miR-27a-3p, and miR-106a-5p, etc.). Cadherins act as an intracellular signal transducer in various signaling pathways including the adherens junctions and the Hippo pathway and play a critical role in the development and cellular function (109).

Adherens junctions are cadherin-based protein complexes, which exist at intercellular adhesions of endothelial and epithelial tissues and play a critical role in embryogenesis and cortical development (110). Evidence confirmed the links between adherens junction and ASD (111). Interestingly, a functional local RNA interference system has been recently found in the epithelial adhesion junctions (112), which affects miRNAs and mRNAs, indicating that miRNAs play an important role in regulating epithelial and endothelial cell functions in development and disease (113). For example, miR-155 adversely affected brain–blood-barrier function during neuroinflammation by targeting cell–cell complex molecules, such as AA2, claudin-1, and molecules that are critical in cell-to-extracellular matrix (ECM) interactions including dedicator of cytokinesis 1 and syntenin-1 (114). Thus, miRNAs may contribute to adherens junctions dysfunction and brain–blood-barrier and intestinal epithelial barriers impairment in ASD (115, 116).

The extracellular matrix and its receptors are essential for neuronal migration during brain development and are involved in the maintenance of stable neuronal connections and regulation of synaptic plasticity (117). ASD risk genes including *RELN*, *LAMB1*, and *THBS1* are involved in ECM-receptor interaction. However, there is still a lack of literature on ECM-receptor interaction in ASD.

Adherens junctions, extracellular matrix, or cadherins could also regulate the Hippo pathway activity (118, 119), which plays a critical role in regulating development and organ size (120). Hippo signaling also cross-talks with innate immunity and regulates inflammation (121). Several ASD risk genes are implicated in Hippo signaling, including *CTNNB1*, *SMAD4*, and *PPP2R1B*. However, there is still little research in the literature regarding the Hippo signaling pathway in ASD.

Thyroid hormones are important regulators of development, growth, and metabolism and crucial for normal brain development. Thyroid hormones regulate AMPK activity and fatty acid metabolism in the central nervous system (122). Maternal thyroid disorders during pregnancy were found to be associated with an increased risk of ASD in offspring (123), and brain genes were altered (124). However, few studies have examined whether postnatal thyroid hormone levels influence the risk of ASD and the underlying mechanisms remain unclear (125). The thyroid hormone and its receptor-related genes (including *THRA*, *TPO*, *TRIP12*, and *NR4A2*) have been linked with ASD, and miRNAs are necessary for thyroid hormone production (126). Further studies are needed to investigate the microRNA regulation of thyroid hormones in ASD during pregnancy and postnatal.

Estrogen and estrogen receptors play critical roles in brain development and functions, including synaptogenesis, corticogenesis, cognition, and learning (127). Evidence showed that prenatal and postnatal estrogen signaling impairment may contribute to ASD (128, 129). Consistent with this, several ASD-miRNAs (including miR-142-3p, miR-151a-3p, miR-19b-3p, miR-32-5p, miR-221-3p, miR-320a, miR-338-5p, and miR-423-5p) target ASD risk genes including *PIK3R2*, *ADCY3*, *ITPR1*, and *GNAI1* (**Supplementary Materials 7–9**), which are also implicated in estrogen signaling.

Interestingly, the ASD-miRNAs target circadian rhythm genes including *RORA, RORB, PER1, PER2, BTRC, NPAS2, NR1D1,* and *CSNK1E*, which are also ASD risk genes (**Supplementary Materials 7–9**). Circadian rhythm disorders are associated with neurological (130) and other functional impairments in ASD (131). Evidence suggests that circadian rhythm genes may be correlated with ASD (132) and cause sleep disorders that are common in ASD (133). Moreover, salivary microRNAs (miR-24-3p, miR-200b-3p, miR-203a-3p, and miR-26a-5p) are associated with sleep disorders in children with ASD, so microRNAs in biofluids such as saliva could have diagnostic and therapeutic values in circadian rhythm disorder and ASD (134).

Collectively, this study clarified overlapping altered microRNAs in ASD and explored their target genes and associated pathways. We also highlight pathways that are significantly enriched while being less studied in ASD, such as adherens junctions, ECM receptor interaction, FoxO, Hippo, and TGF-beta signaling pathway.

Some limitations of this study exist. First, as microRNA profiling and analysis methods are heterogeneous among studies and much raw data are not available, it is difficult to perform a quantitative meta-analysis. Second, various diagnostic approaches were employed, and six studies did not report diagnostic tools (10, 14, 18, 33, 35, 53). The change of diagnostic criteria, for example, from DSM-IV to DSM-V, may lead to the inconsistency of research participants. The heterogeneity of diagnostic criteria and participants made comparisons between studies challenging. Subgroup analysis stratifying by homogeneous characteristics dimensionally or categorically within ASD would be helpful. However, this was not practicable as the included studies did not give sufficient phenotype/behavioral information on ASD individuals. It should also be noticed that some dysregulated microRNAs are located on the X chromosome and miR-29c-3p (27), miR-4732-5p, and miR-423-3p show gender differences (32). However, this link has not been identified in other studies (19, 22, 33, 38, 39). In addition,

some research only included male or female ASD participants. Thus, it is unclear if particular miRNA dysregulation in ASD is gender-specific. Given the heterogeneity and gender differences in ASD, future microRNA expression research with more female participants and stratification by ASD subtype or gender will be valuable.

CONCLUSION

In conclusion, our systematic review on miRNA expression profiling studies identified a number of altered microRNAs in ASD, especially miR-23a-3p, miR-27a-3p, miR-106b-5p, miR-93-5p, miR-7-5p, miR-146a-5p, and miR-155-5p. Some of these microRNAs have the potential to serve as biomarkers for ASD. Each of these altered microRNAs was validated to target many ASD risk genes. The target genes of these microRNAs are implicated in various pathways associated with ASD and form very rich networks, which further highlights the importance of these microRNAs in ASD etiology. However, few studies have reported the implication of microRNAs and some associated pathways in ASD pathogenesis, such as adherens junctions, ECM receptor interaction, FoxO, Hippo, TGF-beta signaling pathway, etc. More research is needed to examine microRNA expression in ASD and their associated target genes and pathways to better understand ASD pathogenesis. This may contribute to the use of microRNAs as potential personalized biomarkers and therapeutic targets.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

Z-XH: conceptualization, methodology, software, formal analysis, bioinformatic analysis, investigation, resources, data extraction, data curation, writing - original draft, writing - review and editing, visualization, and funding acquisition (Qihang Funds of Fujian Medical University). YC: conceptualization, methodology, writing - review and editing, supervision, project administration, and funding acquisition. H-RG: data extraction and data curation. G-FC: data extraction. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.630876/full#supplementary-material

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