GENE AND ENVIRONMENT INTERACTIONS IN NEURODEVELOPMENTAL DISORDERS

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GENE AND ENVIRONMENT INTERACTIONS IN NEURODEVELOPMENTAL DISORDERS

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EDITORIAL
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Editorial: Gene and Environment Interactions in Neurodevelopmental Disorders

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Keywords: gene environment interaction, development, early adversity, prenatal stress, genetics

Editorial on the Research Topic

Gene and Environment Interactions in Neurodevelopmental Disorders

The knowledge that both the genetic patrimony and lifetime environmental exposures define disease risk is well-accepted. However, the so-called Gene-Environment effects—when the consequences of an environmental exposure vary according to the genetic background—are less understood (Kloke et al., 2013). This is especially true in early life when individuals undergo a series of dynamic and tightly linked developmental processes (Miguel et al., 2019a). The brain and the periphery reorganize during specific developmental time periods to adapt to changes in the environment the subjects are being raised. These are known as "critical periods" (Hensch, 2004).

There has been an increased interest in unraveling how certain types of exposure occurring during these critical periods affect developmental trajectories. Characterizing relevant genes and proteins involved and their precise timing of action in relationship with the type and severity of the environmental exposure is critically needed and was the main aim of this Research Topic. The articles enclosed in this Research Topic represent important advances in the understanding of these relationships.

Focusing on main genetic effects, Zhongling et al. reported a case of Joubert syndrome associated with a new mutation in IFT74, a gene responsible for regulating cilia composition. Wang Y. et al. identified increased allele frequencies of single nucleotide polymorphisms (SNPs) from the Interleukin-23 (IL-23) gene in children with cerebral palsy compared to healthy controls. The perspective from Malatesta et al. focuses on the environmental stimulus, proposing the existence of a critical period during which caregiver's postural and motor lateral biases influence offspring hemispheric lateralization.

During gestation, fetal environment is defined by the maternal metabolic milieu, which influences fetal development. Wang H. et al. investigated the effects of high maternal estradiol on proliferation and differentiation of fetal hypothalamic neural stem/progenitor cells (NSC/NPCs) in mice and identified critical effects on neurogenesis related genes. Sandoval et al. explored another maternal internal state during pregnancy: immune activation, which usually results from infections. Though maternal immune activation induced behavioral alterations compatible with autism spectrum disorders and schizophrenia in the offspring, this was not exacerbated by the loss of vesicular zinc, another known risk factor for neurodevelopmental disorders.

Szekely et al. compiled five candidate polymorphisms (one per gene: DAT1, DRD4, DRD2, COMT, BDNF) in a multilocus score, to explore their interaction with prenatal adversity and postnatal parenting behavior on the development of attentional competence skills in 18- and

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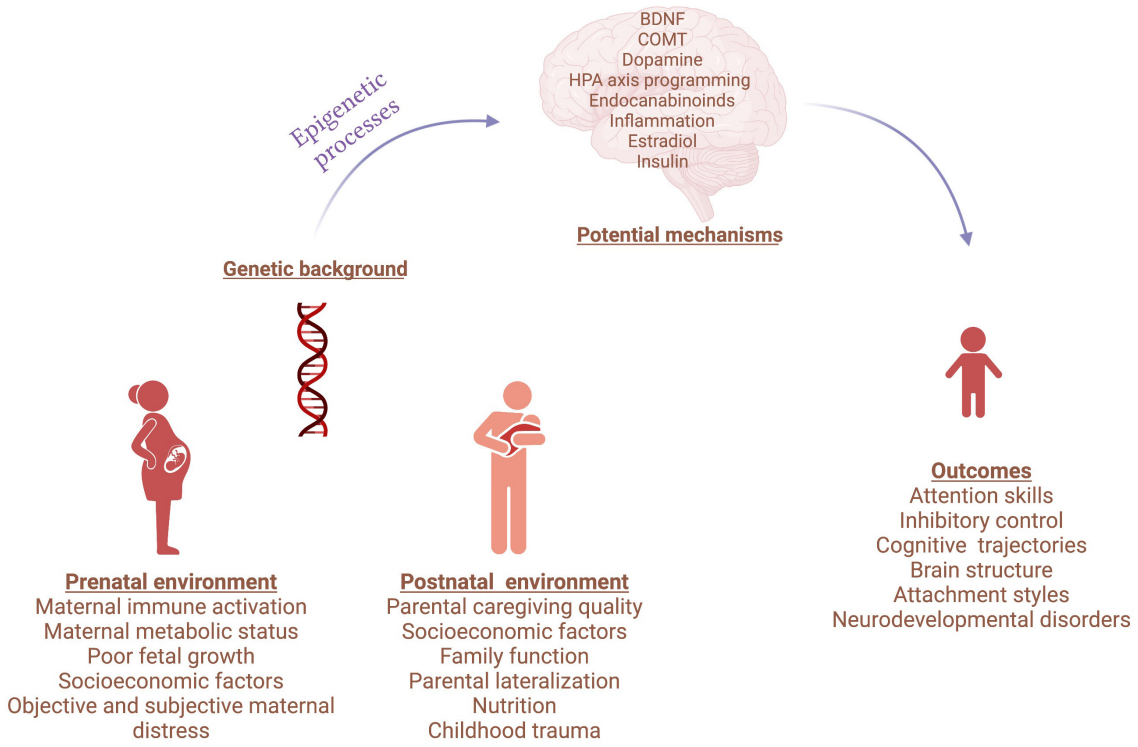

FIGURE 1 | Gene and environment interactions in neurodevelopmental disorders. Created with BioRender.com.

24-months children. The same candidate polymorphisms representing COMT and BDNF were used in the study from Cao-Lei et al., investigating the effects of prenatal maternal stress (defined by exposure to a natural disaster during pregnancy) on hippocampal volumes at 11–12 years of age. The SNP located on COMT gene moderated the effect of maternal distress on hippocampal volumes, suggesting that gene-environment interactions have long-term effects on brain neuroanatomical features.

de Mendonça Filho et al. used a novel approach to genomic profiling (Silveira et al., 2017; Hari Dass et al., 2019; Miguel et al., 2019b), representing variations in a prefrontal cortex-specific BDNF gene co-expression network in children, and show that this biological mechanism moderates the effect of prenatal adversity on cognitive developmental trajectories between 6 and 36 months. Intriguingly, epigenetics-related components of the BDNF gene network moderate the effects of prenatal adversity on gray matter content in cortical regions later in childhood. The same approach (Silveira et al., 2017; Hari Dass et al., 2019; Miguel et al., 2019b) was employed by Potter-Dickey et al. to investigate if prefrontal, striatal and hippocampal Cannabinoid Receptor 1 (CNR1) gene co-expression networks moderate the effect of parental caregiving quality on infant attachment styles. Finally, Batra et al. demonstrated that the genetic background associated with higher fasting insulin regulates the effects of early adversity on the development of inhibitory control in young children, corroborating the programming effects of insulin on executive functions in response to early adversity (Batra et al., 2021).

The Research Topic compiles evidence that gene-environment interactions influence neurodevelopment, proposing mechanisms by which this moderation occurs (**Figure 1**). The studies illustrate novel techniques that can uncover biological targets and pathways with important implications for early detection, prevention, and treatment of neurodevelopmental disturbances.

AUTHOR CONTRIBUTIONS

All authors wrote and approved the submitted version.

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The Association Study of *IL-23R* Polymorphisms With Cerebral Palsy in Chinese Population

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Background: Cerebral palsy (CP) is a syndrome of non-progressive motor dysfunction caused by early brain development injury. Recent evidence has shown that immunological abnormalities are associated with an increased risk of CP.

Methods: We recruited 782 children with CP as the case group and 770 healthy children as the control group. The association between *IL-23R* single nucleotide polymorphisms (SNPs; namely, rs10889657, rs6682925, rs1884444, rs17375018, rs1004819, rs11805303, and rs10889677) and CP was studied by using a case–control method and SHEsis online software. Subgroup analysis based on complications and clinical subtypes was also carried out.

Results: There were differences in the allele and genotype frequencies between CP cases and controls at the rs11805303 and rs10889677 SNPs (Pallele = 0.014 and 0.048, respectively; Pgenotype = 0.023 and 0.008, respectively), and the difference in genotype frequency of rs10889677 remained significant after Bonferroni correction (Pgenotype = 0.048). Subgroup analysis revealed a more significant association of rs10889677 with CP accompanied by global developmental delay (Pgenotype = 0.024 after correction) and neonatal encephalopathy (Pgenotype = 0.024 after correction).

Conclusion: The present results showed a significant association between *IL-23R* and CP, suggesting that *IL-23R* may play a potential role in CP pathogenesis.

Keywords: cerebral palsy, inflammatory cytokines, interleukin, gene polymorphism, IL23R

INTRODUCTION

Cerebral palsy (CP) is a central motor disorder syndrome that manifests with abnormal muscle tension and motor function (Cheng et al., 2011; Wu et al., 2011). Individuals with CP often exhibit sensory, perceptual, cognitive, communication, and behavioral disorders, as well as epilepsy and secondary musculoskeletal problems (Beliakoff and Sun, 2006). Although perinatal medicine has

developed rapidly in recent years, epidemiological studies have shown that the incidence of CP has remained stable at 2–3.5 children out of every 1,000 children (Pennington et al., 2013). CP has been a prominent disease among children's disabilities for a long time due to a lack of effective treatments (Tator et al., 2007; Tatla et al., 2013). CP seriously affects the quality of life of individuals and also brings a heavy financial burden to families and society (Duval et al., 2003; Yunus and Lima, 2009). The identification of its etiology and pathogenesis is essential to the prevention and control of CP.

CP is caused by non-progressive brain damage during the development of the fetus or infant, which can be divided into congenital and acquired damage. Congenital non-progressive injuries are caused by prenatal developmental defects, such as genetic defects, developmental defects, malformations, intrauterine infections, etc. Acquired injuries are caused by postpartum acquired factors, such as premature labor, asphyxia, hypoxic–ischemic encephalopathy (HIE), low birth weight (LOW), and pathogenic jaundice (Sachdev et al., 2001; Jacobsson and Hagberg, 2004). Increasing evidence now indicates that genetic factors are likely to play an important role in CP pathogenesis. In general, CP is regarded as the result of the combined effects of multiple genes and environmental factors. Moreover, CP has been reported to have multiple susceptibility genes, including *IL-6*, *NOS1*, *OLIG2*, *ATG5*, and *ATG7* (Xu et al., 2017; Yu et al., 2018; Sun et al., 2019; Xia et al., 2019).

A great deal of evidence suggests that neuroinflammation has been found to participate in, modulate, and even induce the pathological process of immature brain injury and various cytokines have been associated with CP and neurodevelopmental disability. Previous studies have found that immature brain injury induced by secondary inflammation is one of the important pathological mechanisms of hypoxic–ischemic brain injury (Elovitz et al., 2011; Albertsson et al., 2014; Marshall and Plotkin, 2019). Abnormal activation of cytokines can cause brain damage, and fetuses are more likely to produce inflammatory reactions or brain damage after being affected by inflammatory cytokines due to immature brain development, thus further affecting the normal development of the brain. At present, many inflammatory cytokines have been reported to be significantly associated with CP or neurodevelopmental disorders, such as *IL-6*, *IL-8*, *IL-10*, and *IL-17* (Strle et al., 2001; Chiricozzi et al., 2011; Chen et al., 2013; Magalhaes et al., 2019).

Interleukin-23 (*IL-23*), also known as p19, is a member of the *IL-12* heterodimer cytokine family (Oppmann et al., 2000), which is mainly produced by activated dendritic cells, macrophages, and monocytes. *IL-23* plays an important role in the regulation of tissue homeostasis and congenital or adaptive immunity. IL-23 is involved in the pathogenesis of many chronic inflammatory diseases, such as psoriasis, arthritis, inflammatory bowel disease, and multiple sclerosis (MS). Drugs targeting IL-23 have been used in clinical research regarding immunologic diseases (Wiekowski et al., 2001; Schon and Erpenbeck, 2018). IL-23 binds to the IL-23 receptor (*IL-23R*) through its N-terminal immunoglobulin domain, which activates downstream signaling pathways and exerts biological functions. Human IL-23 receptors (*IL-23R*) are mainly expressed in activated memory T cells, natural killer (NK) cells, and intrinsic immune cells (ILCs). Its extracellular domain contains a signal sequence, one N-terminal Ig-like domain, and two cytokine receptor domains. In a genome-wide association study in 2006, *IL-23R* was significantly associated with Crohn's disease, an inflammatory bowel disease. The A allele of rs11209026, a low-frequency *IL-23R* variant, was negatively correlated with Crohn's disease (Bloch et al., 2018). At the same time, some studies have shown that *IL-23R* mutation is significantly correlated with inflammatory demyelinating diseases, such as MS (Li et al., 2016).

Based on the above information, we speculate that *IL-23R* may be associated with susceptibility to CP, but no relevant studies have been reported thus far. Then, we used a case–control study to explore the possible association of *IL-23R* with CP, which will provide genetic evidence for evaluating the role of *IL-23R* in the etiology of CP and its related potential mechanisms.

MATERIALS AND METHODS

Participants

In this study, 782 children with CP and 771 healthy controls were recruited from the centers for CP rehabilitation and Child Healthcare Departments in the Third Affiliated Hospital of Zhengzhou University, Zhengzhou Children's Hospital. This study was approved by the ethics committee of Zhengzhou University. The guardians of these participants provided written informed consent. The case group comprised 542 males (69.3%) and 240 females (30.7%), and the mean age was 18.5 ± 15.4 months. The control group comprised 771 healthy children, including 515 males (66.8%) and 256 females (33.2%), and the mean age was 19.3 ± 16.8 months (**Table 1**).

TABLE 1 | Clinical characteristics of all participants.

Characteristic	CP cases (n = 782)	Controls (n = 771)
Sex (male:female)	542:240	515:256
Preterm (<37 weeks)	47	10
<2,500 g	40	2
Birth asphyxia	234	13
Type of CP		
Spastic CP	522	NA
CP with quadriplegia	284	NA
CP with diplegia	126	NA
Complications		
CP with PVL	67	NA
CP with HIE	108	NA
CP with GDD	299	NA
Type of CP	310	NA
Maternal factors		
PROM	71	26
TPL	58	0
PIH	26	7

CP, cerebral palsy; PVL, periventricular leukomalacia; HIE, hypoxic–ischemic encephalopathy; GDD, global developmental delay; PROM, premature rupture of membrane; TPL, threatened premature labor; PIH, pregnancy-induced hypertension.

CP Diagnosis, Classification, and Exclusion Criteria

In the case group, we excluded children diagnosed with congenital metabolic diseases and myopathy as well as children with a family history of nervous system diseases. Pediatric rehabilitation specialists confirmed the CP diagnosis using standard criteria related to non-progressive disorders of movement control and posture (Rosenbaum et al., 2007). All participants received a detailed clinical evaluation with comprehensive pre-test counseling.

The available clinical information included demographic variables, such as sex, gestational age, mode of delivery, singletons, and twins, as well as the known risk factors [such as pregnancy-induced hypertension (PIH), perinatal asphyxia, and threatened premature labor], CP complications [such as global developmental delay (GDD)], and neonatal complications (such as HIE).

GDD diagnosis is limited to individuals under the age of 5 years old when the clinical severity level cannot be reliably assessed during early childhood. GDD is diagnosed when an individual fails to meet the expected developmental milestones in several areas of intellectual functioning and applies to individuals who are unable to undergo systematic assessments of intellectual functioning, including children who are too young to participate in standardized testing. Neonatal encephalopathy (NE) is a clinical syndrome that includes HIE, intracranial hemorrhage, various metabolic disorders, neurodegenerative diseases, and so on; its diagnosis requires at least two senior neonatologists.

Genotyping and Statistical Analysis

Peripheral blood samples were obtained from the subjects for genomic DNA extraction. According to the single nucleotide polymorphism (SNP) location in *IL-23R*, a minor allele frequency (MAF) >0.1, and potential function, we selected seven SNPs (rs10889657, rs6682925, rs1884444, rs17375018, rs1004819, rs11805303, and rs10889677, **Figure 1**) as candidates and genotyped them by the MassARRAY system. Shanghai Perchant Biotechnology Co., Ltd. synthesized primers and probes.

We performed statistical analysis with SHEsis, an online program (http://analysis.bio-x.cn/) that can test Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) and calculate allele frequencies, genotype frequencies, and haplotype frequencies for each SNP locus in the case group and control group. The *P* values were two-tailed, and we considered $P < 0.05$ to be significant. We also calculated the odds ratio (OR) and its 95% confidence interval (CI). We employed the Bonferroni correction to account for multiple testing on each individual SNP and haplotype. We used the G*power 3.1 software to evaluate the statistical efficacy.

RESULTS

Overall Analysis

By performing power calculations, the sample size utilized in the present study has >85% power to detect a significant association ($\alpha < 0.05$) when using an effect size index of 0.1. The genotype distributions of rs17375018 among control subjects showed a significant deviation from HWE ($P = 0.011$); therefore, we only analyzed the remaining six SNPs, namely, rs10889657, rs6682925, rs1884444, rs1004819, rs11805303, and rs10889677. There were two linked LD blocks with coefficient D′ value more than 0.8 (**Figure 2**). Block 1 included rs10889657, rs6682925, and rs1884444, whereas block 2 comprised rs1004819, rs11805303, and rs10889677.

For all subjects, the allele frequencies of rs11805303 ($P = 0.014$) and rs10889677 ($P = 0.048$) and the genotype frequencies of rs10889657 ($P = 0.028$), rs11805303 ($P = 0.023$), and rs10889677 ($P = 0.008$) were significantly different between CP patients and controls. After Bonferroni correction, only the rs10889677 AA genotype frequency was significantly more enriched in CP children than in controls (OR = 1.178, 95% CI = 1.002–1.386, $Pc = 0.048$) (**Table 2**).

Haplotype analysis is a powerful strategy to determine whether or not the above-mentioned CP-associated SNPs have a greater effect when analyzed together. Hence, we performed a haplotype analysis of rs11805303 and rs10889677 SNPs. The haplotypes CC ($P = 0.036$) and TA ($P = 0.013$) presented a significant association with CP; the positive association of TA with CP was significant even after Bonferroni correction ($P = 0.039$). Furthermore, there was a statistically significant global effect of the haplotype ($P = 0.039$) (**Table 3**).

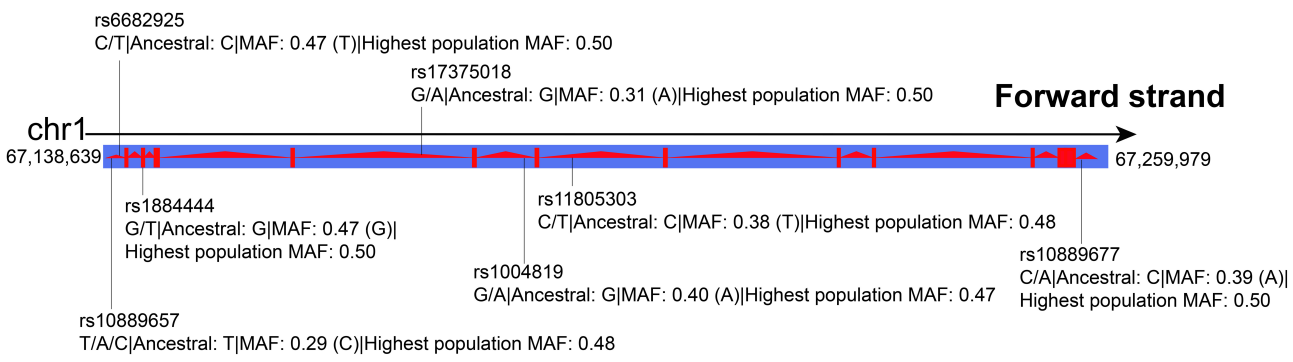

FIGURE 1 | SNPs in *IL-23R*. Highest population minor allele frequency: highest minor allele frequency observed in any population including 1,000 Genomes Phase 3, ESP, and genomAD.

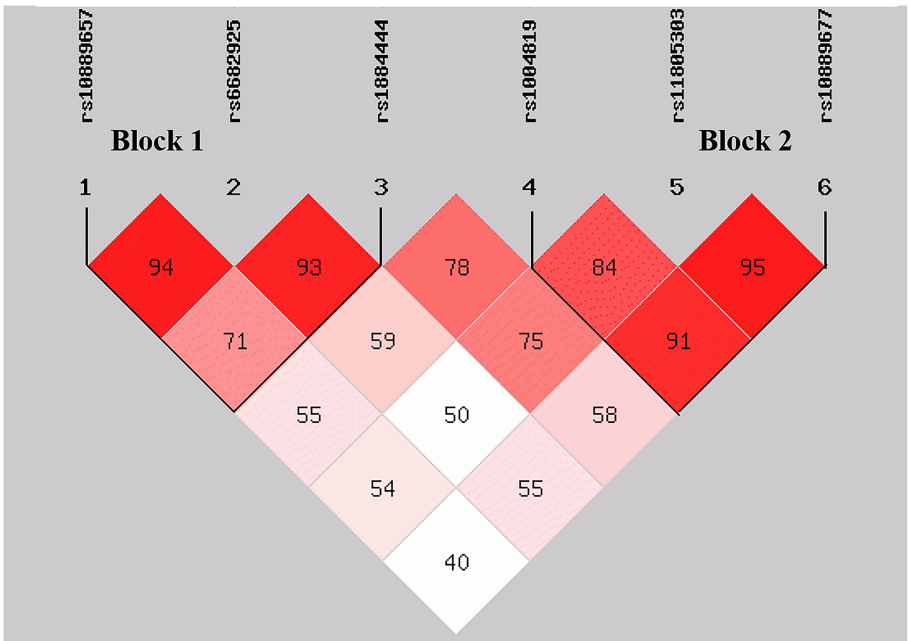

FIGURE 2 | Distribution of blocks defined by linkage disequilibrium scores of six SNPs in *IL-23R*. The data indicate D′ values.

Subgroup Analysis

CP is a highly heterogeneous condition that likely has multiple etiologies. It is reasonable to speculate that the broad clinical spectrum of CP can be at least partially attributed to considerable genetic heterogeneity. Therefore, we conducted subgroup analysis according to clinical phenotypes. There was a significant association of CP + GDD with rs10889657, rs1004819, rs11805303, and rs10889677, and the association of rs10889677 met the Bonferroni correction cut-off for multiple testing (OR = 1.293, 95% CI = 1.034–1.61, $P = 0.024$, **Table 4**).

Furthermore, we analyzed the associations between *IL-23R* and CP subtypes with various risk factors. There were significant differences in the rs11805303 allele frequency and the rs10889677 genotype frequency between controls and CP patients with NE; the association of rs10889677 with CP + NE remained significant even after Bonferroni correction ($OR = 1.176$, 95% CI = 0.947–1.460, $P = 0.024$, **Table 5**). There were no significant differences in either allele or genotype frequencies of the other SNPs in the other CP subgroups defined by clinical phenotypes and available risk factors (**Supplementary Table 1**).

DISCUSSION

CP is a heterogeneous condition with multiple causes (Badawi and Keogh, 2013). The etiology in an individual patient is often multifactorial. These known CP causes, such as periventricular leukomalacia (PVL), NE, infarct, and premature delivery, account for only a minority of the total cases (Cowan et al., 2003; Yildiz et al., 2012; Colver et al., 2014; Chang et al., 2015). A single severe adverse event can be sufficient to cause CP, but much more often it is not a single cause, rather multiple concurrent risk factors that precede CP (Hankins and Speer, 2003; Djukic et al., 2009). Secondary neuroinflammation is associated with many CP risk factors. Findings from animal and clinical studies suggested that persistent neuroinflammation might prevent regeneration or exacerbate brain damage (Elovitz et al., 2011). Altered inflammation is one of the common causes of CP (Chiricozzi et al., 2011; Chen et al., 2013; Xia et al., 2018). Although the exact cause of CP is largely unknown, it is thought to be due to a combination of an altered fetal inflammatory response and primary brain damages.

Abnormal inflammation is one of the important pathological causes of immature brain injury (Djukic et al., 2009; Du et al., 2011), which is involved in the pathogenesis of central nervous system diseases, such as epilepsy, Parkinson's disease, cerebral ischemia, and hemorrhage, and may easily lead to secondary brain insult. During cerebral ischemia–reperfusion injury, inflammatory cells, such as microglia, astrocytes, and leukocytes, are activated. The activated inflammatory cells synthesize and secrete inflammatory mediators, phenomena that, in turn, further activate inflammatory cells and aggravate brain injury (Moon et al., 2009; Albertsson et al., 2018).

Some inflammatory cytokines are involved in immune responses, which cause brain injury through an inflammatory mechanism (McAdams and Juul, 2012). Several inflammatory cytokines have been identified as being involved in CP or other neurodevelopmental disorders, such as *IL-6*, *IL-8*, and *IL-17* (Chiricozzi et al., 2011; Chen et al., 2013). Our previous studies found that rs1800795 (G174C), located in the *IL-6* promoter region, was significantly associated with CP, and that the risk of spastic hemiplegia and quadriplegia in carriers of the rs1800795 C allele also significantly increased (Djukic et al.,

TABLE 2 | Allele and genotype frequency analysis of SNPs of *IL-23R*.

Group	Allele frequency		*p* value	OR [95% CI]	Genotype frequency			*p*-value	H-W
rs10889657	C	T			C/C	C/T	T/T		
CP	347 (0.267)	953 (0.733)	0.09	0.861 [0.725–1.023]	59 (0.091)	229 (0.352)	362 (0.557)	0.028[a]	0.011
Control	372 (0.297)	880 (0.703)			53 (0.085)	266(0.425)	307 (0.490)		0.665
rs6682925	C	T			C/C	C/T	T/T		
CP	625 (0.422)	855 (0.578)	0.406	0.940 [0.813–1.088]	133 (0.180)	359 (0.485)	248 (0.335)	0.681	0.876
Control	634 (0.437)	827 (0.563)			139 (0.189)	365 (0.497)	231 (0.314)		0.807
rs1884444	G	T			G/G	G/T	T/T		
CP	517 (0.344)	985 (0.656)	0.169	0.900 [0.776–1.046]	91 (0.121)	335 (0.446)	325 (0.433)	0.275	0.744
Control	556 (0.368)	954 (0.632)			97 (0.128)	362 (0.479)	296 (0.392)		0.402
rs1004819	A	G			A/A	A/G	G/G		
CP	532 (0.566)	408 (0.434)	0.05	1.194 [1.000–1.425]	168 (0.357)	196 (0.417)	106 (0.226)	0.063	0.001
Control	544 (0.522)	498 (0.478)			150 (0.288)	244 (0.468)	127 (0.244)		0.16
rs11805303	C	T			C/C	C/T	T/T		
CP	628 (0.415)	884 (0.585)	0.014[b]	0.835 [0.723–0.964]	150 (0.198)	328 (0.434)	278 (0.368)	0.023[c]	0.003
Control	696 (0.460)	818 (0.540)			167 (0.221)	362 (0.478)	228 (0.301)		0.304
rs10889677	A	C			A/A	A/C	C/C		
CP	1121 (0.750)	373 (0.250)	0.048[d]	1.178 [1.002–1.386]	429 (0.574)	263 (0.352)	55 (0.074)	0.008[e]	0.099
Control	1079 (0.718)	423 (0.282)			378 (0.503)	323 (0.430)	50 (0.067)		0.085

OR, odds ratio; CI, confidence interval.The P values corrected by Bonferroni are [a]0.168, [b]0.084, [c]0.138, [d]0.288, [e]0.048.

TABLE 3 | Haplotype analysis of rs11805303 and rs10889677 SNPs.

Haplotype	Case (frequency)	Control (frequency)	*P* value	OR (95% CI)
CA	250.91 (0.171)	271.19 (0.183)	0.413244	0.924 (0.765–1.117)
CC	356.09 (0.242)	410.81 (0.277)	0.036346	0.838 (0.711–0.989)
TA	850.09 (0.578)	793.81 (0.536)	0.013013	1.203 (1.040–1.392)
Fisher's *P* value			0.039	
Pearson's *P* value			0.039	

2009; Wu et al., 2009). Central nervous system inflammation is often characterized by microglia activation, and active microglia will mediate neurotoxicity by secreting inflammatory cytokines, proteins, or other bioactive substances, resulting in secondary brain injury (Tang and Le, 2016).

A recent study demonstrated that inflammatory factors are related to microglia activation (Zhao et al., 2020). IL-23 mainly acts as a pro-inflammatory cytokine and has potential anti-tumor and anti-infection effects. IL-23R can activate Janus kinase (JAK). IL-23 bound IL-23R can active downstream JAKs and phosphorylate the signal transducer and activator of transcription (STAT) binding site in the intracellular region of the receptor. STAT dimerizes and is phosphorylated by JAKs. The phosphorylated STAT dimers enter the nucleus and act on downstream target genes. *IL-23R* has been identified to associate with multiple diseases, including alopecia areata (rs10889677) and nephropathy (rs10805303) (**Figure 3**) (Safrany et al., 2011; Yu et al., 2012; Huang et al., 2016; Qin et al., 2016; Poomarimuthu et al., 2018; Sode et al., 2018; Zhong et al., 2018; Kramer et al., 2019; Loures et al., 2019; Ruyssen-Witrand et al., 2019; Tabatabaei-Panah et al., 2020).

Our results showed that *IL-23R* rs10889677 increases susceptibility to CP at the overall level and in some subgroups. These findings suggest that *IL-23R* is a potential susceptibility gene for CP. Furthermore, studies have shown that rs10889677 is related with different diseases in different races, such as Crohn's disease in both Jewish and non-Jewish populations, ankylosing spondylitis in Caucasian patients, Graves' disease in North Americans, and ulcerative colitis in Europeans (Duerr et al., 2006; Wellcome Trust Case Control et al., 2007; Brown, 2008; Huber et al., 2008; Silverberg et al., 2009). Therefore, we have sufficient reason to assume the positive association of *IL-23R* gene with the CP etiology. Whether *IL-23R*, as a gene associated with inflammatory bowel disease, can also affect the brain development of children by affecting intestinal flora remains to be seen.

Given that *TNF-α* and *IL-6* are significantly increased after brain injury (Leviton and Dammann, 2004; Xie et al., 2014), we hypothesize that IL-23R, like other inflammatory factors *TNF-α* and *IL-6*, may cause brain damage and lead to CP through the following steps. (1) Increased inflammatory cytokines promote the release of nitric oxide synthase and free radicals and excitatory amino acids, which have toxic effects on neurons, especially developing brain tissue. (2) They will trigger a systemic inflammatory response that leads to brain damage when undergoing intrauterine infection. (3) Endothelial cell damage can cause thrombosis. Inflammatory factors will activate platelets, lead to their aggregation, activate coagulation factors, and damage white matter neurons. (4) The damage will increase

TABLE 4 | Allele and genotype frequencies of *IL-23R* in CP with GDD and the control group.

Group	Allele frequency	*P* value	OR (95% CI)	Genotype frequency	*P* value	Group	Allele frequency	*P* value	OR (95% CI)
rs10889657	C	T			C/C	C/T	T/T		
CP	132 (0.253)	390 (0.747)	0.06	0.801 (0.635–1.009)	23 (0.088)	86 (0.330)	152 (0.582)	0.026[a]	0.038
Control	372 (0.297)	880 (0.703)			53 (0.085)	266 (0.425)	307 (0.490)		0.665
rs6682925	C	T			C/C	C/T	T/T		
CP	239 (0.424)	325 (0.576)	0.578	0.946 (0.777–1.151)	54 (0.191)	131 (0.465)	97 (0.344)	0.608	0.412
Control	634 (0.437)	827 (0.563)			139 (0.189)	365 (0.497)	231 (0.314)		0.807
rs184444	G	T			G/G	G/T	T/T		
CP	196 (0.343)	376 (0.657)	0.279	0.894 (0.731–1.094)	38 (0.133)	120 (0.420)	128 (0.448)	0.201	0.246
Control	556 (0.368)	954 (0.632)			97 (0.128)	362 (0.479)	296 (0.392)	0.402	
rs1004819	A	G			A/A	A/G	G/G		
CP	219 (0.592)	151 (0.408)	0.021[b]	1.328 (1.044–1.688)	71 (0.384)	77 (0.416)	37 (0.200)	0.051	0.06
Control	544 (0.522)	498 (0.478)			150 (0.288)	244 (0.468)	127 (0.244)		0.16
rs11805303	C	T			C/C	C/T	T/T		
CP	232 (0.403)	344 (0.597)	0.019[c]	0.793 (0.652–0.963)	53 (0.184)	126 (0.438)	109 (0.378)	0.051	0.124
Control	696 (0.460)	818 (0.540)			167 (0.221)	362 (0.478)	228 (0.301)	0.304	
rs10889677	A	C			A/A	A/C	C/C		
CP	439 (0.767)	133 (0.233)	0.024[d]	1.293 (1.034–1.619)	174 (0.608)	91 (0.318)	21 (0.073)	0.004[e]	0.067
Control	1,079 (0.718)	423 (0.282)			378 (0.503)	323 (0.430)	50 (0.067)		0.084

P value after Bonferroni [a]0.077, [b]0.126, [c]0.114, [d]0.144, [e]0.024.

TABLE 5 | Allele and genotype frequencies of IL-23R in CP with NE and the control group.

Group	Allele frequency	*P* value	OR (95% CI)	Genotype frequency	*P* value	Group	Allele frequency	*P* value	OR (95% CI)
rs10889657	C	T			C/C	C/T	T/T		
CP	146 (0.271)	392 (0.729)	0.271	0.881 (0.703–1.104)	25 (0.093)	96 (0.357)	148 (0.550)	0.163	0.109
Control	372 (0.297)	880 (0.703)			53 (0.085)	266 (0.425)	307 (0.490)		0.665
rs6682925	C	T			C/C	C/T	T/T		
CP	257 (0.425)	347 (0.575)	0.619	0.952 (0.787–1.154)	48 (0.159)	161 (0.533)	93 (0.308)	0.434	0.116
Control	634 (0.437)	827 (0.563)			139 (0.189)	365 (0.497)	231 (0.314)		0.807
rs184444	G	T			G/G	G/T	T/T		
CP	219 (0.369)	375 (0.631)	0.984	1.002 (0.823–1.220)	36 (0.121)	140 (0.495)	114 (0.384)	0.891	0.276
Control	556 (0.368)	954 (0.632)			97 (0.128)	362 (0.479)	296 (0.392)		0.402
rs1004819	A	G			A/A	A/G	G/G		
CP	201 (0.552)	163 (0.448)	0.322	1.128 (0.888–1.434)	62 (0.341)	77 (0.423)	43 (0.236)	0.391	0.051
Control	544 (0.522)	498 (0.478)			150 (0.288)	244 (0.468)	127 (0.244)		0.16
rs11805303	C	T			C/C	C/T	T/T		
CP	244 (0.405)	358 (0.595)	0.023[a]	0.801 (0.661–0.970)	52 (0.173)	140 (0.465)	109 (0.362)	0.083	0.541
Control	696 (0.460)	818 (0.540)			167 (0.221)	362 (0.478)	228 (0.301)		0.304
rs10889677	A	C			A/A	A/C	C/C		
CP	450 (0.750)	150 (0.250)	0.141	1.176 (0.947–1.460)	177 (0.590)	96 (0.320)	27 (0.090)	0.004[b]	0.011
Control	1,079 (0.718)	423 (0.282)			378 (0.503)	323 (0.430)	50 (0.067)		0.084

P value after Bonferroni [a]0.138, [b]0.024.

the permeability of the blood–brain barrier, thereby allowing peripheral bacteria and inflammatory factors to enter the brain, aggravating brain damage. (5) They promote the release of prostaglandins and other substances, resulting in pregnancy's advance labor, leading to an increased risk of CP.

Our study has some limitations. First, this is a study based on a single gene for susceptibility to CP. Given the genetic heterogeneity and gene–gene interaction involved in the CP etiology, other candidate genes that are part of the IL-23R signaling pathway need to be analyzed together. Second, we were unable to measure IL-23R protein expression in the brains of the subjects in the current study; future studies are encouraged to examine the inflammatory cytokine alteration in the brain. Third, although our study demonstrated an association between the *IL-23R* rs10889677 SNP and CP, further functional and replicated studies are necessary to verify the association of *IL-23R* with

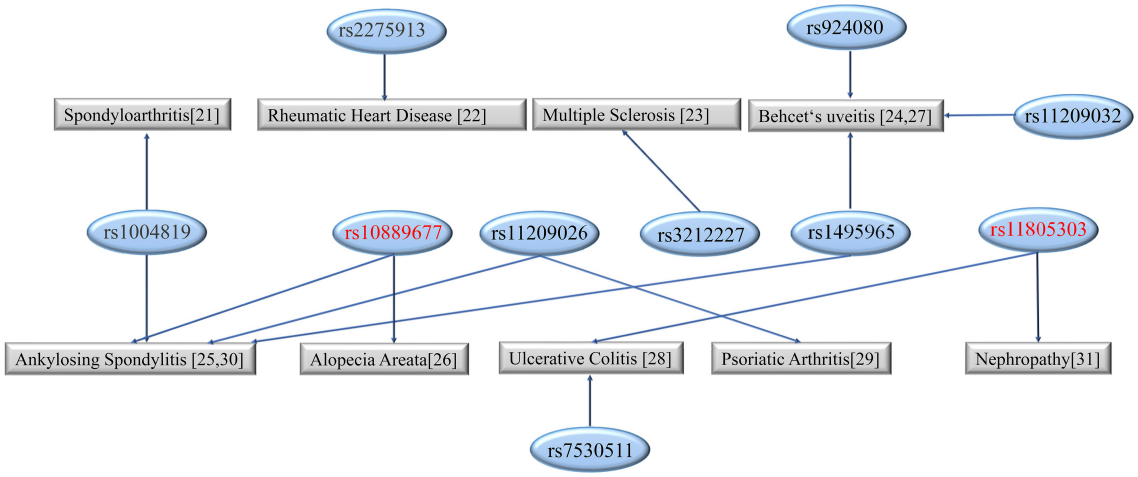

FIGURE 3 | The diseases linked to different sites of *IL-23R*. The SNPs studied in the present study are marked in red.

CP, which is of great significance to identify the CP etiology and pathogenesis.

In summary, a significant association between *IL-23R* and CP was firstly detected in Han Chinese, suggesting that the *IL-23R* gene has a significant effect on the risk of CP, especially in subjects with GDD or NE. The inflammatory response and cytokine cascade are likely to play a role in the occurrence and development of CP. This result needs to be further validated with well-designed studies with large sample sizes and in other populations. We should also pay attention to the possibility of increased risk of CP if the fetus is found to carry the *IL-23R* risk genotypes before or after delivery.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

QX and CZ conceived and designed the study. YX, JS, LX, QS, CG, and DZ recruited subjects and sorted out clinical information. YW and DB performed all of the laboratory work. YW and YF performed all data and statistical analyses. YF drafted the manuscript, and QX, CZ, XW, YW, and YX revised the manuscript critically for important intellectual content. YQ and JS provided data, developed models, reviewed results, and provided guidance on the methods. All authors contributed and critically reviewed the final version of the manuscript. All authors have 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/fnins.2020.590098/full#supplementary-material

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Human Lateralization, Maternal Effects and Neurodevelopmental Disorders

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In humans, behavioral laterality and hemispheric asymmetries are part of a complex biobehavioral system in which genetic factors have been repeatedly proposed as developmental determinants of both phenomena. However, no model solely based on genetic factors has proven conclusive, pushing towards the inclusion of environmental and epigenetic factors into the system. Moreover, it should be pointed out that epigenetic modulation might also account for why certain genes are expressed differently in parents and offspring. Here, we suggest the existence of a sensitive period in early postnatal development, during which the exposure to postural and motor lateral biases, expressed in interactive sensorimotor coordination with the caregiver, canalizes hemispheric lateralization in the "typical" direction. Despite newborns and infants showing their own inherent asymmetries, the canalizing effect of the interactive context owes most to adult caregivers (usually the mother), whose infant-directed lateralized behavior might have been specifically selected for as a population-level trait, functional to confer fitness to offspring. In particular, the case of the left-cradling bias (LCB; i.e., the population-level predisposition of mothers to hold their infants on the left side) represents an instance of behavioral trait exhibiting heritability along the maternal line, although no genetic investigation has been carried out so far. Recent evidence, moreover, seems to suggest that the reduction of this asymmetry is related to several unfavorable conditions, including neurodevelopmental disorders. Future studies are warranted to understand whether and how genetic and epigenetic factors affect the lateralization of early mother-infant interaction and the proneness of the offspring to neurodevelopmental disorders.

Keywords: laterality, hemispheric asymmetry, mother-infant interaction, cradling-side bias, behavioral epigenetics, autism spectrum disorders

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BEHAVIORAL EPIGENETICS AND THE DEVELOPMENT OF LATERALIZATION

Studies on lateralization have progressed at a remarkable pace in recent decades, gathering multiple levels belonging to different disciplines and traditions of research. Neural, behavioral and genetic aspects of asymmetries are becoming more and more connected to each other in the all-encompassing framework of biological evolution. Theoretical models suggest that interactive behaviors are key to the evolution of population-level lateral biases (e.g., Ghirlanda and Vallortigara, 2004): a stable equilibrium in the asymmetrical distribution of lateralized behavioral phenotypes of a

given species might be reached through the fitness contribution of both antagonistic and synergistic interactions occurring among its members (Ghirlanda et al., 2009). Empirical evidence seems also to suggest that early development is a crucial context in which synergistic interactions affect lateralization (Karenina et al., 2017). However, only rarely evolutionary accounts of lateralization including developmental plasticity as a determining factor have been suggested (e.g., see Michel et al., 2018).

In humans, the ontogeny of lateralization emerges from the multifaceted interaction between genetic and environmental factors that have not been understood in full detail (Güntürkün and Ocklenburg, 2017). Structural asymmetries of the brain are but a small fraction of the *Bauplan* of neural lateralization—the largest part being expressed in the form of functional asymmetries—and they consist in the allocation of different roles to two structurally similar brain hemispheres (Corballis, 2017). Functional asymmetries are ubiquitous in the nervous system especially in the neocortex, and they emerge in many behavioral and mental functions, including action (Guiard, 1987; Serrien and Sovijärvi-Spapé, 2015), imagination (Marzoli et al., 2011a,b, 2013, 2017a; Prete et al., 2016b; Altamura et al., 2020), perception (Marzoli and Tommasi, 2009; Brancucci and Tommasi, 2011; Prete et al., 2015d, 2018b; Prete and Tommasi, 2018), emotion (Prete et al., 2014a, 2015a,c; Wyczesany et al., 2018), attention (Yamaguchi et al., 2000; Chen and Spence, 2017) and memory (Iidaka et al., 2000; Penolazzi et al., 2010; D'Anselmo et al., 2016). Language can be considered the most emblematic case of functional asymmetry, also because the history of discoveries on brain lateralization (and localization) began precisely with aphasia studies (Leblanc, 2017). Nevertheless, it must be noted that motor functions deserve a special place in this list, particularly because of the peculiar status of handedness as a function that is lateralized both behaviorally and neurologically from early childhood (Bondi et al., 2020): around 90% of humans show a preference for using the right hand, which is controlled by the left brain hemisphere (McManus, 2002; Tommasi, 2009). Additionally, footedness should also be granted a special position in the field of human laterality, having been shown to share similarities with handedness both in behavioral and neuropsychological terms, and to be less influenced by cultural and social factors than handedness (Elias and Bryden, 1998; Tran et al., 2014; Packheiser et al., 2020a,c). Population-level motor asymmetries which seem to be precursors of handedness are observed already during fetal life (Hepper et al., 1990; Hepper, 2013; see also Baciadonna et al., 2010 for analogous early predictors of limb laterality in a non-human species), speaking in favor of a substantial genetic contribution. In this regard, the search for genetic factors of human functional lateralization has been characterized by single- or multiple-gene theories aimed to explain handedness, and continues nowadays within molecular genetics studies addressed to the identification of specific loci (Cuellar-Partida et al., 2021). Interestingly, these studies also suggest a partly common ground among genetic variants influencing the development of brain functional laterality and the emergence of neurodevelopmental disorders (Wiberg et al., 2019). However, no evidence has proven strong enough to exactly explain the statistical frequencies of hand preference observed in families (Medland et al., 2009; McManus et al., 2013; Armour et al., 2014). Environmental factors have been therefore implicated, from the effect of hormones (Geschwind and Galaburda, 1985; Berretz et al., 2020) and fetus position *in utero* (Previc, 1991), to the visual experience of own and others' hands during early infancy (Michel and Harkins, 1986; Fagard and Lemoine, 2006). Michel et al. (2018) suggested that the development of lateralization begins prenatally, and progresses postnatally as a head orientation preference, predominantly right-biased in infants (Michel and Harkins, 1986). Such an early rightward postural asymmetry would have the effect of placing their right hand in their visual field more than their left hand, thus causing cascading feedback-based proprioceptive effects during movement, possibly facilitating the gradual emergence of right-handedness. This suggestion was also confirmed by the observation of children with congenital muscular torticollis, whose restricted early visual experience affected the later development of handedness (Ocklenburg et al., 2010). On the other hand, right-handedness might also be fostered by children imitating adult's manual preferences (Fagard and Lemoine, 2006). Similar mechanisms might be involved not only in the development of handedness, but also in the attentional bias toward the right side of others' body observed in both right- and left-handers (Marzoli et al., 2015, 2017a,b, 2019; Lucafò et al., 2016, 2021; see also Marzoli et al., 2014), which in turn could account for the left-handers' advantage in fighting and sports (e.g., Groothuis et al., 2013). Although the relative weight of genetic and environmental determinants of handedness has not been established yet, epigenetic effects have been hypothesized at both the molecular (Leach et al., 2014) and the behavioral level (Schmitz et al., 2017), and the same should be true for other instances of functional asymmetries.

In addition to prenatal processes occurring *in utero* (e.g., Ocklenburg et al., 2017), behavioral epigenetics could play a major role during postnatal life, specifically because of parental care: humans, as many mammalian species, are indeed characterized by altriciality, that is an extended period after birth during which the newborn is helpless and depends on external sources (i.e., adults) for survival (Gubernick, 2013). This means that the social and behavioral environment is crucial—through an extraordinarily complex matrix of variables—for development. This "epigenetic niche" exerts an effect on the offspring's endophenotype, bringing about the expression of the genes in an environment shared with the caregivers. Importantly, the social bonding between parent and offspring is an environment in and of itself, and since the attachment behavioral system is the predisposed motivational structure that brings the infant and the mother to seek proximity to each other (Simpson and Belsky, 2008; Norholt, 2020), it may well constitute a very powerful context for the development of laterality. In this frame, lateralization research might take advantage of an important example of epigenetic niche: in the last decades, in fact, "cradling behavior" emerged as a specific case of lateralized social behavior involving parent (in particular the mother) and child, potentially modulating the development of hemispheric lateralization (Packheiser et al., 2019b).

FIGURE 1 | An example of left-cradling bias (LCB).

CRADLING-SIDE BIAS AS MATERNAL EFFECT

Cradling behavior has been consistently reported as left-lateralized at the population level, especially in women (65–70% of women cradle infants to the left of their body midline; see **Figure 1**; Packheiser et al., 2019b), and the bias has been causally linked to the development of the right hemisphere (Manning and Chamberlain, 1991; Harris et al., 2001; Bourne and Todd, 2004).

Indeed, it has been shown that the left-cradling bias (LCB) sets the postural conditions that facilitate an optimal emotional attunement between adult and infant because the right brain hemispheres of both are predominantly engaged during interactions in which the infant is held on the left side of the adult (Harris et al., 2010). This bias can be supposed to provide the infant with what Gilbert Gottlieb called "experiential canalization" (Gottlieb, 1991), a form of supervised narrowing of experience that the infant is predisposed to receive during a precise period. This is supported by a great amount of evidence: (i) in adults, cradling behavior is more strongly left-biased during the first year of life of the child and then declines in strength (Dagenbach et al., 1988); (ii) adults are selectively biased to the left when cradling (or even imagining cradling) infants or dolls rather than when holding or carrying inanimate objects (Harris et al., 2000); (iii) females are significantly more left-biased than males (Packheiser et al., 2019b); and (iv) the LCB seems to be transmitted from mother to daughter as a sex-linked inherited trait (Manning and Denman, 1994). In light of this evidence, it could be argued that the adult genes encode for the presence of an "obligatory" behavior in the mother-infant attachment during a "sensitive period" of the infant's development, and for a population-level predisposition to implement it asymmetrically on the left side. The experiential side of the story would consist of the interaction and sensorimotor coordination between adults and infants arising from the LCB. From this perspective, such an experience might modulate epigenetically the direction of the development of typical brain lateralization, triggered and scaffolded by the parent or the caregiver. Interestingly, the stronger LCB in females and the related maternal intergenerational transmission might be consistent with epigenetic studies indicating that certain genes are expressed differently in parents and offspring, as occurs in the case of differential parental imprinting (e.g., maternally derived duplications of a specific portion of chromosome 15 lead to an increased risk of autism and schizophrenia more than analogous paternally derived duplications; Cook et al., 1997; Isles et al., 2016).

A further aspect of this epigenetic view is that the LCB could be advantageous from an evolutionary perspective, because it is correlated to fitness-related traits in mothers, and possibly in children. For instance, research has shown that the correlates of cradling are indirectly evident when comparing women showing different degrees of left (typical) or right (atypical) cradling (Malatesta et al., 2019a,b, 2020b), bringing to the hypothesis that an atypical trajectory in maternal cradling might be one early sign of interference of dyadic socio-emotional communication, and thus of potential neurodevelopmental dysfunctions (Malatesta et al., 2020a,d). The fact that this left-sided population-level asymmetry goes in the direction opposite to that of a majority of right-handers, moreover, provides an important hint that it possibly attained a special functional status during evolution, and this speculation is further supported by the presence of an LCB also in left-handers. In this regard, it should be noted that the bias is detectable also in left-handers, indicating that it does not depend upon the fact that holding on the left would free the adult's dominant hand (Packheiser et al., 2019b). As such, the epigenetic niche represented by the mother cradling the baby would consist, in strictly biological terms, in a genuine maternal effect (Maestripieri and Mateo, 2009). This is supported by evidence of sex- and side-dependent effects of social perception obtained in previous works—for instance, the fact that the well-known left-face bias seems to be stronger for female faces, suggesting a greater sensitivity for the female face in the right hemisphere (Parente and Tommasi, 2008; Prete et al., 2016a, 2017), and the fact that females showing an LCB are more likely attracted by the left rather than right profile of a baby compared to females showing the opposite bias (Malatesta et al., 2020c).

Among the main explanations suggested for the LCB, the right-hemisphere hypothesis—the most accredited one today—revolves around the interaction and the socio-emotional information exchanged between the cradling and the cradled individual (Manning and Chamberlain, 1991; Harris et al., 2001; Bourne and Todd, 2004; for similar considerations in non-human species see Giljov et al., 2018). According to this

hypothesis, the right hemisphere should be mainly involved in emotional processing (Levy et al., 1983; Gainotti, 2012; Prete et al., 2014b, 2015b, 2018a), leading to a left hemibody and hemiface superiority in both the expression and the encoding of emotions. Similarly, evidence confirming the right-hemisphere hypothesis has been collected also for other lateralized social behaviors such as embracing and kissing (Ocklenburg et al., 2018; Packheiser et al., 2019a, 2020b). Therefore, cradling might represent a specific interactional framework benefiting both the mother and the infant, whose lateralization has unlikely been left to chance by evolutionary pressures. From the mother's point of view, the left-side positioning might facilitate the monitoring of her infant's well-being cues through her left visual and auditory fields, which project more directly to her right hemisphere (i.e., the one more involved in social and emotional processing; Brancucci et al., 2009; Prete et al., 2020a,b). Consistently, left-cradling individuals exhibit a stronger leftward bias for the processing of emotions from faces (Harris et al., 2001, 2010; Bourne and Todd, 2004). Moreover, the discovery of a preference for the left profile of infants in women showing a left-cradling bias (Malatesta et al., 2020c) suggests that a further adaptive function of the LCB might consist in a facilitated monitoring of the left hemiface of the infant, which is considered more expressive (Mendolia and Kleck, 1991) and whose emotional valence is identified more accurately, especially when a negative emotion is displayed (Kleck and Mendolia, 1990). Similarly, the LCB might expose the right hemisphere of children to the more expressive side of the mother's face (Hendriks et al., 2011). It is also possible to suppose that this double interaction (**Table 1**) gave an important advantage to both mothers and infants during the evolution by fostering typical neurodevelopment in the cradled infants.

In this regard, it has been shown that individuals cradled on the mother's right side during infancy showed a significant decrease of the typical left bias for emotional faces compared to left-cradled individuals, suggesting that mothers' cradling laterality has crucial outcomes on their children's development of socio-emotional abilities, such as the ability to perceive facial emotions later in life (Vervloed et al., 2011).

CRADLING BEHAVIOR AND NEURODEVELOPMENTAL DISORDERS

The role of the LCB in facilitating emotional communication is supported by findings suggesting that a reduction or inversion of the typical cradling lateralization is associated with several factors that might interfere with the quality of the mother-infant relationship and be a sign of a lack of wellbeing in the cradling woman. In previous studies, we showed that a reduction of the LCB is related to: (i) reduced empathy and increased depressive symptoms in mothers (Malatesta et al., 2019b); (ii) non-optimal patterns of attachment styles in females (Malatesta et al., 2019a); and (iii) prejudiced attitudes towards the cradled individual's ethnic group in females (Malatesta et al., 2020b). Similarly, the negative association between atypical (right) cradling and the quality of the mother-infant relationship seems to be confirmed by the fact that stress and negative affective states reduce the leftward asymmetry (Bogren, 1984; Weatherill et al., 2004; Suter et al., 2007, 2011; Reissland et al., 2009; Scola et al., 2013; Boulinguez-Ambroise et al., 2020; Pileggi et al., 2020). Furthermore, a link between this population-level bias and the later development of a typical cognitive and socio-emotional functioning has been suggested by recent findings associating developmental disorders—such as autism spectrum disorder (ASD)—and atypical patterns of lateralization in cradling (Jones, 2014; Pileggi et al., 2015; Forrester et al., 2019, 2020; Herdien et al., 2020; Malatesta et al., 2020a,d). This link is also highlighted by evidence unveiling that ASD constitutes a group of neurodevelopmental disorders that, besides entailing chronic and severe impairment in socio-communicative and empathic relationships, are also characterized by an early hypolateralization of brain functions (e.g., Escalante-Mead et al., 2003; Stroganova et al., 2007), including a reduced left bias for faces (Ashwin et al., 2005; Dundas et al., 2012). Furthermore, given that parents of children with ASD exhibit autistic traits to a greater extent compared with controls (Bishop et al., 2004; Ruta et al., 2012; Bora et al., 2017) and given that autistic traits in adults are associated with a reduced LCB (Fleva and Khan, 2015), we have hypothesized an association between reduced left-cradling preference in mothers and later diagnosis of ASD in children (Malatesta et al., 2020a,d). This perspective is in line with research on other forms of systematic deviation from the typical behavioral lateralization such as left-handedness. For example, although the issue is still debated (McManus, 2019), left-handedness has been related to several impairments (e.g., in cognitive abilities such as intelligence and spatial abilities; Gibson, 1973; Johnston et al., 2009; Nicholls et al., 2010; Papadatou-Pastou and Tomprou, 2015; Somers et al., 2015) and has been considered as a cue of reduced fitness (e.g., for evidence in favor of a relation between reduced right-handedness and decreased academic and socioeconomic success see Deary et al., 2007; Strenze, 2007), along with other negative predictors of fitness (e.g., fluctuating asymmetries such as ear, digit, or wrist asymmetries; Manning et al., 1997) which have been related to atypical brain asymmetries (Thoma et al., 2002) and left-handedness itself (Kobyliansky and Micle, 1986).

TABLE 1 | Table summarizing the double interaction of left-cradling bias (LCB) functions from the perspective of mother and infant.

Mother	Infant
Monitoring the infant through the left visual and auditory fields.	Exposure to the mother's left-hemiface.
Exposure to the infant's left-hemiface.	Monitoring the mother through the left visual and auditory fields.

CONCLUSION

We propose the idea that human caregivers play a canalizing role during a sensitive period of developmental plasticity *via* their own lateralized motor patterns. These would give rise in the infant to lateralized experiences in multiple sensory

modalities, due to the bidirectional nature of interactive behavior at very close contact. Of all biases, the case of cradling would be extremely interesting to examine with such an approach because its obligatory and simple nature could qualify it as a major epigenetic determinant of neural lateralization. Moreover, the LCB could be the access point to a wider pattern of lateralized adult-infant interactive and social behaviors (embracing, caressing, kissing, cuddling, tickling, whispering, et cetera) acting as epigenetic niches for typical development. Further studies are needed to establish associations among the lateralized experience provided by those interactive behaviors, hemispheric asymmetries, and motor, cognitive and socioemotional development. Given the role of the attachment system as a regulator of proximity seeking (Simpson and Belsky, 2008), and the previous evidence linking the cradling side to attachment in adults (Malatesta et al., 2019a), a major target should be the search for links among the observed patterns of infant attachment and the aforementioned motor, neural and developmental variables. Furthermore, cradling behavior has coevolved with the infant's proclivity to actively cling onto the caregiver (Berecz et al., 2020), and being held or carried on the left or the right side of the adult's body imposes complementary degrees of freedom on the infant's left and right upper limbs. Thus, a direct effect of adult-infant postural laterality is expected to be manifested in the differential use of arms and hands by the infant. More specifically, it is possible to predict that left-sided cradling favors the development of right-handedness in the infant, an effect already assessed in nonhuman primates (Hopkins, 2004) and investigated only partially in humans (Scola and Vauclair, 2010).

Based on the state-of-the-art on the cradling-, embracing- and kissing-side bias research, a better understanding of the adaptive role of these behavioral asymmetries appear desirable to verify their potential function. For example, although research carried out since 1960 has examined the possible correlations between typical/atypical cradling lateralization and several variables in different populations, we do not know much about its association with typical brain organization and increased fitness, and the possible outcomes on the offspring of being cradled on the left or the right during infanthood. Compared to other asymmetrical patterns of brain organization (e.g., handedness), cradling behavior necessarily involves the joint participation of two individuals: one cradling and another being cradled. In this regard, it is plausible that lateral cradling preferences are strongly associated with affective functioning, which is known to be strongly impaired in disorders such as autism, schizophrenia, and alexithymia (Tordjman, 2008).

To conclude, this perspective aims to encourage the detailed study of the nature and effects of the motor and sensory lateral biases expressed in the context of adult-infant interactive behavior. Due to the difficulties in directly manipulating such a dyadic interaction to show possible causal effects in humans, the involvement of animal models might be a useful approach (Manning et al., 1994; Karenina et al., 2017; Giljov et al., 2018; Boulinguez-Ambroise et al., 2020). Moreover, the lateral preference stability over time has received little attention to date, with conflicting findings (Dagenbach et al., 1988; Manning, 1991; Scola et al., 2013; Todd and Banerjee, 2016; Malatesta et al., 2020a). Therefore, the dynamics and spatiotemporal progression of the active and passive biases of the dyad over time should be investigated with a microgenetic approach, and their directionality and strength should be associated with longitudinal assessments of hemispheric asymmetries, cognitive development, and the pattern of attachment between parent and infant.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

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

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Neurogenesis Potential Evaluation and Transcriptome Analysis of Fetal Hypothalamic Neural Stem/Progenitor Cells With Prenatal High Estradiol Exposure

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High maternal estradiol is reported to induce metabolic disorders by modulating hypothalamic gene expression in offspring. Since neurogenesis plays a crucial role during hypothalamus development, we explored whether prenatal high estradiol exposure (HE) affects proliferation and differentiation of fetal hypothalamic neural stem/progenitor cells (NSC/NPCs) in mice and performed RNA sequencing to identify the critical genes involved. NSC/NPCs in HE mice presented attenuated cell proliferation but increased neuronal differentiation *in vitro* compared with control (NC) cells. Gene set enrichment analysis of mRNA profiles indicated that genes downregulated in HE NSC/NPCs were enriched in neurogenesis-related Gene Ontology (GO) terms, while genes upregulated in HE NSC/NPCs were enriched in response to estradiol. Protein-protein interaction analysis of genes with core enrichment in GO terms of neurogenesis and response to estradiol identified 10 Hub mRNAs, among which three were potentially correlated with six differentially expressed (DE) lncRNAs based on lncRNA profiling and co-expression analysis. These findings offer important insights into developmental modifications in hypothalamic NSC/NPCs and may provide new clues for further investigation on maternal environment programmed neural development disorders.

Keywords: prenatal exposure, estradiol, neural stem/progenitor cells, neurogenesis, RNA sequencing, gene set enrichment analysis, protein-protein interaction, interaction network

INTRODUCTION

The theory that the intrauterine environment can influence prenatal development and the future health of offspring (Bateson et al., 2004) has resulted in increased interest in the developmental origin of chronic disease. Ovulation induction clinically used in assisted reproductive technology generates a supraphysiologic level of blood estradiol, which may predispose offspring to an abnormal intrauterine environment after fresh embryo transfer (Hu et al., 2014).

Our previous study reported a programming effect by high maternal estradiol during early pregnancy on the hypothalamic glucoregulatory system of male mouse offspring, which induces adult metabolic disorders (Wang et al., 2018). This discovery indicates that high prenatal estradiol levels alter hypothalamus development, though the underlying mechanism is not yet well understood.

Neural stem/progenitor cells (NSC/NPCs) generate neurons during a process called neurogenesis (Bond et al., 2015). In the central nervous system (CNS), neurogenesis begins in the embryonic stage and continues throughout life. NSC/NPCs are preprogrammed to form specific types of functional neurons even before birth (Fuentealba et al., 2015); therefore, the study of prenatal neurogenesis may help researchers understand the mechanisms underlying adult neural disorders.

The hypothalamus regulates the metabolic homeostasis of the whole body and is sensitive to adverse prenatal environment (Ralevski and Horvath, 2015), so the development of hypothalamic neurons could affect metabolism in later life. Hypothalamic NSC/NPCs are first generated between embryonic day 10.5 (E10.5) and E14.5 in rodents (Padilla et al., 2010), and can proliferate to form neurospheres and differentiate into neurons *in vitro* (Desai et al., 2011). Substantial evidence has shown that dysfunctions of hypothalamic NSC/NPCs are associated with metabolic disorders, such as obesity and glucose intolerance (Li et al., 2012, 2014; Livesey, 2012), but the corresponding whole genomic features of these NSC/NPCs are rarely reported.

RNA sequencing (RNA-Seq) is an effective approach to revealing genome alterations which provides expression information for all transcripts, including mRNAs and non-coding RNAs. LncRNAs may play a considerable regulatory role by interacting with mRNAs, so exploring the link between them may provide more clues for elucidating molecular pathways. Gene set enrichment analysis (GSEA) is a robust and tractable analytical method for interpreting RNA-Seq data, as it can detect differential enrichment of biological functions across an entire network of genes (Subramanian et al., 2005), without the limitations associated with the single-gene method.

Because a prenatally programmed adult hypothalamic disorder resulting from high maternal estradiol has been identified (Wang et al., 2018), here we explore whether neurogenesis potential is affected in fetal hypothalamic NSC/NPCs and attempt to identify the key lncRNA-mRNA network through integrated bioinformatic analysis. These findings may help us to understand molecular modifications of fetal hypothalamic neurogenesis resulting from an adverse intrauterine environment.

MATERIALS AND METHODS

Animal Model and 5-Bromodeoxyuridine Labeling in Fetal Brain

A mouse model of prenatal high estradiol exposure was created based on our previously published method (Wang et al., 2018). Briefly, 8-week-old pregnant C57BL/6 mice received 100 μg/kg/d estradiol valerate [Sigma; high estradiol (HE) group] or an equal amount of blank solvent [corn oil; control (NC) group] *via* gavage from E5.5 to E11.5. For bromodeoxyuridine (BrdU) labeling, pregnant mice at E14.5 received a single intraperitoneal injection of 100 mg/kg BrdU (Sigma) 2 h before euthanized; then, the fetuses were extracted and decapitated. Male fetuses were identified by visual identification of testes next to the bladder using a dissection microscope. The brains of male fetuses were removed and fixed in 4% paraformaldehyde (PFA) for 24 h and then infiltrated with 20–30% sucrose. Brain sections of 20 μm were made using a freezing microtome (Leica) for immunofluorescence staining.

Tissue Immunofluorescence

Brain sections were blocked with 5% bovine serum albumin/0.3% Triton X-100 for 1 h at room temperature and incubated with primary antibodies mouse anti-Nestin (1:200, Millipore, catalog no. MAB353) and rabbit anti-BrdU (1:100, Abcam, catalog no. ab152095) overnight at 4°C, followed by reaction with secondary antibodies anti-rabbit Alexa Fluor 488 (1:200, Invitrogen, catalog no. A-11008) and anti-mouse Alexa Fluor 594 (1:200, Invitrogen, catalog no. A-11005) for 2 h at room temperature before counterstaining with 4',6-diamidino-2-phenylindole. The $BrdU^+Nestin^+$ cells were counted in five serial sections across the hypothalamus in each mouse.

NSC/NPC Isolation and Neurosphere Assay

The brains of E14.5 male fetuses were dissected quickly on ice to remove the hypothalami, which were then fragmented in Neurobasal-A (Gibco), digested with TrypLE (Gibco) in 37°C for 15 min, and gently triturated into single cells with tips. The cells were then washed twice in Neurobasal-A and suspended in a proliferation medium containing Neurobasal-A, 2% B27 (Gibco), 10 ng/ml EGF (PeproTech), 10 ng/ml bFGF (PeproTech), and 1% GlutaMAX (Gibco), seeded in ultralow adhesion 6-well plates at a density of 10^5/ml (Li et al., 2012), and incubated in 5% CO_2 at 37°C. The neurospheres were photographed under a microscope for 4 days (Marshall et al., 2007), and the number was counted and diameter measured using the software ImageJ on the fourth day after isolation.

NSC/NPC Proliferation and Differentiation Assay

To assess the proliferation ability of NSC/NPCs, the primary neurospheres were digested to count single cells and passaged at a density of 10^5/ml in ultralow adhesion 6-well plates. The total cell number in each of the first four passages was calculated based on the assumption that all of the cells from the previous passage were replated. For the BrdU incorporation assay, primary NCS/NPCs were plated on Matrigel (BD)-coated coverslips at a density of 10^5/ml in proliferation medium and cultured for 24 h. Then, the cells were treated with 10 μm BrdU for 2 h before immunofluorescence staining.

For induced differentiation, primary NSC/NPCs were seeded as single cells at a density of 3×10^5/ml in Matrigel-coated coverslips placed in 24-well plates in differentiation medium containing Neurobasal-A, 2% B27, 1% fetal bovine serum (Gibco), and 1 μm retinoid acid (Sigma). The medium was changed every second day for 10 days, and then, the coverslips were removed to receive immunofluorescence detection of target neuron markers.

NSC/NPC Immunofluorescence

For immunofluorescence staining of NSC/NPCs, neurospheres were moved using tips to seed on Matrigel-coated coverslips for 20 min before detection, and cells on coverslips were fixed with 4% PFA for 15 min and blocked with 5% bovine serum albumin/0.3% Triton X-100 for 1 h at room temperature. Cells were then incubated with primary antibodies rabbit anti-Sox2 (1:400, Cell Signaling Technology, catalog no. 23064), mouse anti-Nestin (1:200, Millipore, catalog no. MAB353), rabbit anti-BrdU (1:100, Abcam, catalog no. ab152095), or mouse anti-Tuj1 (1:200, Cell Signaling Technology, catalog no. 4466) overnight at 4°C and with secondary antibodies anti-rabbit Alexa Fluor 488 (1:200, Invitrogen, catalog no. A-11008), anti-mouse Alexa Fluor 594 (1:200, Invitrogen, catalog no. A-11005), or anti-mouse Alexa Fluor 488 (1:200, Invitrogen, catalog no. A-11001) for 2 h at room temperature before counterstaining with 4',6-diamidino-2-phenylindole. The BrdU/Nestin and Tuj1 positive cells were counted in each group.

RNA-Seq Analysis

Three samples of first-passage NC and HE NSC/NPCs were harvested separately, each containing cells obtained from two mice. RNA was extracted with TRIzol (Invitrogen), its quality valued by spectrophotometer, and its integrity checked by Agilent 2,100 bioanalyzer. Total RNA was enriched by oligo beads, fragmented into small pieces, and reverse transcribed into cDNA. Second-strand cDNA was synthesized by DNA polymerase I with dUTP to construct a strand-specific library. The cDNA was then purified, end-repaired, poly A-added, and ligated to Illumina adaptor. The libraries were size-selected by agarose gel electrophoresis, PCR-amplified, and sequenced by Illumina NextSeq 500 by Personal Bio Co. (Shanghai, China).

The raw RNA-Seq data were filtered by removing low-quality and adaptor-related reads. The clean reads were then aligned to the mouse reference genome (10 mm) using Tophat2. Coding and non-coding transcripts were distinguished by Coding Potential Calculator, Coding-Non-Coding Index, and Pfam-scan. Non-coding RNAs with length >200 nt and exon number ≥2 were considered to be lncRNAs. Expression values were expressed as reads per kilobase per million reads. Differential expression analysis was conducted using DESeq2 (Love et al., 2014). LncRNAs and mRNAs with a $\log_2$ (fold change) ≥1 or ≤−1 and FDR < 0.05 were considered differentially expressed.

Bioinformatics Analysis

We conducted enrichment analyses using GSEA with the standard procedure obtained from the GSEA Web site.[1] The number of permutations was set to 1,000, and FDR < 0.25 with $p < 0.05$ was considered statistically significant. We downloaded gene sets needed for Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analysis from the GSEA Web site. The enrichment bubble diagrams were made with R software (version 4.0.3).

A protein-protein interaction (PPI) network of selected mRNAs was constructed using STRING 11.0,[2] and the Hub mRNAs [the top 10 nodes ranked by Maximal Clique Centrality (MCC); Chin et al., 2014] were identified using the plugin cytoHubba in Cytoscape software.[3]

To explore the lncRNA-mRNA regulatory network, Pearson's correlation coefficient (PCC) between DE lncRNAs and Hub mRNAs was calculated and plotted using R software, and gene pairs with PCC ≥0.990 or ≤−0.990 and $p < 0.05$ were considered to be potentially correlated. The interaction network was visualized using Cytoscape software.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA of NSC/NPCs was extracted using TRIzol (Invitrogen) and reverse-transcribed into cDNA using Primer Script RT Reagent Kit (Takara) and amplified with QuantiNova SYBR Green PCR Kit (QIAGEN) according to the manufacturer's instructions. The thermocycling conditions were 95°C for 2 min, followed by 40 cycles of 95°C for 5 s and 60°C for 10 s. The primers are listed in **Table 1**. GAPDH was used as an endogenous control, and the relative expression level was analyzed using the $2^{-\triangle\triangle CT}$ method.

Statistical Analysis

Data aside from RNA-Seq and bioinformatics analysis were analyzed using the Statistical Package for Sciences Software, version 21.0 (IBM), and are presented as the mean ± standard error of the mean. Unpaired Student's *t*-tests were used for comparisons between two groups, and $p < 0.05$ was considered statistically significant.

RESULTS

Prenatal High Estradiol Affects Neurogenesis Potential of Fetal Hypothalamic NSC/NPCs

Serum estradiol after gavage in the HE pregnant mice reaches a peak value of four times that of the control group (Wang et al., 2018), forming a high maternal estradiol environment. Fetal brain sections were made on E14.5, and hypothalamic NSC/NPCs were isolated at the same time (**Figure 1A**). Authentic biomarkers Sox2 and Nestin were used to label NSC/NPCs (Suh et al., 2007; Gilyarov, 2008). Tissue immunofluorescence presented decreased number of $BrdU^+Nestin^+$ cells in HE fetal hypothalami after BrdU injection (**Figures 1B,C**), indicating a reduction of

[1]http://www.gsea-msigdb.org/gsea/index.jsp

[2]https://string-db.org

[3]http://www.cytoscape.org

TABLE 1 | Primer sequences for quantitative real-time polymerase chain reaction (qPCR).

Gene	Primer type	Primer sequence
Tbr1	Forward	GCAGCAGCTACCCACATTC
	Reverse	GTCCTTGGAGTCAGGAAAATTGT
Six3	Forward	TCAACAAACACGAGTCGATCC
	Reverse	TGGTACAGGTCGCGGAAGT
Foxg1	Forward	GAAGGCCTCCACAGAACG
	Reverse	CAAGGCATGTAGCAAAAGAGC
Pou3f2	Forward	GCAGCGTCTAACCACTACAGC
	Reverse	GCGGTGATCCACTGGTGAG
Dlx2	Forward	GGCTCCTACCAGTACCACG
	Reverse	GTAGCCCAGGTCGTAGCTG
Fezf2	Forward	GCAAAGGCTTTCACCAAAAA
	Reverse	GCATGTGGAAGGTCAGATTG
Dlx1	Forward	ATGCCAGAAAGTCTCAACAGC
	Reverse	AACAGTGCATGGAGTAGTGCC
Nkx2-1	Forward	ATGAAGCGCCAGGCTAAGG
	Reverse	GGTTTGCCGTCTTTGACTAGG
Sox1	Forward	TTTTCCGGGGTTTACTTCC
	Reverse	GCTCGAGGTCCGTCACTC
Notch1	Forward	TGCCACAATGAGATCGGCTC
	Reverse	GGGCACATAGGGCAGTTCA
Egfr	Forward	ATGAAAACACCTATGCCTTAGCC
	Reverse	TAAGTTCCGCATGGGCAGTTC
Fgfr1	Forward	ACTCTGCGCTGGTTGAAAAAT
	Reverse	GGTGGCATAGCGAACCTTGTA
Fgfr2	Forward	GCCTCTCGAACAGTATTCTCCT
	Reverse	ACAGGGTTCATAAGGCATGGG
Fgfr3	Forward	CCGGCTGACACTTGGTAAG
	Reverse	CTTGTCGATGCCAATAGCTTCT
Fgfr4	Forward	GCTCGGAGGTAGAGGTCTTGT
	Reverse	CCACGCTGACTGGTAGGAA

proliferating NSC/NPCs *in vivo*. Immunofluorescence staining of Sox2 and Nestin in neurospheres was performed for NSC/NPC identification after cell isolation (**Figure 1D**). The neurosphere assay showed a decreased amount of neurospheres with shorter average diameters in HE NSC/NPCs compared with NC on the fourth day (D4) of the first passage (**Figures 1E,F**), and the proliferation curve presented the accumulated NC NSC/NPC number significantly exceeded that in the HE group from the second to fourth passage (P2 to P4; **Figure 1G**), indicating attenuated proliferation ability in HE NSC/NPCs. We also performed BrdU incorporation assay in primary NSC/NPCs and found the decreased proportion of proliferating NSC/NPCs *in vitro* in HE group (**Figures 1H,I**).

NSC/NPCs from two groups were induced to differentiate into neurons, and the neuronic marker Tuj1 (Lee et al., 1990) was stained for quantification. The results showed neurons formed in both groups after a 10-day induction (**Figure 1J**); however, in contrast with proliferation assay, the number of neurons significantly increased in HE NSC/NPCs (**Figure 1K**).

Transcriptional Analysis of NSC/NPCs Reveals Hub mRNAs Involved in Neurogenesis

To elucidate the transcriptional changes related to altered neurogenesis, we compared the transcriptional profile of HE and NC NSC/NPCs by RNA-Seq. Heatmap of mRNAs showed distinctly different clustering between NSC/NPCs from two groups (**Figure 2A**). mRNAs with a $\log_2$ (fold change) ≥ 1 or ≤ -1 and FDR < 0.05 were considered DE mRNAs, the volcano plot presented a total of 117 DE mRNAs, including 45 upregulated and 72 downregulated in HE NSC/NPCs compared with NC (**Figure 2B**). We conducted GSEA afterward, aiming to find neurogenesis-related gene sets (**Figures 2C–F**). The results revealed that genes downregulated in HE NSC/NPCs were enriched in neurogenesis-related GO biological processes (BP), such as neuroblast division, neuroblast proliferation, stem cell division, and neuron fate commitment, while genes upregulated in HE NSC/NPCs were enriched in response to estradiol (**Figure 2D**). The enrichment plots of these gene sets are presented in **Figure 2G**.

To further explore key mRNAs in the gene sets above and their interactions, mRNAs with core enrichment in each gene set (found in GSEA details) were picked for PPI analysis in STRING followed by Hub gene identification using Cytoscape. The top 10 genes ranked by MCC score were identified as Hub mRNAs, including *Tbr1*, *Six3*, *Foxg1*, *Pou3f2*, *Dlx2*, *Fezf2*, *Dlx1*, *Nkx2-1*, *Sox1*, and *Notch1* (**Figure 2H**).

To validate the mRNA profiling and Hub gene identification results, the expression of the Hub mRNAs above was screened by quantitative real-time polymerase chain reaction with NSC/NPC samples used in RNA-Seq (six NC mice and six HE mice). The relative gene expression indicated all 10 Hub mRNAs decreased in the HE NSC/NPCs compared to the NC group and were identical to expression trends in RNA-Seq (**Figure 3**).

Identification of DE lncRNA-Hub mRNA Interaction Network

Epigenetic modification is recognized to regulate early life neurodevelopment (LaSalle et al., 2013; Yao et al., 2016), and dysregulation of lncRNAs leads to impaired development or neural dysfunction (Ng et al., 2013); therefore, we investigated whether lncRNA profiles were affected in NSC/NPCs with prenatal high estradiol stimulation. The resultant heatmap showed separated clustering of lncRNA transcripts in two groups (**Figure 4A**). Transcripts with a $\log_2$ (fold change) ≥ 1 or ≤ -1 and FDR < 0.05 were considered DE lncRNA transcripts, and the volcano plot showed a total of 85 DE lncRNA transcripts, including 58 upregulated and 27 downregulated transcripts in the HE NSC/NPCs compared with the NC group (**Figure 4B**). The correlation between these DE lncRNA transcripts and the Hub mRNAs identified above were evaluated by constructing an expression matrix and calculating the PCC of each gene pair (**Figure 4C**). Gene pairs with a PCC ≥ 0.990 or ≤ -0.990 and $p < 0.05$ were considered potentially correlated (**Figure 4D**). The co-expression network of these correlated genes was constructed (**Figure 4E**), including 6 lncRNA transcripts (ENSMUST00000037953, ENSMUST00000136217, ENSMUST00000138077, ENSMUST00000145804, ENSMUST 00000170557, and ENSMUST00000189763) and 3 Hub mRNAs (*Sox1*, *Fezf2*, *Foxg1*). Among them, ENSMUST00000189763, ENSMUST00000037953, and ENSMUST00000145804 were

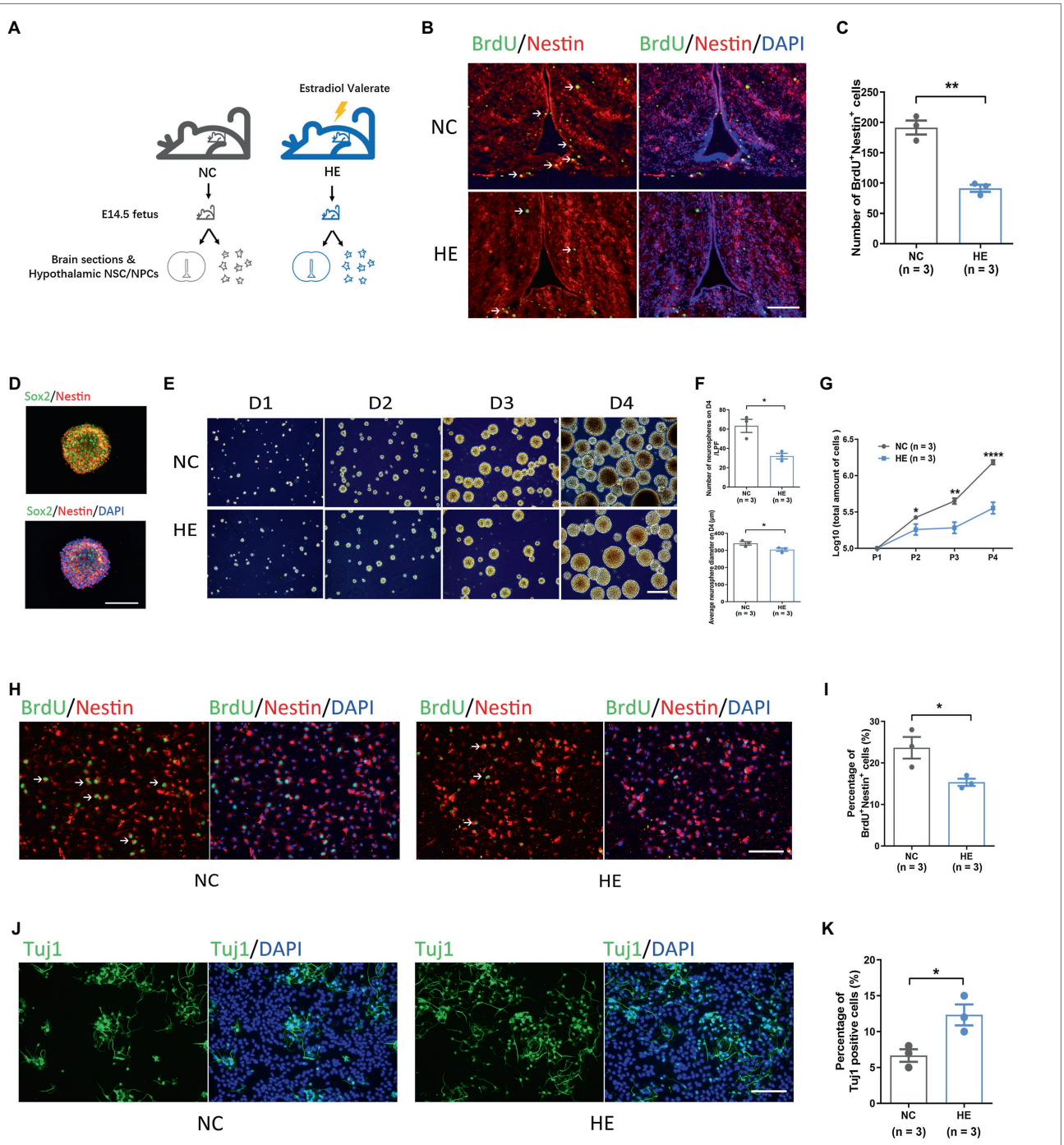


FIGURE 1 | Isolation and evaluation of fetal hypothalamic neural stem/progenitor cell (NSC/NPC) neurogenesis potential in a mouse model. **(A)** Schematic of the method used to generate fetal brain sections and hypothalamic NSC/NPCs in a mouse model. **(B)** Representative images of bromodeoxyuridine (BrdU) and Nestin immunofluorescence in fetal hypothalamic tissue; scale bar: 200 μm. Arrows show $BrdU^+Nestin^+$ cells. **(C)** Quantification of $BrdU^+Nestin^+$ cells in five serial fetal hypothalamic tissue sections (n = 3 mice per group). **(D)** Representative images of Sox2 and Nestin immunofluorescence in neurospheres; scale bar: 100 μm. **(E)** Representative images of neurosphere formation across 4 days in the two experimental groups; scale bar: 400 μm. **(F)** Top: quantification of neurospheres on D4 of passage in two groups (n = 3 mice per group). Bottom: measurement of average diameters of neurospheres on D4 of passage in two groups (n = 3 mice per group). **(G)** Accumulated cell number of NSC/NPCs from P1 to P4 in the two experimental groups (n = 3 mice per group). **(H)** Representative images of BrdU and Nestin immunofluorescence in NSC/NPCs; scale bar: 100 μm. Arrows show $BrdU^+Nestin^+$ cells. **(I)** Quantification of $BrdU^+Nestin^+$ cells (%) in NSC/NPCs (n = 3 mice per group). **(J)** Representative images of Tuj1 immunofluorescence in neurons differentiated *in vitro* in the two experimental groups; scale bar: 100 μm. **(K)** Quantification of Tuj1 positive cells (%) in the two experimental groups (n = 3 mice per group). Error bars represent the standard error of the mean. Significance was determined by Student's t-test. $^*p < 0.05$; $^{**}p < 0.01$; and $^{****}p < 0.0001$.

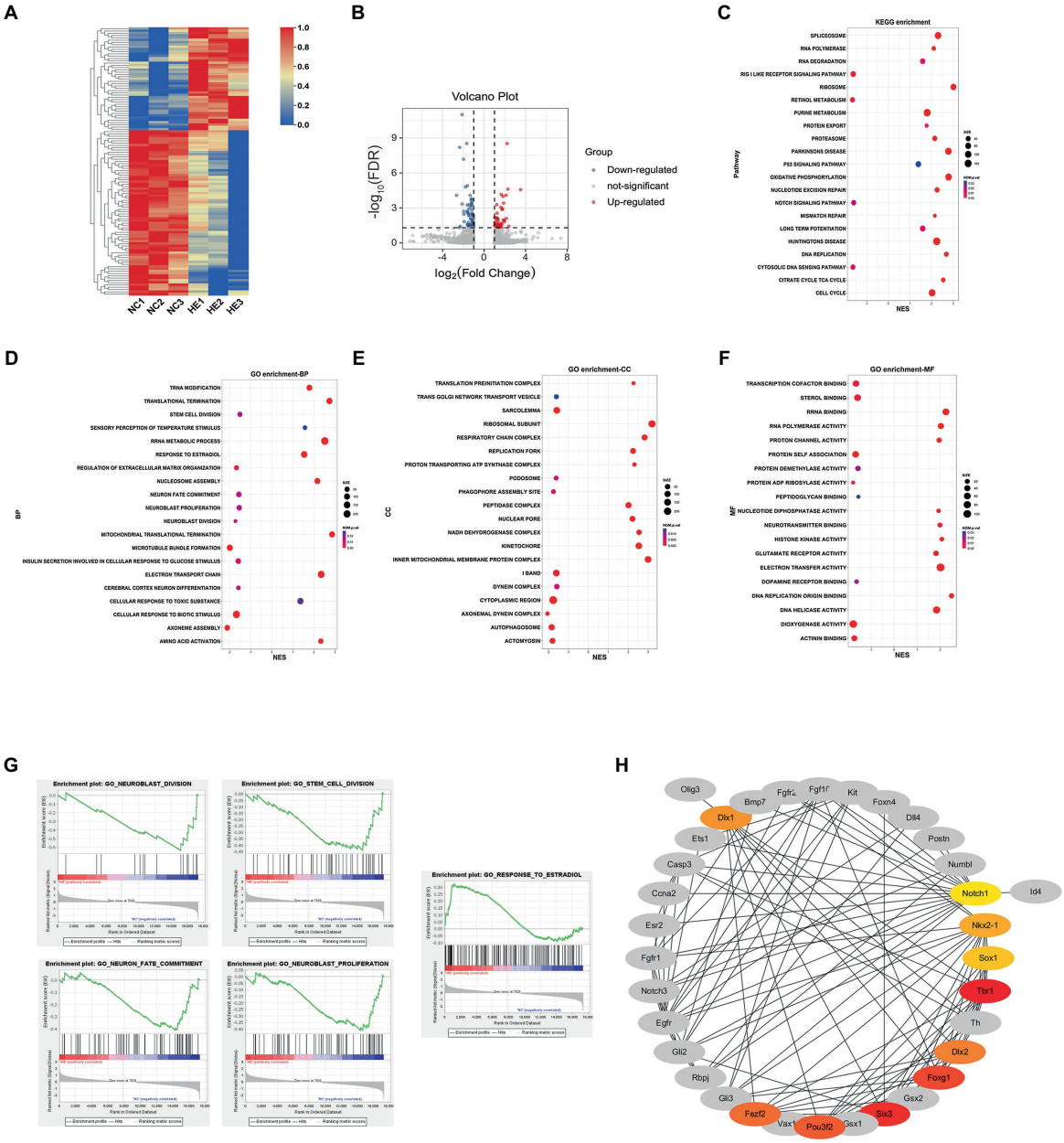


FIGURE 2 | mRNA profiling and Hub mRNA identification. **(A)** Heatmap of 117 DE mRNAs in HE NSC/NPCs compared to NC. Red indicates upregulation, and blue indicates downregulation; row scale is from 0 to 1. **(B)** Volcano plot of DE mRNAs in HE NSC/NPCs compared with NC, red dots represent 45 upregulated mRNAs, and blue dots represent 72 downregulated mRNAs. **(C)** Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment results from gene set enrichment analysis (GSEA). The *x*-axis represents normalized enrichment score (NES), and the *y*-axis represents KEGG terms. The size of the dot indicates gene count, and the color indicates normalized value of *p*. Positive and negative NES indicate upregulation and downregulation in HE. **(D-F)** GO BP, CC (cellular component), and MF (molecular function) enrichment results from GSEA. The *x*-axis represents NES, and the *y*-axis represents GO terms. The size of the dot indicates gene count, and the color indicates normalized value of *p*. Positive and negative NESs indicate upregulation and downregulation in HE, respectively. **(G)** The enrichment plot of gene sets involved in neurogenesis and estradiol response. **(H)** Protein-protein interaction analysis of genes with core enrichment and Hub mRNA identification. Colored nodes indicate Hub mRNAs, with their shade positively correlated with the Maximal Clique Centrality score.

upregulated and the rest were downregulated. Therefore, we estimated these lncRNA-mRNA interactions may play a part in mediating the less proliferative and more neurogenic potential of fetal hypothalamic NSC/NPCs resulting from high maternal estradiol exposure.

DISCUSSION

Mounting evidence suggests that an adverse intrauterine hormonal environment could impair the health of offspring. High maternal estradiol is usually induced by ovarian stimulation

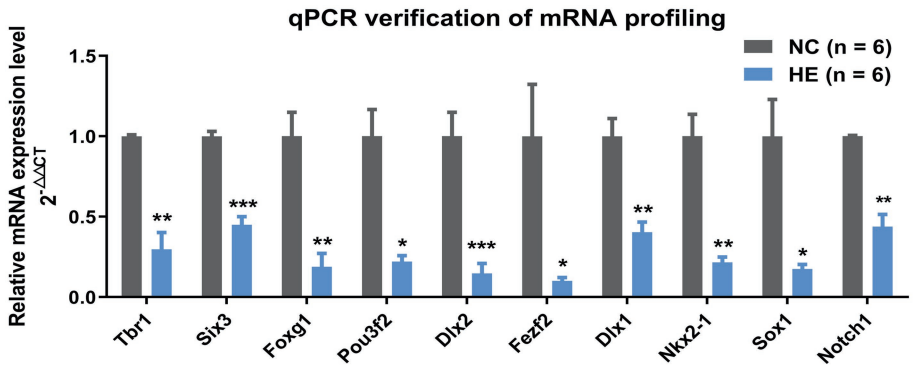


FIGURE 3 | Verification of selected mRNAs by qPCR. Fold change of 10 Hub mRNAs in HE NSC/NPCs compared with NC (n = 6 mice per group). Significance was determined by Student's t-test. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

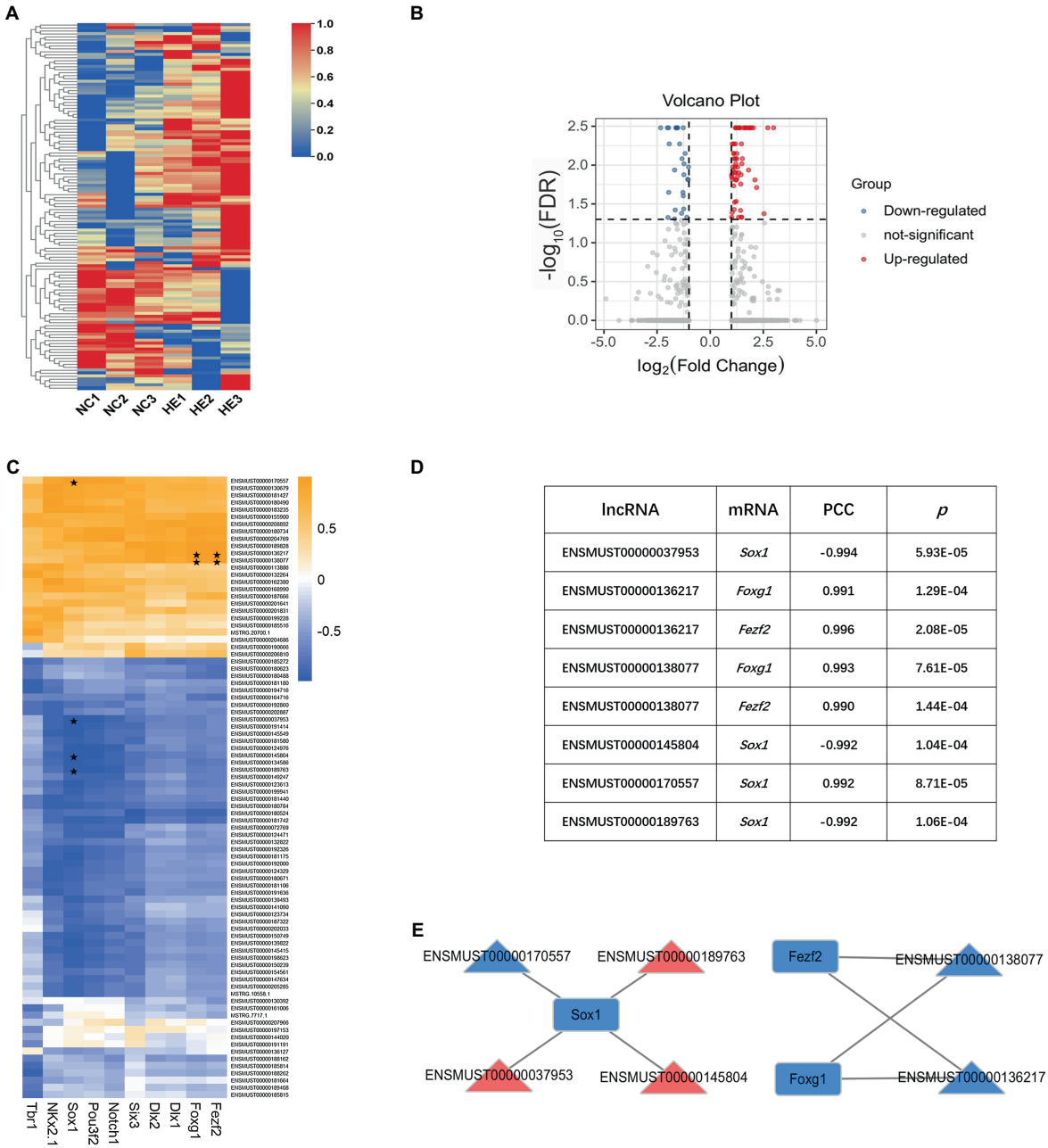
in assisted reproductive technology, and it can disrupt neurodevelopment, resulting in metabolic disorders and diminished verbal abilities in offspring (Wang et al., 2018; Zhou et al., 2020). In our previous study, high maternal estradiol led to insulin resistance and disordered eating in mouse offspring due to decreased insulin receptor and elevated neuropeptide Y expression in the hypothalamus (Wang et al., 2018). That these alterations were detected postnatally prompted us to search for corresponding events in earlier developmental stages.

Because fetal NSC/NPCs proliferate and differentiate actively, they are vulnerable to exogenous stimulators like high maternal estradiol, which can pass through the placental barrier (Gude et al., 2004) and may directly affect their biological properties. We isolated primary neural stem cells from the fetal hypothalamus and cultured them as neurospheres. Actually, neurospheres may derive from mixed cells with dynamic markers, and even the purified cells remain heterogeneous (Pastrana et al., 2011), resulting in limitations in neurosphere assay if applied alone. Therefore, we examined proliferation in Sox2 and Nestin-positive cells both *in vivo* and *in vitro*, which are recognized to be NSCs and NPCs (Bani-Yaghoub et al., 2006; Ernst and Christie, 2006; Hernandez et al., 2007; Campbell et al., 2015). Since the vast majority of the primary cells were Sox2 and/or Nestin positive, as shown in **Figures 1D,H**, we consider the researches were performed mainly in the same population of cells in two groups. Although a small proportion of other type of cells might exist, their effect on gene expression appeared insignificant when compared with NCS/NPCs. In consideration of the limitations of our study, we believe single-cell RNA-Seq should make a more precise method in future research. In fact, the cell composition in neurospheres would possibly change during different passages, and we speculate that the diminished proliferation might be a continuation of properties in primary NSC/NPCs, and further studies are expected to reveal whether increased cell apoptosis and senescence could occur.

The proliferation medium contained EGF and bFGF, which are essential factors for NSC/NPC growth. To explore whether cell proliferation changed due to different levels of EGF and bFGF receptors, we examined the mRNA expressions of them in primary NSC/NPCs and found no significant difference between the two groups (**Supplementary Figure S1**). This result indicated that the decreased proliferation in HE NSC/NPCs was not attributed to different levels of growth factor receptors in the culture medium, but more likely to the intrauterine programming effect.

Neurogenesis is a complex process, and how NSC/NPCs are maintained, divided, and differentiated remain controversial (Lazutkin et al., 2019). Our results indicated decreased proliferative activity and increased neuronal production in HE NSC/NPCs; however, whether or not this resulted from premature exhaustion of the stem cell pool requires further study. A comprehensive evaluation of hypothalamic neurogenesis from the prenatal period to adulthood may show us a more precise effect of high maternal estradiol on neurodevelopment. It should be noted that the NSC/NPC differentiation assay was carried out *in vitro*, which generates early and immature neurons rather than functional neurons, and an *in vivo* labeling of NSC/NPCs at the embryonic stage followed by detections like lineage tracing may provide a more accurate indication of their differentiation directions.

Several published studies show that estrogen stimulates both proliferation and differentiation of NSC/NPCs (Okada et al., 2010; Li et al., 2020) and attenuates damage to neurogenesis in the developing brain caused by chemical drug exposure (Li et al., 2019); however, another study shows that 10 nM estradiol increases NSC/NPCs proliferation and stimulates differentiation into neurons *in vitro*, but 50 nM estradiol markedly decreases NSC/NPCs proliferation (Zhang et al., 2019a). Thus, the effects of maternal estradiol on neurogenesis of fetal hypothalamic NSC/NPCs may be dose dependent, and the dose that caused metabolic disorder in our mouse model exerted a different effect on proliferation and differentiation. The stimulative effect on neuron formation may explain our previous finding that hypothalamic neuropeptide Y increases in HE offspring (Wang et al., 2018); that is, prenatal high estradiol probably promotes orexigenic neuron generation, leading to disordered eating.



lncRNA	mRNA	PCC	*p*
ENSMUST00000037953	*Sox1*	-0.994	5.93E-05
ENSMUST00000136217	*Foxg1*	0.991	1.29E-04
ENSMUST00000136217	*Fezf2*	0.996	2.08E-05
ENSMUST00000138077	*Foxg1*	0.993	7.61E-05
ENSMUST00000138077	*Fezf2*	0.990	1.44E-04
ENSMUST00000145804	*Sox1*	-0.992	1.04E-04
ENSMUST00000170557	*Sox1*	0.992	8.71E-05
ENSMUST00000189763	*Sox1*	-0.992	1.06E-04

FIGURE 4 | LncRNA profiling and DE lncRNA-Hub mRNA interaction network construction. **(A)** Heatmap of 85 DE lncRNA transcripts in HE NSC/NPCs compared to NC. Red indicates upregulation, and blue indicates downregulation; row scale is from 0 to 1. **(B)** Volcano plot of DE lncRNA transcripts in HE NSC/NPCs compared to NC. Red dots represent 58 upregulated transcripts, and blue dots represent 27 downregulated transcripts. **(C)** Heatmap of DE lncRNA-Hub mRNA Pearson's correlation coefficient (PCC) score. Orange indicates positive PCC, and blue indicates negative PCC. PCC ≥0.990 or ≤−0.990 and $p < 0.05$ are labeled with ★. **(D)** List of correlated DE lncRNA transcript-Hub mRNA pairs, with their PCC and value of *p*. **(E)** The interaction network of six DE lncRNA transcripts and three Hub mRNAs. Red indicates upregulation, and blue indicates downregulation in HE NSC/NPCs compared to NC. Triangles represent lncRNAs, and rectangles represent mRNAs.

We used $\log_2$ (fold change) ≥1 or ≤−1 and FDR < 0.05 as the cutoffs to define DE genes in our study, and looser criteria of $\log_2$ (fold change) ≥1 or ≤−1 with $p < 0.05$ were also tried to identify DE genes and predict DE lncRNA-Hub mRNA regulatory network. This method presented 567 DE mRNAs (383 upregulated and 184 downregulated) and 148 DE lncRNA transcripts (89 upregulated and 59 downregulated) in HE NSC/NPCs compared with NC group (**Supplementary Figures S2A–D**). In spite of this, the potentially correlated DE lncRNA-Hub mRNA pairs remained

unchanged compared with those marked in **Figure 4C** (**Supplementary Figure S2E**).

Gene set enrichment analysis (GSEA) revealed both enrichment and expression of mRNA profiles in two groups; positive NES indicated upregulation in HE compared with NC NSC/NPCs, and negative NES indicated downregulation. Although KEGG enrichment did not reveal items directly involved in cell proliferation and differentiation, it showed differential enrichment of cell cycles, DNA replication, nucleotide excision repair, and RNA polymerase. Moreover, we found upregulated mRNAs that are enriched in Huntington's and Parkinson's disease in the HE NSC/NPCs, suggesting an increased risk of neurodegenerative disease in offspring exposed to high maternal estradiol, which requires further validation. The GO CC and MF enrichment revealed downregulated genes enriched in autophagosome and dopamine receptor binding, and upregulated genes enriched in neurotransmitter binding and glutamate receptor binding in HE NSC/NPCs. These discoveries may help illuminate the mechanisms of maternal estradiol-induced neurodevelopmental disorders.

The GO BP enrichment result revealed downregulated genes in HE NSC/NPCs enriched in stem cell division, neuroblast proliferation, and neuroblast division, which consisted with their less proliferative potential observed. However, genes enriched in neuron fate commitment also decreased in HE NSC/NPCs, which seemingly contradicted the more neurogenic activity *in vitro*. To find a rational explanation for this, we focused on Hub mRNAs of core enrichment in these gene sets.

The Hub mRNAs were identified based on a PPI network consisting of genes enriched in neurogenesis and response to estradiol; however, the top 10 Hub mRNAs ranked by MCC in Cytoscape were all genes involved in neurogenesis, and they were all downregulated in HE NSC/NPCs. This result may be explained by the fact that Hub genes are highly connected genes in a co-expression network, and genes enriched in response to estradiol failed to present such close connections with those enriched in altered neurogenesis, implying a probable indirect effect of estradiol stimulation on neurogenesis in our study. Most of these Hub mRNAs are transcription factors except *Notch1*. *Pou3f2* influences multiple stages of neurogenesis by promoting neural transcription factor *Tbr1* (Dominguez et al., 2013), which regulates cell differentiation and migration and involves glutamatergic neurogenesis (Mihalas and Hevner, 2017). *Six3*, *Foxg1*, and *Sox1* maintain the balance between proliferation and neuronal differentiation of NSC/NPCs. Specifically, upregulation of *Six3* plays a role in keeping NSC/NPCs in an undifferentiated state (Appolloni et al., 2008); *Foxg1* deficiency leads to premature differentiation of neurons, and its overexpression increases the NSC/NPC pool (Hanashima et al., 2004; Brancaccio et al., 2010); and *Sox1* loss induces depletion of proliferating NSC/NPCs with increased cell cycle exit (Bylund et al., 2003). *Dlx1* and *Dlx2* drive GABAergic neuron generation (Lindtner et al., 2019; Barretto et al., 2020), and *Fezf2* is involved in the dopaminergic neuron generation (Eckler and Chen, 2014); moreover, knockdown of *Fezf2* leads to decreased *Foxg1* and *Six3* in mouse embryonic stem cells (Wang et al., 2011). *Notch1* signaling is reported to promote NSC/NPC proliferation but decrease neuronal differentiation during meningitis and spinal cord injury (Peng et al., 2019; Zhang et al., 2019b). *Nkx2-1* is a critical factor maintaining the anorectic gene *Pomc* expression from early development to adulthood (Orquera et al., 2019). To sum up, the published information above supports our findings that decreased *Six3, Foxg1*, *Sox1*, *Fezf2*, and *Notch1* in HE NSC/NPCs directly correlated with decreased proliferation and enhanced neuronal generation, which probably reflected a premature differentiation. As the upstream regulator of *Pomc*, decreased *Nkx2-1* in HE offspring could contribute to the verified orexigenic phenotype in later life (Wang et al., 2018). Since *Pou3f2*, *Tbr1*, *Dlx1*, and *Dlx2* are involved in the generation of glutamatergic or dopaminergic or GABAergic neurons, figuring out the neuron types that these NSC/NPCs tended to form would help validate the effect on neurogenesis of these genes in our experiment.

The published studies fail to specify the effect of estradiol on Hub mRNAs above during neurogenesis. One research shows estradiol stimulation does not affect *Notch1* expression during hippocampus development but reduces the level of its transcriptionally active domain (Bender et al., 2010). Since we previously found DNA methylation programs hypothalamic gene expression in HE offspring (Wang et al., 2018), it may support the hypothesis that expression changes of these Hub mRNAs could be attributed to epigenetic regulators, such as DNA methylation and non-coding RNAs.

LncRNAs are important components of regulatory networks in CNS development whose dysregulation leads to neurological disorders (Ng et al., 2013). Since lncRNAs exert functions mainly through regulating mRNA expression, we constructed the interaction network between DE lncRNAs and Hub mRNAs, aiming to discover lncRNAs possibly connected with the altered neurogenesis. Our study predicted six DE lncRNA transcripts correlated with three Hub mRNAs based on their expression level. These lncRNA transcripts (ENSMUST00000037953, ENSMUST00000136217, ENSMUST00000138077, ENSMUST00000145804, ENSMUST00000170557, and ENSMUST00000189763) are coded by genes *2810032G03Rik*, *Prdm16os*, *Gm13110*, *Ppp1r18os*, *Gm17035*, and *D130058E05Rik*, respectively, according to Ensemble genome browser.[4] We also checked the expression correlations between DE lncRNAs and all mRNAs in **Figure 2H** (mRNAs with core enrichment in neurogenesis and response to estradiol, core mRNAs for short), which predicted a potential regulatory network of 14 DE lncRNA transcripts and 11 core mRNAs (**Supplementary Figures S3A–C**). In consideration of our limited sample size, we repeated the co-expression analysis of these lncRNAs and mRNAs using the pubic dataset GSE65487 in Gene Expression Omnibus, which assessed the RNA profiles of proliferating progenitors, differentiating progenitors and neurons from E14.5 mouse cortex. However, five of DE lncRNAs were not detected in GSE65487 (**Supplementary Figure S3D**), and four of the rest presented potential correlations with three mRNAs each,

[4]http://asia.ensembl.org/index.html

when the cutoffs were set to be PCC ≥0.900 or ≤−0.900 and $p < 0.05$ (PCC ≥0.990 or ≤−0.990 identified no significant correlation; **Supplementary Figures S3E,F**). Although we failed to discover the same lncRNA-mRNA pair as demonstrated in our study by using this public dataset, it still reflected possible connections of these lncRNAs with neurogenesis. Since there are not yet any published literatures about the functions of these lncRNAs, future work focused on their specific roles is expected to help answer questions regarding development-originated neuroendocrine disorders.

In short, our research presents the cytologic changes in early neural development under a high maternal estradiol environment and reveals the corresponding whole genomic features with a prediction of the underlying molecular modifications. This study demonstrates comprehensive information about fetal hypothalamic NSC/NPCs with prenatal high estradiol exposure and contributes to our understanding of the fetal-programmed adult diseases.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in an online repository. The name of the repository and accession number can be found at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE168075.

ETHICS STATEMENT

This animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University.

AUTHOR CONTRIBUTIONS

HW and CZ designed the experiments, collected the data, analyzed the data, and drafted the manuscript. MH, HH, and YS revised the final manuscript. All authors have reviewed the manuscript before submitting it and approved the final 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/fgene.2021.677935/full#supplementary-material

Supplementary Figure S1 | mRNA expression of EGF and bFGF receptors examined by qPCR. Fold change of EGF and bFGF receptor mRNAs in HE NSC/NPCs compared with NC ($n = 6$ mice per group). Significance was determined by Student's *t*-test; ns, not significant.

Supplementary Figure S2 | DE gene identification and DE lncRNA-Hub mRNA interaction network analysis using $\log_2$ (fold change) ≥1 or ≤−1 with $p < 0.05$ as the cutoff. **(A)** Heatmap of 567 DE mRNAs in HE NSC/NPCs compared to NC. Red indicates upregulation, and blue indicates downregulation; row scale is from 0 to 1. **(B)** Volcano plot of DE mRNAs in HE NSC/NPCs compared to NC, red dots represent 383 upregulated mRNAs, and blue dots represent 184 downregulated mRNAs. **(C)** Heatmap of 148 DE lncRNA transcripts in HE NSC/NPCs compared to NC. Red indicates upregulation, and blue indicates downregulation; row scale is from 0 to 1. **(D)** Volcano plot of DE lncRNA transcripts in HE NSC/NPCs compared with NC. Red dots represent 89 upregulated transcripts, and blue dots represent 59 downregulated transcripts. **(E)** Heatmap of DE lncRNA-Hub mRNA PCC score. Orange indicates positive PCC, and blue indicates negative PCC. PCC ≥0.990 or ≤−0.990 and $p < 0.05$ are labeled with ★.

Supplementary Figure S3 | DE lncRNA-core mRNA interaction network construction and verification with public dataset. **(A)** Heatmap of DE lncRNA-core mRNA PCC score. Orange indicates positive PCC, and blue indicates negative PCC. PCC ≥0.990 or ≤−0.990 and $p < 0.05$ are labeled with ★ (DE lncRNA-Hub mRNA) or ● (DE lncRNA-non Hub mRNA). **(B)** List of correlated DE lncRNA transcript-core mRNA pairs, with their PCC and value of *p*. **(C)** The interaction network of 14 DE lncRNA transcripts and 11 core mRNAs. Red indicates upregulation, and blue indicates downregulation in HE NSC/NPCs compared to NC. Triangles represent lncRNAs, and rectangles represent mRNAs. **(D)** List of lncRNAs in panel B with the corresponding lncRNA genes found in GSE65487. **(E)** Heatmap of lncRNA-mRNA PCC score graphed using data from GSE65487. Orange indicates positive PCC, and blue indicates negative PCC. PCC ≥0.900 or ≤−0.900 and $p < 0.05$ are labeled with ★. **(F)** List of correlated lncRNA-mRNA pairs presented in panel E, with their PCC and value of *p*.

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Associations Among Parental Caregiving Quality, Cannabinoid Receptor 1 Expression-Based Polygenic Scores, and Infant-Parent Attachment: Evidence for Differential Genetic Susceptibility?

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Attachment is a biological evolutionary system contributing to infant survival. When primary caregivers/parents are sensitive and responsive to their infants' needs, infants develop a sense of security. Secure infant attachment has been linked to healthy brain and organ-system development. Belsky and colleagues proposed the term differential susceptibility to describe context-dependent associations between genetic variations and behavioral outcomes as a function of parenting environments. Variations in the Cannabinoid Receptor Gene 1 (CNR1) are associated with memory, mood, and reward and connote differential susceptibility to more and less optimal parental caregiving quality in predicting children's behavioral problems.

Aim: To determine if parental caregiving quality interacts with children's expression-based polygenic risk score (ePRS) for the CNR1 gene networks in the prefrontal cortex, striatum, and hippocampus in predicting the probability of attachment security and disorganized attachment.

Design: Prospective correlational methods examined maternal-infant pairs (n = 142) from which infants provided DNA samples at 3 months. Parental caregiving quality was assessed via the Child Adult Relationship Experiment (CARE)-index at 6 months, and attachment security via the Strange Situation Procedure at a mean age of 22 months. The CNR1 ePRSs include genes co-expressed with the CNR1 genes in the prefrontal cortex, striatum, or hippocampus, and were calculated using the effect size of the association between the individual single nucleotide polymorphisms

from those genes and region-specific gene expression (GTEx). Logistic regression was employed (alpha < 0.05, two-tailed) to examine the main and interaction effects between parental caregiving quality and ePRSs in predicting attachment patterns. Interpretation of results was aided by analyses that distinguished between differential susceptibility and diathesis-stress.

Results: Significant interactions were observed between (1) maternal sensitivity and ePRS in the striatum in predicting attachment security, (2) maternal unresponsiveness with the ePRS in the hippocampus in predicting disorganization, and (3) maternal controlling with the ePRS in the hippocampus in predicting disorganization.

Conclusion: These findings offer support for genetic differential susceptibility to the quality of maternal sensitivity in the context of the ePRS in the striatum. However, the significant interactions between hippocampal ePRS and maternal unresponsiveness and controlling in predicting the probability of disorganization were more suggestive of the diathesis-stress model.

Keywords: expression-based polygenic risk score (ePRS), cannabinoid receptor gene 1 (CNR1), parent–child relationship quality, CARE-index, strange situation procedure, APrON study, attachment security, attachment disorganization

INTRODUCTION

Since psychiatrist John Bowlby first considered the importance of infants' secure attachments with their caregivers to later mental health, research on attachment patterns has exploded (Sroufe et al., 2005; Cassidy, 2016). Attachment theory has not only provided a basis for international research programs but has also become an influential perspective on child development in clinical and welfare practice (Sroufe et al., 2005; Kozlowska and Elliott, 2014; Teti and Kim, 2014). The most fundamental aspect of attachment theory is that a child's attachment behavior has social-biological underpinnings promoting a vulnerable infant's proximity to the attachment figure, improving their chance of survival (Simpson, 1999; Cassidy, 2016). When primary caregivers/parents are available and responsive to their infants' needs, infants develop a sense of security, making them feel safe, secure, and protected (Bowlby, 1982; Benoit, 2004; Solomon and George, 2016). Infants anticipate their parents' responses to their distress and shape their attachment behaviors accordingly (Benoit, 2004). When observed and scored, infant attachment behavior typically is classified into one of four attachment patterns: secure, insecure-avoidant, insecure-resistant, and disorganized (Ainsworth et al., 1978; Main and Solomon, 1986). A growing body of evidence links infant secure attachment patterns to healthy brain and organ-system development and insecure and disorganized attachment to increased levels of all-cause morbidity, chronic inflammation, coronary artery disease, and an array of mental health disorders (Schore, 2000, 2001; Sroufe, 2005; Puig et al., 2013).

Parental caregiving quality, typically characterized by qualities of maternal sensitivity, control, and responsiveness, predicts infants' attachment pattern (De Wolff and van Ijzendoorn, 1997; van Ijzendoorn et al., 1999; Madigan et al., 2006; Crittenden, 2010; Bailey et al., 2017). Sensitivity is a caregiver's ability to perceive, accurately interpret, and respond promptly and accurately to an infant's cues (Ainsworth et al., 1978). High maternal sensitivity involves responding to infant/child cues that signal needs or distress, such as fussiness due to hunger or fatigue, in a timely fashion, while low maternal sensitivity is indicated by low responsiveness (Barnard and Guralnick, 1997). High maternal sensitivity also denotes behaviors contrary to overtly or covertly hostile behaviors or attempts to excessively control infant behavior in routine interactions (Kelly et al., 2008). While sensitive caregiving may support the development of acceptable emotional expressions and optimal regulation, harsh, controlling caregiving behaviors may undermine children's emotional development. Therefore, high-quality parental caregiving is typically characterized by sensitive and responsive interactions attentive to infant needs while mitigating excessive intrusion and control. A greater degree of sensitivity shows the infant that the caregiver is dependable, which creates a secure base for the child then to explore the world (Thompson, 2016). Parental sensitivity is regarded as one of the most important determinants of infant attachment security (Fearon et al., 2006; Bakermans-Kranenburg and van Ijzendoorn, 2007; Colmer et al., 2011), while traumatic events thought to undermine parental caregiving predict disorganized attachment (Lyons-Ruth, 2015).

However, despite being an important factor in predicting attachment patterns, parental caregiving quality does not explain as much variance as one might expect (De Wolff and van Ijzendoorn, 1997; van Ijzendoorn et al., 1999; Bailey et al., 2017). There has been evidence to support associations between attachment patterns and several sociodemographic factors such as maternal age (Esma et al., 2018), socioeconomic status (Acevedo et al., 2012), migration background (Keller, 2018), infant sex (Weinberg et al., 1999; David and Lyons-Ruth, 2005), infant gestational age (Wille, 1991), descriptive factors such as maternal depression (Kohlhoff and Barnett, 2013), social support (Jacobson and Frye, 1991), and infant birth weight (Wille, 1991; Weiss et al., 2000). In addition, there is a growing body of

evidence suggesting that individuals' genetics may influence attachment patterns (Lakatos et al., 2000, 2002; Belsky and Beaver, 2011; Luijk et al., 2011; Belsky et al., 2015; Pappa et al., 2015; Golds et al., 2020). Specifically, disorganized attachment patterns have been linked to genetic variations of the genes responsible for regulating dopamine (DA; Lakatos et al., 2000, 2002; Gervai et al., 2005, 2007; van Ijzendoorn and Bakermans-Kranenburg, 2006).

The majority of the literature examining the roles of parental caregiving behavior and genetics in predicting attachment relates to neurotransmitters, particularly those implicated in reward processing (Bakermans-Kranenburg and van Ijzendoorn, 2016; Feldman, 2017). DA is a neurotransmitter associated with motivation or pleasure necessary to promote a response to environmental cues that signal reward and depend on carrying out a specific action or behavior to receive it (Du Hoffmann and Nicola, 2014). The endocannabinoid system (ECS) is implicated in a wide variety of brain functions, such as reward processing as well as memory, mood, and motor control. The Type 1 Cannabinoid Receptor (CB1), encoded by the Cannabinoid Receptor 1 (CNR1) gene, is a key component of the endocannabinoid system and is expressed in both the central and peripheral nervous systems, particularly on axon terminals in the cerebellum, hippocampus, basal ganglia, frontal cortex, amygdala, hypothalamus, and midbrain (Romero-Fernandez et al., 2013; Brzosko et al., 2015). The CB1 receptor is an important component of the ECS in the nervous system, regulating synaptic transmission by modulating neurotransmitters' release, including DA (Tao et al., 2020). Two of the most commonly studied CNR1 polymorphisms include rs1049353 (Agrawal et al., 2012) and rs7766029 (Juhasz et al., 2009) in relation to different phenotypic outcomes, especially in the rs1049353 genotype, A allele. Previous studies support the notion that outcomes can vary with the different polymorphic variants of these genes. For example, when the CNR1-A allele is absent, the caregiving environment's impact on children's externalizing behaviors is attenuated. Higher levels of negative caregiver control, in the presence of the CNR1-A gene, predicted parent-report of more externalizing behaviors in children. In comparison, lower levels of negative caregiver control predicted the report of less externalizing behaviors in a differentially susceptible manner (Letourneau et al., 2019).

Genetic variations can have varying functional effects in different biological contexts; thus, specific genes may produce different observable outcomes in response to either stressful or protective environments (Del Giudice, 2016). Belsky and Pluess (2009a) proposed utilizing the term differential susceptibility when describing genes associated with both adaptive and maladaptive changes in phenotypes in response to "supportive" and "unsupportive" parental caregiving environments. Parental caregiving quality incorporates constructs such as nurturing, acceptance, and cohesion, and involves behaviors toward the child (e.g., praising, encouraging, and giving physical affection), which signal to the child love, support, and acceptance (Barnes et al., 2000). In short, genetic differential susceptibility theory may explain why some infants appear to have increased susceptibilities to parental caregiving qualities. Genetic variation leading to neurobiological and temperamental traits characterized by highly sensitive and responsive stress physiology may determine increased susceptibility to stress and adversity (Del Giudice, 2016). Highly genetically susceptible children have disproportionately high morbidity rates when raised in adverse stressful environments; in addition, children with a higher degree of genetic susceptibility more frequently exhibit mental health symptoms in adolescence (Essex et al., 2011), exhibit epigenetic modifications (i.e., decreased DNA methylation; Goodman et al., 2018), and are more likely to exhibit behavioral problems under circumstances of low caregiver support (Skowron et al., 2014; Letourneau et al., 2019). In contrast, children with a high degree of genetic susceptibility become more socially integrated, have the lowest levels of illness (Boyce et al., 1995), and highest school engagement levels when receiving high-quality parental caregiving (Obradović et al., 2010). This dichotomy in children with a high degree of genetic susceptibility suggests a unique opportunity to identify individuals who could be at risk for poor health outcomes by assessing children's genetic differential susceptibility to parental caregiving quality. However, a rival explanation for some of these associations is diathesis-stress, in which poor developmental experiences (e.g., low-quality parenting) are most likely to impact the development of individuals who carry vulnerability factors that result in maladaptation. Ascertaining whether parenting interacts with genetic factors in either a differential susceptibility or diathesis-stress manner is a subject of ongoing exploration (Garmezy et al., 1984; Roisman et al., 2012; Portella et al., 2020).

Novel genomic metrics that either predict gene expression in tissue-specific regions or use gene co-expression information may provide a more comprehensive view of a specific gene or a gene network's role in modulating an individual's response to environmental variations, compared to that provided by the single candidate gene approach (Gamazon et al., 2015; Barth et al., 2020). Expression-based polygenic risk scores (ePRS) offer one such approach to understand the underlying genetic background linked to behavioral outcomes (Hari Dass et al., 2019). ePRS is a genomic risk profiling method that recognizes a gene network contribution to a particular condition or outcome derived from a combination of small effects from many genetic variants. ePRS scores are derived based on transcriptional co-expression profiles from specific regions of the mouse (GeneNetwork) and human (Brainspan) brains, used to identify Single Nucleotide Polymorphisms (SNPs) functionally associated with gene expression in the human brain (GTEx). ePRS analyses provide a new paradigm to identify gene-by-environment interactions (McGrath et al., 2013; Plomin, 2013; Iyegbe et al., 2014; Silveira et al., 2017; Belsky et al., 2019; De Lima et al., 2020).

When attachments form in early infancy, activation and closer links are observed among neurobiological brain systems underpinning affiliation, reward, and stress management (Ulmer-Yaniv et al., 2016). Functional magnetic resonance imaging (fMRI) has been used to investigate the brain activity associated with humans' various social attachments (Feldman, 2017). These fMRIs provide evidence for three main inter-connected neural systems that integrate to establish, maintain, and

enhance our attachments to others, including the reward-motivation system (Berridge and Robinson, 1998), the embodied simulation/empathy network (Gallese, 2014), and mentalizing processes (Frith and Frith, 2006). The reward-motivation system comprises the striatum (nucleus accumbens, caudate, and putamen), amygdala, ventral tegmental area, orbitofrontal cortex, ventromedial prefrontal cortex, and anterior cingulate cortex (ACC). The existence of convergent projections from the cortex to the striatum, along with hippocampal and amygdala-striatal projections, places the striatum as a central entry port for processing emotional/motivational information supporting human attachment (Haber and Knutson, 2010; Robinson et al., 2012; Pauli et al., 2016). The reward-motivation system employs DA and oxytocin rich pathways (Schultz, 2000; Berridge et al., 2009; Haber and Knutson, 2010) and supports multiple attachment-related motivational behaviors, such as social orienting, social seeking, and maintaining contact (Acevedo et al., 2012; Chevallier et al., 2012). Attachments have an intrinsic motivational value that combine immediate hedonic responses with approach motivation, goal-directed behavior, and learning (Berridge and Robinson, 1998).

The embodied simulation/empathy network includes the insula, ACC, inferior frontal gyrus, inferior parietal lobule, and supplementary motor area. Embodied simulation is an ancient evolutionary mechanism essential to grounding a 'shared world' in the brain and underpins the human capacity to build and maintain attachments (Craig, 2009; Gallese, 2014). Finally, the formation and maintenance of attachment bonds also rely on higher-order mentalizing processes that involve complex top-down inferences (Frith and Frith, 2006; Van Overwalle, 2009). Mentalizing processes underpin attachment and reinforce attachment formation by building on the individual's ability to appreciate multiple perspectives, understand others' goals and motives, and keep in mind their values and concerns (Ciaramidaro et al., 2014; Hari et al., 2015). The mentalizing system consists of frontotemporal–parietal structures, particularly the superior temporal sulcus, posterior cingulated cortex, temporoparietal junction, temporal pole, and medial prefrontal cortex (Feldman, 2017).

To the best of our knowledge, this is the first study that seeks to investigate if infant genetic susceptibility interacts with the quality of parental caregiving in predicting attachment patterns using observational measures. This understanding could offer empirical evidence of infants' physiological responsivity to positive (and negative) parental caregiving (Barth et al., 2020). We propose utilizing the innovative approach of ePRS to determine if parental caregiving quality (i.e., sensitivity, unresponsiveness, and controlling) interacts with children's ePRS for the prefrontal cortex, striatum, and hippocampus CNR1 gene networks in predicting the probability of secure and/or disorganized attachment. Previous studies examining various polymorphic variants, including CNR1, in relation to children's behavior have suggested that they have the potential to interact with environmental influences in a differentially susceptible manner (Young et al., 2002; Letourneau et al., 2019). Due to the activation of the neurobiological systems associated with the ECS that underpin affiliation, reward, stress management, responsiveness to the environment, and mood (Lupica et al., 2004; Ranganathan and D'Souza, 2006; Hill et al., 2009; Zuurman et al., 2009; Zanettini et al., 2011; Feldman, 2017), and thus potential to relate to attachment pattern formation in infancy (Berridge and Robinson, 1998; Acevedo et al., 2012; Chevallier et al., 2012), we chose this specific gene (CNR1) and tissue-specific networks for study. We focused on the prefrontal cortex due to its association with cognitive, emotional functions, impulse control, and adaptive behaviors (Morecraft and Yeterian, 2002; Bechara and Van Der Linden, 2005), and the striatum for its involvement in the reward motivation system and potential to relate to attachment formation in infancy specifically (Feldman, 2017). Convergent projections from the cortex to the striatum, along with hippocampal and amygdala-striatal projections, places the striatum as a central entry port for processing emotional/motivational information supporting human attachments (Haber and Knutson, 2010; Robinson et al., 2012; Pauli et al., 2016; Feldman, 2017). Finally, as part of the limbic system, the hippocampus was chosen for its spatial and emotional memory involvement. The hippocampus plays an essential role in social memory and consolidating declarative or explicit memories of facts or events that enable conscious recall from long-term memory (Campbell and Macqueen, 2004). The ability to recognize and memorize familiar conspecifics (social memory) is a critical aspect of social interactions in animals (McGraw and Young, 2010; Okuyama et al., 2014, 2016). As the hippocampus develops, the infant can recognize and remember their caregiver and begin to feel a sense of pleasure with them during engaging interactions (Chambers, 2017).

We hypothesize that within the three selected brain regions (i.e., prefrontal cortex, striatum, and hippocampus): (1) higher maternal sensitivity will interact with ePRS for the CNR1 gene networks in predicting a higher probability of secure attachment and reduced probability of disorganization, (2) higher maternal controlling will interact with ePRS for CNR1 gene networks in predicting a reduced probability of secure attachment and higher probability of disorganization, and (3) higher maternal unresponsiveness will interact with ePRS for CNR1 gene networks in predicting a reduced probability of secure attachment and higher probability of disorganization.

MATERIALS AND METHODS

This secondary analysis employs data from the Fetal Programming Study (Giesbrecht et al., 2017), a sub-study derived from the larger Alberta Pregnancy Outcomes and Nutrition (APrON) longitudinal cohort study (Kaplan et al., 2014), which ended enrollment in 2012. The Fetal Programming Study aimed to examine biomarkers of maternal stress during pregnancy and collect data on parent–infant interaction quality and attachment (Kaplan et al., 2014; Giesbrecht et al., 2017; Letourneau et al., 2017). Ethics approval was obtained from the Conjoint Health Research Board at the University of Calgary in Alberta, Canada. All participants in the study completed a process of informed consent prior to participating. For this

project's scope, relevant data were collected at study visits during pregnancy and 3, 6, and 22 months postpartum.

Participants and Recruitment

Recruitment of 294 pregnant women into the Fetal Programming study took place between 2011 and 2012 in a large western Canadian city. Expectant mothers were recruited through media advertisements and maternity, ultrasound, family medicine, and obstetric clinics (Kaplan et al., 2014). To be eligible at enrollment, mothers: (1) were less than 22 weeks pregnant, (2) were 16 years of age or older, (3) were pregnant with a singleton, (4) reported abstaining from alcohol and tobacco during pregnancy, (5) reported not receiving a glucocorticoid medication during pregnancy, and (6) reported no known fetal complications. Mothers were excluded if they could not answer questions in English or planned to move out of the region during the study's timeframe (Kaplan et al., 2014). Of the 294 recruited participants in the Fetal Programming Study, 142 maternal infant-pairs provided an infant Deoxyribonucleic Acid (DNA) sample in the form of a buccal swab or blood sample with sufficient quantity to calculate ePRS and completed all assessments of maternal-infant relationship quality and attachment patterns (Thomas et al., 2017).

Procedures and Measures

Data were collected on mothers' demographic characteristics at enrollment and infant demographic characteristics at birth. Additional data were collected during pregnancy and postpartum on depression and social support. Blood was drawn, or buccal cells were collected from children at 3 months of age. Observational assessments of maternal-infant interaction quality (predictor) were conducted at 6 months of age and infant attachment pattern (outcome) at 22 months.

Predictors

To measure *parental caregiving quality*, we employed the Child Adult Relationship Experiment (CARE)-Index (Crittenden, 2010). It is valid with infants from birth up to 15 months (Crittenden and Bonvillian, 1984; Crittenden and DiLalla, 1988; Ward and Carlson, 1995; Leadbeater et al., 1996; Leventhal et al., 2004), and inter-rater reliability values range between r = 0.73 and 0.95 (Leventhal et al., 2004; Azar et al., 2007). When the infants were 6 months of age, a 5-min observational procedure was carried out by videotaping the mother–infant pairs engaging in play with age-appropriate toys. Seven aspects of interaction behavior are assessed, including facial expression, verbal expression, positional and body contact, affection, turn-taking, control, and activity choice. Total scores for parental sensitivity, controlling, and unresponsiveness are derived, ranging from 0–14 (Crittenden, 2010). Author Letourneau is a reliable CARE-Index coder and supervised the administration and blinded data coding. Trained, independent designates coded video recordings at Crittenden's laboratory, who achieved a 94.4% inter-rater agreement on the three observable constructs. For each of the CARE-Index subscales (i.e., sensitivity, controlling, and unresponsiveness), three scoring category groups were created, including: "low" which included maternal-child dyads that scored less than one standard deviation below the calculated mean; "mean," which included maternal-child dyads that scored within one standard deviation above or below the calculated mean; and "high" which included maternal-child dyads that scored more than one standard deviation above the calculated mean. These categories enabled data in graphs and figures to be interpreted more readily.

To collect *DNA for analysis*, blood was drawn from infants at a study visit at 3 months of age. All samples were drawn by a certified phlebotomist using either a butterfly needle or a 25-gauge 3/4 inch infant needle. The blood samples were processed within 6 h of collection at the affiliated hospital genetics laboratory. This process involved spinning the vacutainer at 3,000 rotations per minute for 15 min to separate the plasma, buffy coat (i.e., leukocytes and platelets), and erythrocytes. The buffy coat was extracted using a pipette from the collection container, placed into a microcentrifuge tube, and stored at −80°C for DNA extraction at a later date. Buccal epithelial cells (BEC) were also collected from infants if their blood draw yield was low or unobtainable. This was done by rubbing a sterile cytology brush up and down the infant's entire cheek ten times on two different swabs to ensure an adequate sample was obtained. The BEC and processed blood leukocytes were kept in short-term storage at −80°C before DNA extraction. DNA extraction was done by cell lysis, followed by purification using the Gentra Puregene method (Qiagen, Venlo, Limburg, Netherlands). The samples were processed for DNA purification using the Autopure method (Qiagen, Venlo, Limburg, Netherlands) and processed further using the cell lysate program. Samples were left open to air allowing for evaporation of excess ethanol, and low-TE buffer was added to the tubes. After DNA extraction, the isolated DNA samples were stored at 4°C at the affiliated hospital genetics laboratory.

The genetic data were extracted using Illumina HumanCoreExome BeadChipVersion 1 and subjected to quality control (QC) procedure using PLINK 1.9 (Chang et al., 2015). SNPs with missing call rate > 5%, minor allele frequency (MAF) < 5%, or violation of Hardy-Weinberg equilibrium (HWE) with p-value < 1e-30, as well as samples with missing call rate > 5%, outliers on heterozygosity or sex mismatches were removed. This final data set included 179 subjects and 289,296 genotyped SNPs. Then we utilized the Sanger Imputation Service for imputation. After the post-imputation QC and the imputation accuracy filter (INFO-score) > 0.80, the final data set included 23,752,992 SNPs.

To describe the population stratification, we performed principal component analysis using SMARTPCA (Patterson et al., 2006) on this pruned dataset of genotyped SNPs (with r2 < 0.20, sliding window of 50 and an increment of 5 SNPs).

The *ePRS* was created considering genes co-expressed with the Cannabinoid Receptor (ePRS-CNR1) in the prefrontal cortex (see **Figure 1**), striatum (see **Figure 2**), and hippocampus (see **Figure 3**) according to the protocol previously described by Silveira et al. (2017) and Hari Dass et al. (2019). In summary, the genetic score was created using (a) Genenetwork[1], (b) Brainspan[2],

[1]http://genenetwork.org

[2]http://www.brainspan.org/rnaseq/search/index.html

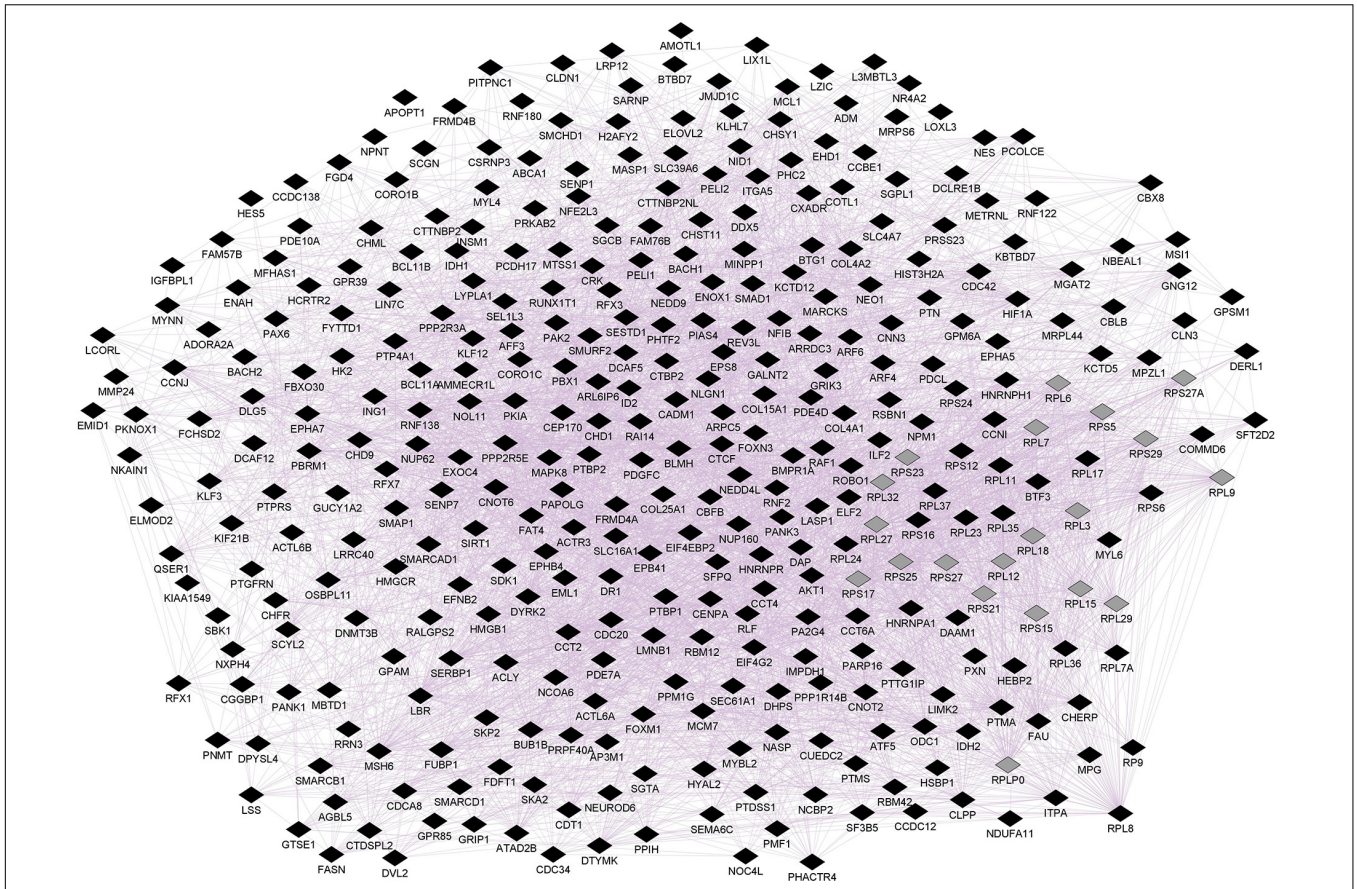

FIGURE 1 | Prefrontal CNR1 gene network using GeneMANIA. Black diamonds indicate query genes, whereas gray diamonds indicate related genes added by GeneMANIA. GeneMANIA converts mRNA expression data from Gene Expression Omnibus (GEO) to functional association networks, connecting co-expressed genes through purple lines. Node sizes represent gene scores, reflecting how often paths that start at a given gene node end up in one of the query genes.

and (c) GTEx[3]. In (a), we identified the transcriptional co-expression profiles of CNR1 (4,704 genes co-expressed with CNR1 in mice prefrontal cortex, 1,717 genes co-expressed with CNR1 in mice hippocampus, and 86 genes co-expressed with CNR1 in mice striatum $r > 0.5$) (GeneNetwork). These genes were filtered by selecting those that were overexpressed during fetal/childhood (up to 5 years of age) at 1.5-fold more than adult gene expression in human postmortem samples (Brainspan). The final list included 343 genes for the CNR1 prefrontal gene network, 12 genes for the striatal network, and 175 genes for the hippocampal network. Based on the functional annotation of these genes in the National Center for Biotechnology Information, United States National Library of Medicine[4] using GRCh37.p13, we gathered all of the existing SNPs from these genes present on our data, merged this list with SNPs that were available on GTEx, and retained the resulting list of SNPs for linkage disequilibrium clumping ($r2$, 0.25). The final lists of SNPs included 8506 independent functional SNPs for CNR1 prefrontal ePRS, 3446 SNPs for the hippocampal ePRS, and 434 SNPs for the striatal network. Based on the children's genotype data, alleles at a given *cis*-SNP were weighed by the estimated effect of the genotype on gene expression (GTEx in which the effect allele is the alternative allele). Final ePRSs were obtained by summation over all SNPs accounting for the sign of correlation coefficient between the genes and CNR1 gene expression in the different regions. For inclusion in modeling, the CNR1 ePRS scores were standardized. Enrichment analysis of the gene networks was done using MetaCore® (Clarivate Analytics[5]). Cytoscape® software (Shannon, 2003) and GeneMANIA app (Franz et al., 2018) were used to visualize the gene networks. The nodes are the elements of a network, and edges are the connection between these elements, that represent co-expression. Further, CNR1 ePRSs were then categorized into two groups, through a median split to characterize children into low or high ePRS groups.

Outcome

Attachment patterns were measured at a mean age of 22 months via the Strange Situation Procedure (SSP), the gold standard assessment for attachment patterns in infancy (Ainsworth et al., 1978). The coding scale was originally designed for children

[3]https://www.gtexportal.org/home/

[4]https://www.ncbi.nlm.nih.gov/variation/view/

[5]https://portal.genego.com/

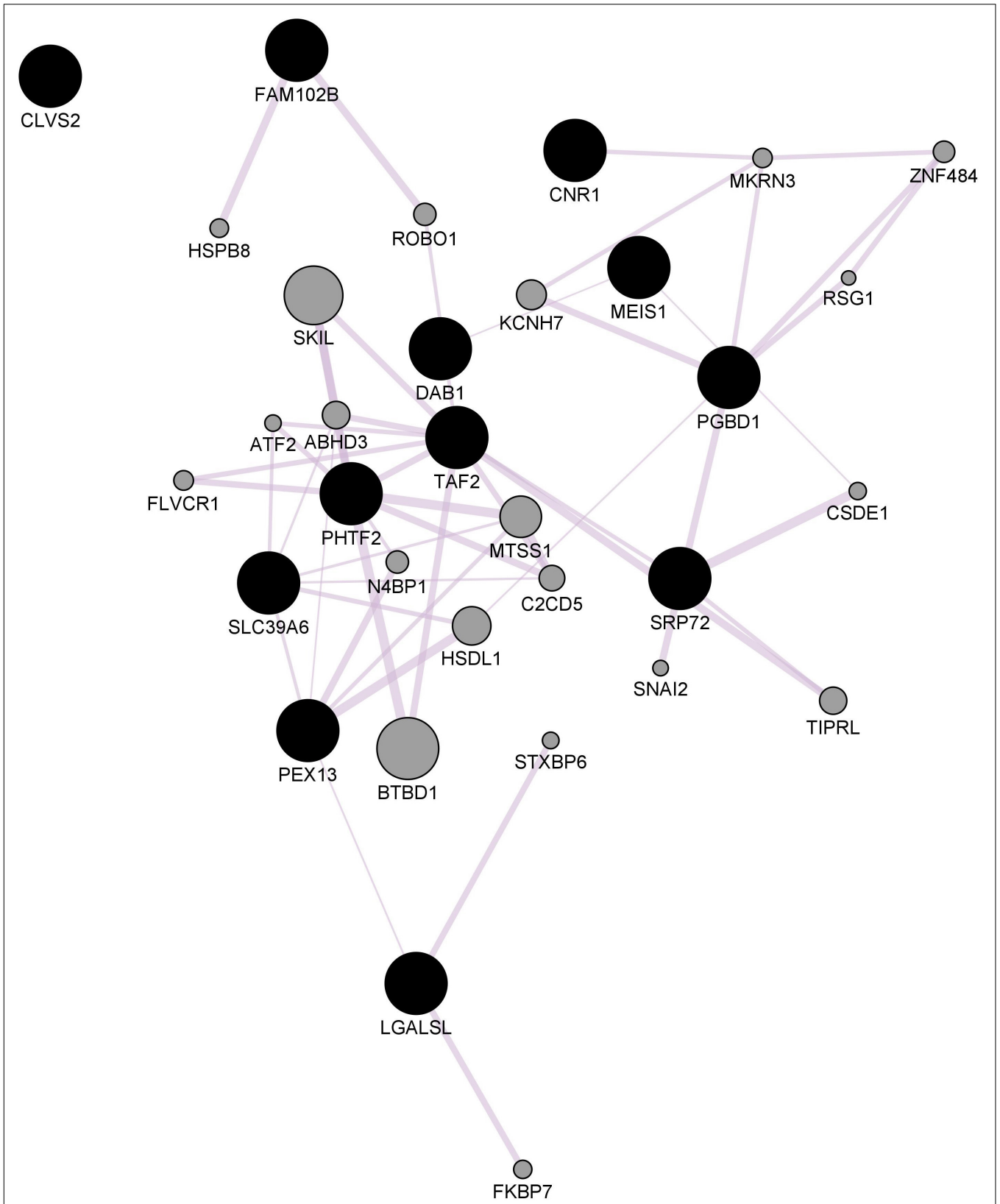

FIGURE 2 | Striatal CNR1 gene network using GeneMANIA. Black circles indicate query genes, whereas gray circles indicate related genes added by GeneMANIA. Node sizes represent gene scores, reflecting how often paths that start at a given gene node end up in one of the query genes.

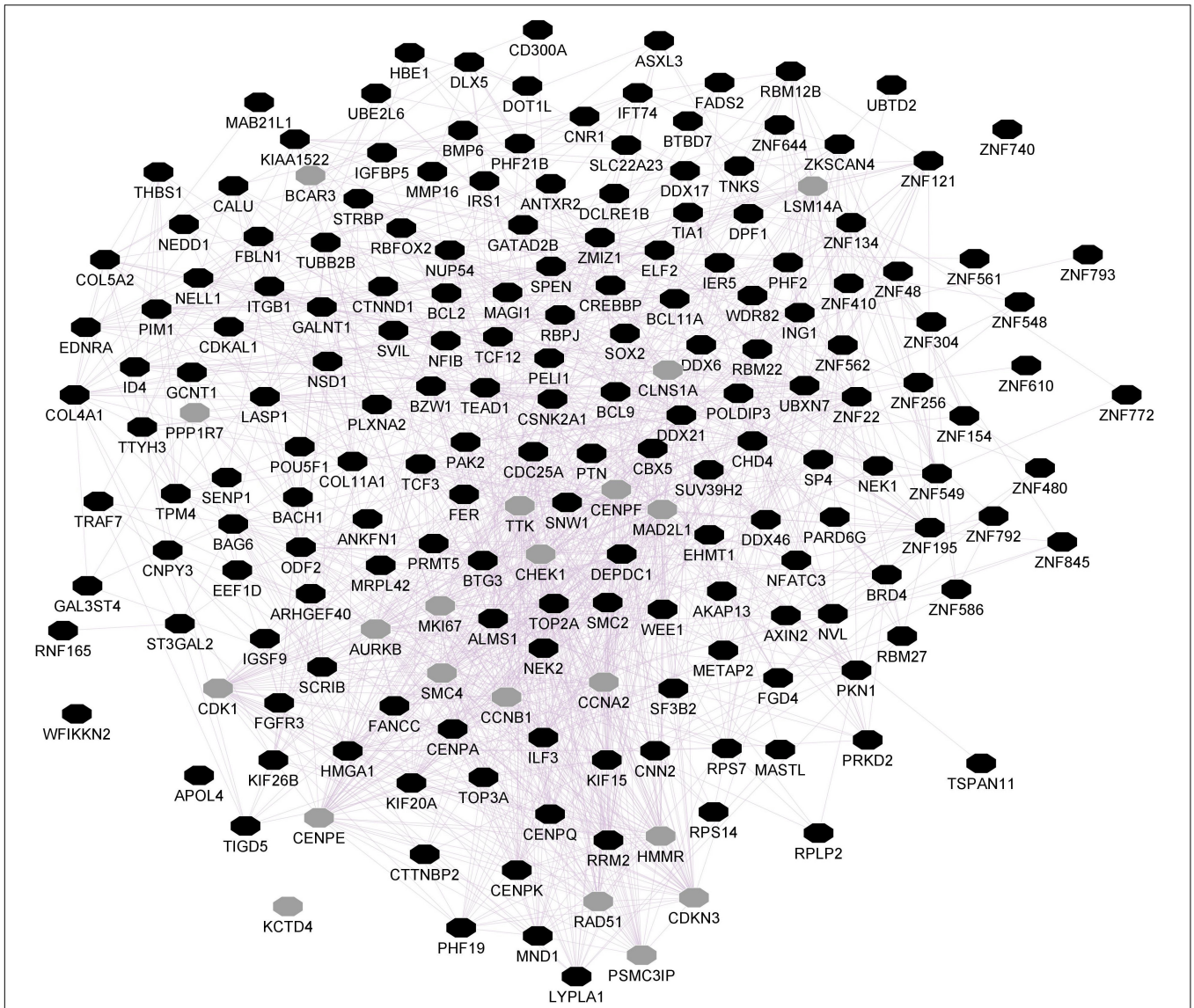

FIGURE 3 | Hippocampal CNR1 gene network using GeneMANIA. Black hexagons indicate query genes, whereas gray hexagons indicate related genes added by GeneMANIA. Node sizes represent gene scores, reflecting how often paths that start at a given gene node end up in one of the query genes.

between 12 and 20 months of age but is commonly used up to 24 months (Ainsworth et al., 1978; van IJzendoorn and Kroonenberg, 1988; Solomon and George, 2016). The SSP procedure contains eight brief episodes designed to activate infant's attachment behaviors by evoking mild levels of stress in children (e.g., seeking proximity to the parent) through a series of mother–child separations and reunions between the infant and mother and interactions between the infant and a 'stranger' (a research confederate), where the child's behaviors were observed through a two-way mirror and video-recorded for coding. A coder (Author Hart) deemed reliable by Alan Sroufe of the Institute of Child Development at the University of Minnesota (ABCD Model) and by Marinus van Ijzendoorn at Cambridge University (Type D) using the Main and Solomon Coding System, coded all SSP videotapes for patterns of attachment using standard categories of secure (B), insecure with subtypes avoidant (A) and resistant (C), and disorganized (D; Ainsworth et al., 1978; Main and Solomon, 1986). To attain a D code, Main's coding scheme was applied (Main and Stadtman, 1981; Main and Solomon, 1990), which assesses the degree of disorganized behavior in an interpretive way regarding conflict (e.g., aggressive outbursts) and/or disruptive behaviors (e.g., immobilized, disoriented, misdirected, behavior, sudden disordered activities, uninterpretable noises or movements) during the SSP. An expert coder at the Institute for Child Development also re-coded a random 15% of recordings. Cohen's kappa for inter-rater reliability was 0.73. Due to the relatively small group sample sizes of insecure category subtypes (A and C) and disorganized (D), we dichotomized the sample into two groups, which is

common in published research (Lewis-Morrarty et al., 2015; Fresno et al., 2018). The dichotomized groups used in the analyses were comprised of infants classified as secure (B) versus insecure (A, C, and D) and disorganized (D) versus organized (A, B, C).

Covariates

Demographic (i.e., maternal age, education, marital status, household income, country of birth; infant birth weight, gestational age, and sex) and descriptive (i.e., depression and social support) variables were considered. Mothers' perceptions of the quality of their partners' social support at 3, 6, and 22 months postnatal were assessed via the Social Support Effectiveness Questionnaire (SSEQ). The SSEQ is a 35-item measure that evaluated the type (i.e., emotional/affirmational, informational, instrumental, and negative) and self-perceived effectiveness of the support mothers received from their partner or another support person. Total scores range from 0 to 80, with higher scores indicating more effective support from partners. The internal consistency for this instrument is strong (Cronbach's alpha = 0.87) when used to distinguish levels of social support for childbearing women (Rini et al., 2006; Stapleton et al., 2011; Giesbrecht et al., 2017). The Edinburgh Postnatal Depression Scale (EPDS) was employed at 3, 6, 12, and 22 months postpartum. On a 10-item self-administered scale, the parent is asked to select the number next to the response closest to how they have felt in the past 7 days. For women, the EPDS has been found to have high sensitivity (83.6%) and specificity (88.3%) for identifying depressive symptoms and the widely accepted cut-off of EPDS $\geq$ 10, indicating at least probable minor depression (Pop et al., 1992; Matthey et al., 2006). We attempted to employ latent class analysis for both covariates to reduce the data collected at multiple time points (three times for social support, four times for depression). Only the analysis of social support revealed latent classes, categorized as high and low support. As no latent classes were identified for depression, we selected the maximum value on the depression scale over the four measurement time points and employed that value in analyses.

Statistical Analyses

First, the sample characteristics were analyzed with descriptive summaries, including frequencies, means, and standard deviations as appropriate. Second, univariate logistic regression associations between sample characteristics and attachment security/insecurity and disorganization/organization were examined to identify significant covariates for inclusion in the modeling that follows. Third, logistic regression modeling was employed to examine the main effects of the CARE-Index (sensitivity, controlling, and unresponsiveness) separately (X variable) using ePRS for CNR1gene networks in the prefrontal cortex, striatum, and hippocampus (Z variable; Model 2) and their interaction terms (Model 3), adjusting for principal components (PCs) for ancestry and sex of the child, along with any identified covariates above. We fitted each model to the data by maximum likelihood and ranked the models by their Akaike Information Criterion (AIC) to control for overfitting (Akaike, 1973). Further, to aid in visualizing the results, we computed the unadjusted predicted probability of attachment pattern for each value of the parental caregiving quality predictors (CARE-Index sensitivity, controlling, and unresponsiveness) considering interaction with ePRS categorized into low (−1SD) and high (+1SD) scores.

Analysis of Differential Susceptibility

An additional step was performed in models with a significant interaction term to ensure that any observed differential susceptibility effects were not an artifact of imposing linear model assumptions on non-linear relationships (Model 4). Following the recommendations outlined by Roisman et al. (2012), additional linear regression models, including X^2 and Z^*X^2 as predictors, were created to verify that neither of these two terms were statistically significant. A *post hoc* analysis for the interaction terms in model 3 included analysis of

TABLE 1 | Sociodemographic and descriptive characteristics of study participants.

Variables	Frequency	Percentages
Maternal age in years [mean (*SD*)]	31.4 [3.90]	
Gestational age at birth in weeks [mean (*SD*)]	39.34 [1.57]	
Birth weight in kilograms [mean (*SD*)]	3.41 [0.51]	
Secure attachment		
Yes	68	47.9%
No	74	52.1%
Disorganized attachment		
Yes	17	12.0%
No	125	88.0%
Sex of child		
Male	72	50.7%
Female	70	49.3%
Household income		
Below $70,000	26	18.3%
$70,000 or more	116	81.7%
Marital status		
Single	2	1.4%
Married	140	98.6%
Ethnicity		
Non-Caucasian	24	16.9%
Caucasian	118	83.1%
Born in Canada		
No	30	21.1%
Yes	112	78.9%
Education level		
Below degree	43	30.3%
Degree or more	99	69.7%
Social support (latent class)		
Class 1 (low social support)	72	50.7%
Class 2 (high social support)	70	49.3%
Depressive symptoms max value (3, 6, 12, and 22 months)		
EPDS < 9	102	71.8%
EPDS $\geq$ 10	40	28.2%

Proportion of Interaction (PoI; i.e., the proportion of the total area represented in the interaction plots uniquely attributable to differential susceptibility) and Proportion Affected (PA; i.e., the proportion of the population that is differentially affected by the moderator–*Z* variable; Roisman et al., 2012). The regions of significance (RoS) analyses were conducted using a Web-based program developed by Fraley[6]. Further, as per Roisman et al. (2012), evidence for differential susceptibility can be confirmed when the RoS analyses are performed to determine whether the moderator (*Z* variable) and the outcome variable are correlated at the low and high ends of the distribution of the predictor (*X* variable). Results should be considered significant only within a certain range of interest, that is ±2*SD* of the observed predictor variable. Values for the PoI index should be approximately within 0.40 and 0.60, and for the PA index should be close to 0.50 (Roisman et al., 2012; Portella et al., 2020).

[6]http://www.yourpersonality.net/interaction

RESULTS

Table 1 presents a descriptive analysis of the study variables. The mean age of mothers was 31.40 (*SD* = 3.90) years. The majority of women were married (98.6%), had attained a university degree or more (69.72%), and had household incomes ≥$70,000 (81.69%). Males made up approximately half of the sample of children (50.7%), and most of the mothers were born in Canada (78.9%). Less than half of children demonstrated a secure (48%) rather than an insecure attachment pattern (52%). **Table 2** presents the results of the bivariate analyses of associations between predictors and attachment pattern, revealing that only birth weight significantly predicts disorganization.

TABLE 2 | Associations between predictors and attachment pattern.

Variables	Secure	Insecure	OR 95% CI	Not disorganized	Disorganized	OR 95% CI
			***P*-value**	**[*n* (%)]**	**[*n* (%)]**	***P*-value**
Maternal age in years [mean (*SD*)]	31.69 (3.97)	31.13 (3.8)	1.04 (0.95, 1.13) $p = 0.393$	31.43 (3.81)	31.17 (4.66)	0.98 (0.86, 1.12) $p = 0.769$
Gestational age at birth Birth in weeks [mean (*SD*)]	39.41 (1.24)	39.28 (1.8)	1.05 (0.85, 1.29) $p = 0.639$	39.41 (1.52)	38.87 (1.88)	0.82 (0.62, 1.10) $p = 0.185$
Birth weight in kg [mean (*SD*)]	3.48 (0.48)	3.34 (0.54)	1.74 (1.89, 3.36) $p = 0.101$	3.45 (0.49)	3.07 (0.57)	0.23 (0.08, 0.65) **$p = 0.005$**
ePRS CNR1	−0.15 (0.90)	0.14 (1.06)	0.74 (0.53, 1.04) $p = 0.087$	−0.07 (0.97)	0.55 (1.08)	1.97 (1.13, 3.43) **$p = 0.017$**
Maternal sensitivity	5.19 (1.75)	5.33 (2.27)	0.96 (0.69, 1.34) $p = 0.806$	5.32 (1.89)	5.0 (3.01)	0.65 (0.38, 1.15) $p = 0.139$
Maternal controlling	2.70 (3.68)	2.61 (3.46)	0.97 (0.70, 1.35) $p = 0.856$	2.52 (3.47)	3.75 (4.02)	1.42 (0.89, 2.25) $p = 0.139$
Maternal unresponsiveness	6.07 (3.45)	6.05 (3.71)	1.05 (0.75, 1.46) $p = 0.783$	6.14 (3.58)	5.25 (3.49)	0.84 (0.51,1.39) $p = 0.510$
Sex						
Male	31 (45.59)	41 (55.41)	1.48 (0.76, 2.87)	64 (51.20)	8 (47.06)	1.16 (0.42, 3.20)
Female	37 (54.41)	33 (44.59)	$p = 0.243$	61 (48.80)	9 (52.94)	$p = 0.748$
Household income						
Below $70,000	12 (17.65)	14 (18.92)	1.09 (0.46, 2.55)	21 (16.80)	5 (29.41)	0.48 (0.15, 1.51)
$70,000 or more	56 (82.35)	60 (81.08)	$p = 0.845$	104 (83.20)	12 (70.59)	$p = 0.207$
Born in Canada						
No	15 (22.06)	15 (20.27)	0.89 (0.40, 2.01)	25 (20.0)	5 (29.41)	0.59 (0.19, 1.84)
Yes	53 (77.94)	59 (79.73)	$p = 0.794$	100 (80.0)	12 (70.59)	$p = 0.372$
Education level						
Below university degree	22 (32.35)	21 (28.38)	0.83 (0.40, 1.69)	38 (30.40)	5 (29.41)	1.03 (0.34, 3.14)
University degree or more	46 (67.65)	53 (71.62)	$p = 0.607$	87 (69.60)	12 (70.59)	$p = 0.934$
Maternal depression (max value)						
Not depressed	47 (69.12)	54 (73.97)	1.27 (0.61, 2.64)	93 (73.81)	9 (56.25)	2.19 (0.76, 6.35)
Depressed	21 (30.88)	19 (26.03)	$P = 0.523$	33 (26.19)	7 (43.75)	$p = 0.148$
Social support (latent class)						
Low social support	36 (53.73)	36 (48.65)	0.82 (0.42, 1.58)	66 (52.80)	6 (35.29)	2.05 (0.71, 5.89)
High social support	31 (46.27)	38 (51.35)	$p = 0.547$	59 (47.20)	11 (64.71)	$p = 0.176$

Bolded indicates a p value of less than 0.05.

ePRS CNR1 Prefrontal Cortex

Hypothesis 1. We hypothesized that higher maternal sensitivity would interact with CNR1 ePRS in the prefrontal cortex in predicting a higher probability of secure attachment and reduced probability of disorganization, controlling for covariates. With respect to the probability of attachment security or disorganization, logistic regression revealed no significant associations in any model (results not shown).

Hypothesis 2. We hypothesized that higher maternal controlling would interact with CNR1 ePRS in the prefrontal cortex in predicting a reduced probability of secure attachment and a higher probability of disorganization. With respect to both attachment security and disorganization, logistic regression revealed no significant interactions (results not shown).

Hypothesis 3. We hypothesized that higher maternal unresponsiveness would interact with CNR1 ePRS in the prefrontal cortex to predict a reduced probability of secure attachment and a higher probability of disorganization. With respect to both attachment security and disorganization, logistic regression revealed no significant interactions (results not shown).

ePRS CNR1 Striatum

Hypothesis 1. We hypothesized that higher maternal sensitivity would interact with CNR1 ePRS in the striatum in predicting a higher probability of secure attachment and reduced probability of disorganization, controlling for covariates. We observed a significant interaction between striatum CNR1 ePRS and maternal sensitivity in predicting the probability of attachment security in the fully adjusted model 3 (see **Table 3**). This model complied with a differential susceptibility assessment, given that the crossover point was within the limits of the *X* variable (maternal sensitivity) and indices were near expected values (PoI = 0.67, PA = 0.63). See **Figure 4** for the graphed associations. With respect to the probability of disorganization, logistic regression revealed no significant associations in any model (results not shown).

Hypothesis 2. We hypothesized that higher maternal controlling would interact with CNR1 ePRS in the striatum in predicting a reduced probability of secure attachment and a higher probability of disorganization. With respect to both attachment security and disorganization, logistic regression revealed no significant interactions (results not shown).

Hypothesis 3. We hypothesized that higher maternal unresponsiveness would interact with CNR1 ePRS in

TABLE 3 | Associations among maternal sensitivity, striatal gene network for CNR1ePRS, covariates, and secure vs. insecure attachment pattern.

Variables	Model 1 Adjusted OR (95% CI)	Model 2 Adjusted OR (95% CI)	Model 3 Adjusted OR (95% CI)	Model 4 Adjusted OR (95% CI)
Maternal sensitivity	0.93 (0.67, 1.31) $p = 0.693$	–	0.93 (0.64, 1.34) $p = 0.929$	4.8- (0.57, 40.31) $p = 0.148$
Maternal sensitivity^2	–	–	–	0.92 (0.83, 1.02) $p = 0.123$
Striatal_ePRS	–	0.90 (0.64,1.26) $p = 0.542$	0.86 (0.59, 1.23) $p = 0.414$	1.05 (0.65,1.70) $p = 0.829$
Maternal sensitivity × hippocampal ePRS	–	–	**0.64 (0.43, 0.96)** $\mathbf{p = 0.031}$	**0.54 (0.32, 0.91)** $\mathbf{p = 0.019}$
Maternal sensitivity^2 × striatal ePRS	–	–	**–**	0.71 (0.44, 1.13) $p = 0.152$
Female	1.51 (0.77, 2.94) $p = 0.227$	0.64 (0.33, 1.25) $p = 0.193$	0.59 (0.29, 1.19) $p = 0.144$	1.64 (0.81, 3.34) $p = 0.167$
PC1	–	0.10 (0.0, 18.51) $p = 0.388$	0.10 (0.00, 18.52) $p = 0.387$	0.06 (0.00, 12.76) $p = 0.303$
PC2	–	165.81 (0.00, 3.15E + 9) $p = 0.410$	244.85 (0.00, 5.05E + 9) $p = 0.378$	168.38 (0.00, inf) $p = 0.417$
PC3	–	0.01 (0.00, 19.42) $p = 0.250$	0.01 (0.00, 11.31) $p = 0.191$	0.0 (0.00, 6.75) $p = 0.141$
AIC	201.07	204.90	203.03	200.39
PoI	–	–	0.67	–
Crossover point		–	–0.34	–
PA index	–	–	0.63	–

CI, confidence interval; OR, odds ratio. PC 1, 2, 3, principal component for ancestry; AIC, Akaike Information Criterion; PoI, proportion of interaction; PA, proportion affected; inf, very large CI and OR; bold refers to p < 0.05. Logistic regression modeling for main effects of the sensitivity (X variable; model 1) using ePRS for CNR1 gene networks in the striatum (Z variable; model 2) and their interaction terms (Model 3), adjusting for principal components (PCs) for ancestry and sex of the child. Model fit statistics (AIC; model) confirmed that Model 3 was optimal. Bolded indicates a p value of less than 0.05.

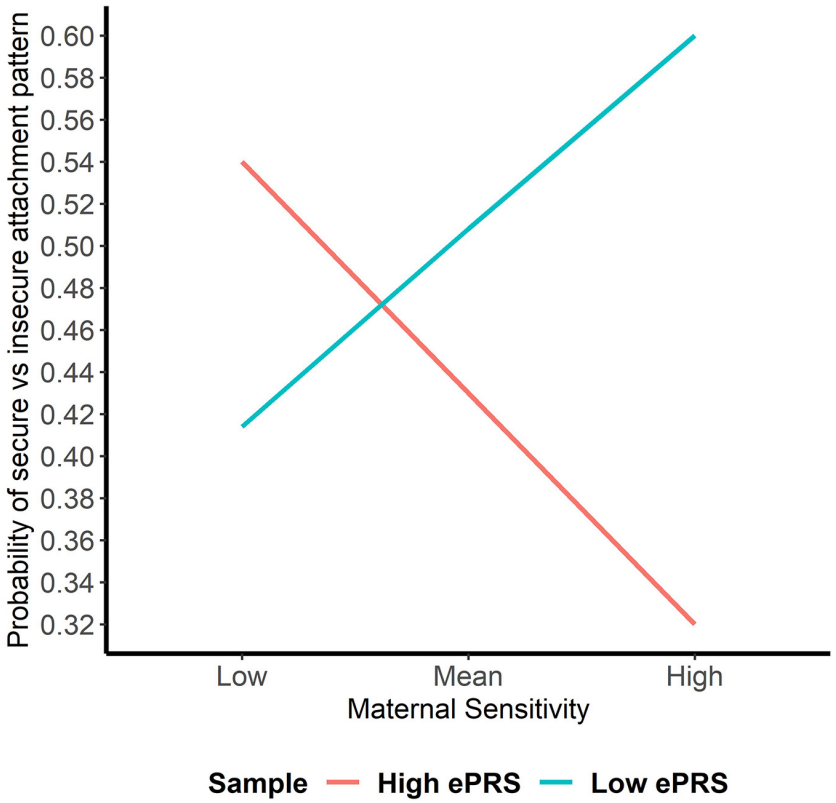

FIGURE 4 | Interaction between Striatal Gene Network and Maternal Sensitivity in Predicting Attachment. Shows that higher maternal sensitivity and a low CNR1 ePRS in the striatum predicts a higher probability of secure attachment. Higher maternal sensitivity and a high CNR1 ePRS in the striatum predicts a lower probability of secure attachment.

the striatum to predict a reduced probability of secure attachment and a higher probability of disorganization. With respect to both attachment security and disorganization, logistic regression revealed no significant interactions (results not shown).

ePRS CNR1 Hippocampus

Hypothesis 1. We hypothesized that higher maternal sensitivity would interact with CNR1 ePRS in the hippocampus in predicting a higher probability of secure attachment and reduced probability of disorganization, controlling for covariates. With respect to both attachment security and disorganization, logistic regression revealed no significant interactions (results not shown).

Hypothesis 2. We hypothesized that higher maternal controlling would interact with CNR1 ePRS in the hippocampus to predict a higher probability of insecure attachment and a higher probability of disorganization, controlling for covariates. With respect to the probability of attachment security (results not shown), logistic regression revealed no significant associations in any model. However, we observed a significant interaction between hippocampal CNR1 ePRS and maternal controlling behavior in predicting the probability of disorganization in the fully adjusted model 3 (see **Table 4**). This model did not comply with the criteria for differential susceptibility, given that the cross over point was not near the midpoint of *X* or even within the limits of the *X* variable (maternal controlling) and indices were outside of expected values (PoI = 0.16, PA = 0.22), suggesting that this interaction is more indicative of diathesis-stress. See **Figure 5** for the graphed associations.

Hypothesis 3. We hypothesized that higher maternal unresponsiveness would interact with CNR1 ePRS in the hippocampus in predicting a higher probability of insecure attachment and higher probability of disorganization, controlling for covariates. With respect to the probability of attachment security, logistic regression revealed no significant associations in any model (results not shown). We observed a significant interaction between hippocampal CNR1 ePRS and maternal unresponsiveness in predicting the probability of disorganization in the fully adjusted model 3 (see **Table 5**). See **Figure 6** for the graphed association. This model did not comply with the criteria for the differential susceptibility model, given that the cross over point was not near the midpoint of *X* or even within the limits of the *X* Variable (maternal unresponsiveness) and indices were outside of expected values (PoI = 0.73, PA = 0.68), suggesting that this interaction is more indicative of diathesis–stress.

Gene Network Analysis

Enrichment analysis demonstrated the prefrontal CNR1 gene network is enriched for gene ontology terms related to nervous system development (FDR q = 7.493e-16), regulation of neuron differentiation (FDR q = 1.958e-13), and neurogenesis (FDR q = 2.514e-13). The hippocampal network is enriched for gene ontology terms related to regulation of transcription (FDR q = 1.757e-21) and regulation of metabolic processes (FDR q = 1.022e-21). The striatal network is enriched for GO terms related to transcription initiation (FDR q = 7.619e-9), histone acetylation (FDR q = 2.124e-5), and the cannabinoid signaling pathway (FDR q = 4.786e-4).

DISCUSSION

This study set out to analyze if parental caregiving qualities (i.e., sensitivity, controlling, and unresponsiveness) interacted with the ePRS for the CNR1 gene networks in the prefrontal cortex, striatum, and hippocampus in predicting the probability of secure or disorganized attachment patterns. We hypothesized that higher sensitivity, lower controlling, and lower unresponsiveness would interact with ePRS for CNR1 in these three brain regions in predicting a higher probability of secure attachment and reduced probability of disorganization. Results for the prefrontal cortex failed to reject the null hypotheses for interaction effects between sensitivity, unresponsiveness, and controlling with CNR1 ePRS on either security of attachment or disorganization. Within the striatum, we observed a significant interaction between maternal sensitivity and CNR1 ePRS in predicting attachment security. We observed that higher maternal sensitivity and a low CNR1 ePRS in the striatum predicted a higher probability of secure attachment. The opposite is true for high CNR1 ePRS; higher maternal sensitivity and a high CNR1 ePRS in the striatum predicts a lower probability of secure attachment. Within the hippocampus, we observed a significant interaction between both unresponsiveness and controlling with the CNR1 ePRS in predicting disorganization. Higher maternal controlling and a higher CNR1 ePRS in the hippocampus predicted a lower probability of disorganization, and higher maternal controlling with a lower CNR1 ePRS predicted a higher probability of disorganization. Finally, we observed that high maternal unresponsiveness coupled with a low CNR1 ePRS in the hippocampus predicted a lower probability of disorganization and higher maternal unresponsiveness with a high CNR1 ePRS predicted a higher probability of disorganization.

In summary, low CNR1 ePRS in the striatum, a region of the brain involved in the reward motivation system, predicted

TABLE 4 | Associations among maternal controlling, hippocampal gene network for ePRS, covariates, and disorganized versus organized attachment pattern.

Variables	Model 1 Adjusted OR (95% CI)	Model 2 Adjusted OR (95% CI)	Model 3 Adjusted OR (95% CI)	Model 4 Adjusted OR (95% CI)
Maternal controlling	1.35 (0.83, 2.21) $p = 0.221$	-	1.58 (0.89,2.81) $p = 0.112$	1.13 (0.61, 2.11) $p = 0.691$
Maternal controlling^2				1.0 (0.94, 1.06) $p = 0.973$
Hippocampal ePRS	–	1.44 (0.79, 2.59) $p = 0.229$	1.79 (0.92, 347) $p = 0.083$	1.21 (0.45, 3.21) $p = 0.700$
Maternal controlling × hippocampal ePRS	–	–	**0.47 (0.25, 0.89)** ***p* = 0.021**	**0.29 (0.10., 0.86)** ***p* = 0.026**
Maternal Controlling^2 × Hippocampal ePRS	–	–	–	1.65 (0.67, 4.08) $p = 0.270$
Female	1.14 (0.39, 3.30) $p = 0.804$	1.23 (0.40, 3.73) $p = 0.715$	0.92 (0.28, 2.95) $p = 0.883$	1.03 (0.31, 3.38) $p = 0.962$
Birth weight (kgs)	**0.25 (0.09, 0.70)** ***p* = 0.008**	**0.24 (0.08, 0.77)** ***p* = 0.016**	**0.20 (0.06, 0.72)** ***p* = 0.014**	**0.22 (0.06, 0.77)** ***p* = 0.018**
PC1	–	0.04 (0.00, 149.96) $p = 0.436$	1.05 (0.00, 224.54) $p = 0.438$	0.02 (0.00, 184.59) $p = 0.401$
PC2	–	Inf (0.06, inf) $p = 0.109$	**Inf (0.72, inf)** ***p* = 0.05**	**Inf (2.17, inf)** ***p* = 0.039**
PC3	–	2.14 (0.01, inf) $p = 0.844$	1.05 (0.00, inf) $p = 0.991$	1.19 (0.00, inf) $p = 0.963$
AIC	102.28	104.63	101.15	103.85
PoI	–	–	0.16	–
Crossover point	–	–	0.78	–
PA index	–	–	0.22	–

CI, confidence interval; OR, odds ratio. PC 1, 2, 3, principal component for ancestry; AIC, Akaike Information Criterion; PoI, proportion of interaction; PA, proportion affected; inf, very large CI and OR; bold refers to $p < 0.05$. Logistic regression modeling for main effects of the controlling (X variable; Model 1) using ePRS for CNR1 gene networks in the hippocampus (Z variable; Model 2) and their interaction terms (Model 3), adjusting for principal components (PCs) for ancestry and sex of the child. Model fit statistics (AIC) confirmed that Model 3 was optimal. Bolded indicates a p value of less than 0.05.

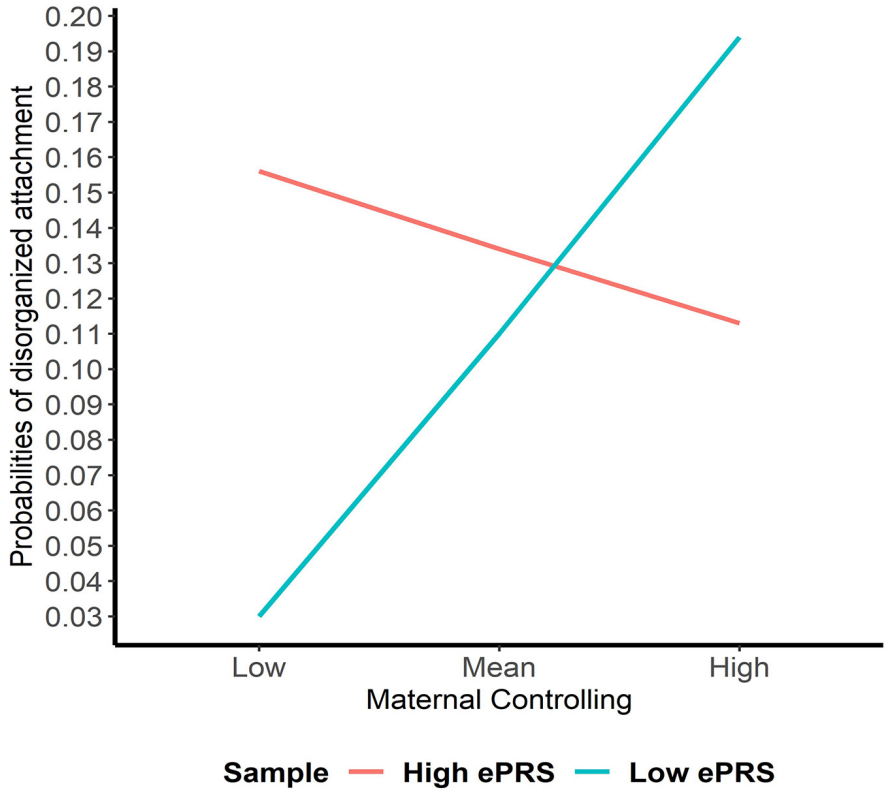

FIGURE 5 | Interaction between Hippocampal Gene Network and Maternal Controlling in Disorganized Attachment. Shows that higher maternal controlling and a high CNR1 ePRS in the hippocampus predicts a lower probability of disorganization. Higher maternal controlling and a low CNR1 ePRS in the hippocampus predicts a higher probability of disorganization.

a greater likelihood of secure attachment in the context of more optimal parental caregiving (i.e., greater sensitivity). Within the hippocampus, a region of the brain known for its involvement in spatial and emotional memory, suboptimal parental caregiving (i.e., greater degrees of controlling and unresponsive parental behavior) predicted a decreased likelihood of disorganized attachment with a high CNR1 ePRS with respect to maternal controlling and a low CNR1 ePRS with respect to maternal unresponsiveness. Our findings offer support for the genetic differential susceptibility to the quality of maternal sensitivity within the context of the CNR1 ePRS in the striatum, as suggested by Belsky (1997), who theorized that children may differ in their receptiveness to parenting influences. However, in the case of the significant interactions between hippocampal CNR1 ePRS and maternal unresponsiveness and controlling in predicting the probability of disorganization, the analyses carried out to confirm differential susceptibility were more suggestive of the diathesis-stress model. The diathesis-stress model suggests that poor developmental experiences (e.g., low-quality parenting) will have the greatest impact on the development of individuals who carry vulnerability factors (e.g., genetic polymorphisms), which are latent diatheses that result in maladaptation when "turned on" by poor environmental experiences (Garmezy et al., 1984; Roisman et al., 2012). These findings are consistent when examining the role that genetics may play in how children form attachments, as other studies have observed that parenting particularly affected children with various polymorphisms of genes that regulate the DA system (i.e., DAT1 9- and 10-repeat and Dopamine Receptor D4 7-repeat) and reward sensitivity (Bakermans-Kranenburg et al., 2008; Bosmans et al., 2020). Our findings further support the notion that multiple genes may make a child more or less susceptible to their caregiving environment (Belsky and Beaver, 2011; Roisman et al., 2012), but in a manner consistent with either differential susceptibility or diathesis-stress, given the brain region under study.

Attachment is a relationship between infants and their caregivers, representing a brain-based biological evolutionary system promoting infant survival (Ainsworth et al., 1978; Chisholm, 1996). In attachment pattern formation, activation is observed among the neurobiological systems underpinning affiliation, reward, and stress management (Ulmer-Yaniv et al., 2016). These observations are likely a result of the intrinsic motivational value that combines the immediate hedonic responses in developing bonds with approach motivation, goal-directed behavior, and learning (Berridge and Robinson, 1998). Our findings related to the CNR1 gene network in the prefrontal cortex, striatum, and hippocampus corroborates the associations between the genetic variations within the ECS and attachment

pattern formations. When primary caregivers/parents provide a supportive environment, infants develop a sense of security, making them feel safe, secure, and protected (Bowlby, 1982; Benoit, 2004; Solomon and George, 2016). In contrast, evidence suggests that disorganized attachment is predicted by sub-optimal parenting and can lead to child behavioral and lifespan mental health problems (Main and Solomon, 1990; Lakatos et al., 2000, 2002; Sroufe, 2005; Puig et al., 2013).

The CNR1 gene networks within the prefrontal cortex, striatum, and hippocampus were chosen to be examined within the context of differential susceptibility, yet findings also pointed to the diathesis-stress model. CNR1 gene has been identified through extensive research as having polymorphisms associated with different observable outcomes (e.g., externalizing behavior and self-regulation) in response to differences in parenting/caregiving qualities (Belsky and Pluess, 2009b; Belsky and Beaver, 2011). In addition, these gene networks were examined within these brain regions because of the existence of convergent projections from the cortex to the striatum, along with hippocampal and amygdala-striatal projections, that places the striatum as a central entry port for processing emotional/motivational information in supporting the development of human attachments (Feldman, 2017). While several studies have focused on the effects of specific variations of these genes in relation to behavior and self-regulation (Belsky and Pluess, 2009b; Belsky and Beaver, 2011; Letourneau et al., 2019), this is the first study to our knowledge that not only examines the associations between these genes and attachment patterns but also utilizes ePRS to predict the probability of disorganized attachment patterns. Our findings suggest that it is important to consider both the ePRS and the brain region when looking at a child's susceptibility to their caregiving environment and provide promise for examining these gene networks in other regions of the brain or other gene networks where a candidate gene approach has been associated with varying attachment patterns and differential susceptibility or diathesis-stress [e.g., dopamine receptor D4 gene (DRD4) and a disorganized attachment pattern; Lakatos et al., 2000; Bakermans-Kranenburg and van Ijzendoorn, 2016].

Attachment theory provides a framework that explains the influence of early social experiences on normal and problematic development (Lakatos et al., 2000). Even in the case of adopted children who are not biologically related to their parents, it was found that early mother–infant interactions and attachment patterns predicted later social-emotional and cognitive development (Stams et al., 2002).

TABLE 5 | Associations among maternal unresponsiveness, hippocampal gene network for ePRS, covariates, and disorganized versus organized attachment pattern.

Variables	Model 1 Adjusted OR (95% CI)	Model 2 Adjusted OR (95% CI)	Model 3 Adjusted OR (95% CI)	Model 4 Adjusted OR (95% CI)
Maternal unresponsiveness	0.88 (0.51, 1.49) $p = 0.629$	–	0.69 (0.36, 1.32) $p = 0.261$	4.45 (0.41, 47.88) $p = 0.218$
Maternal unresponsiveness^2	–	–	–	0.94 (0.88, 1.01) $p = 0.088$
Hippocampal ePRS	–	1.44 (0.79, 2.59) $p = 0.229$	1.57 (0.81, 3.03) $p = 0.182$	1.17 (0.51, 2.68) $p = 0.700$
Maternal unresponsiveness × hippocampal ePRS	–	–	**2.56 (1.29, 5.08)** ***p* = 0.007**	**4.36 (1.78, 10.64)** ***p* = 0.001**
Maternal unresponsiveness^2 × hippocampal ePRS	–	–	–	2.08 (0.95,4.52) $p = 0.065$
Female	1.12 (0.39, 3.19) $p = 0.843$	1.23 (0.40, 3.73) $p = 0.715$	0.96 (0.30, 3.08) $p = 0.949$	0.98 (0.28, 3.40) $p = 0.983$
Birth weight (kgs)	0.24 (0.08, 0.67) $p = 0.006$	0.24 (0.08, 0.77) $p = 0.016$	**0.16 (0.04, 0.61)** ***p* = 0.007**	**0.13 (0.03, 0.53)** ***p* = 0.004**
PC1	–	0.04 (0.00, 149.96) $p = 0.436$	0.05 (0.00, 376.37) $p = 0.509$	(0.00, 196.55) $p = 0.378$
PC2	–	Inf (0.06, inf) $p = 0.109$	Inf (1.99, inf) $p = 0.119$	**Inf (361.76, inf)** ***p* = 0.010**
PC3	–	2.14 (0.01, inf) $p = 0.844$	0.74 (0.001, 2.14 E + 7) $p = 0.940$	0.05 (0.00, 356.35) $p = 0.519$
AIC	103.50	104.64	99.71	98.04
PoI	–	–	0.73	–
Crossover point	–	–	−0.48	–
PA index	–	–	−0.68	–

CI, confidence interval; OR, odds ratio. PC 1, 2, 3, principal component for ancestry; AIC, Akaike Information Criterion; PoI, proportion of interaction; PA, proportion affected; inf, very large CI and OR; bold refers to p < 0.05. Logistic regression modeling for main effects of the unresponsiveness (X variable; Model 1) using ePRS for CNR1 gene networks in the hippocampus (Z variable; Model 2) and their interaction terms (Model 3), adjusting for principal components (PCs) for ancestry and sex of the child. Model fit statistics (AIC) confirmed that Model 3 was optimal. Bolded indicates a p value of less than 0.05.

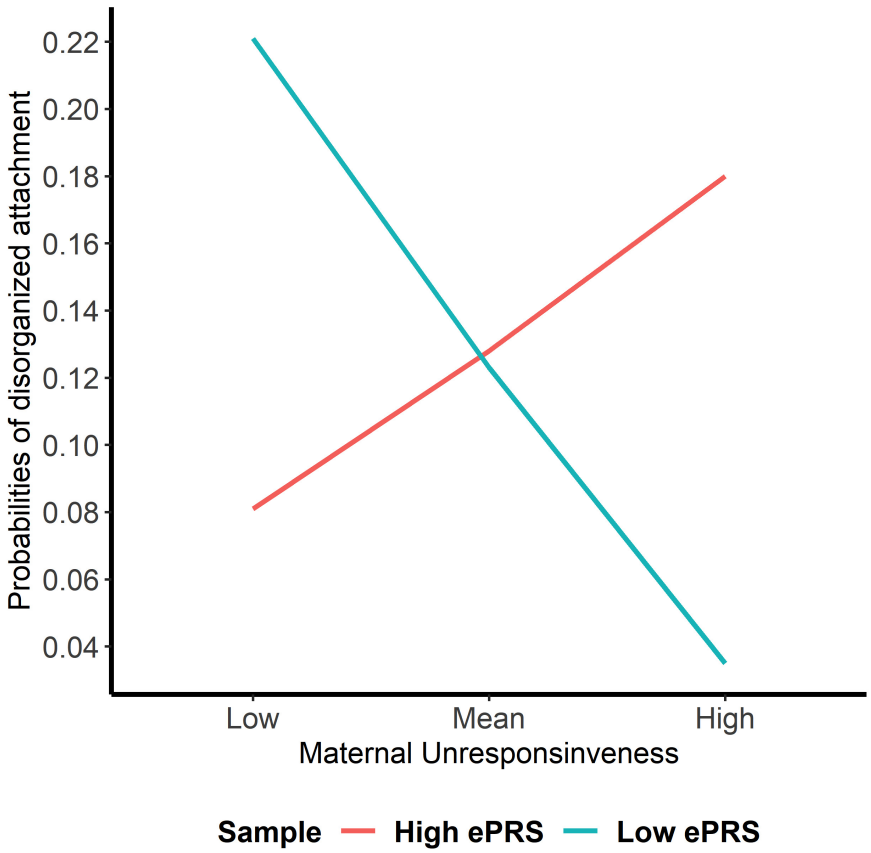

FIGURE 6 | Interaction between Hippocampal Gene Network and Maternal Unresponsiveness in Disorganized Attachment. Shows that high maternal unresponsiveness and a low CNR1 ePRS in the hippocampus predict a lower probability of disorganization. Higher maternal unresponsiveness and a high CNR1 ePRS in the hippocampus predict a higher probability of disorganization.

Disorganized infant-parent attachment has become an area of significant interest to researchers and clinicians due to its clear associations with lifespan developmental and psychological disorders (Newman et al., 2015). We have demonstrated that variations among the CNR1 gene networks in the various brain regions (i.e., prefrontal cortex, striatum, and hippocampus) demonstrated different findings in predicting secure and disorganized attachment (De Wolff and van Ijzendoorn, 1997; van Ijzendoorn et al., 1999; Madigan et al., 2006; Crittenden, 2010; Cyr et al., 2010; Leerkes, 2011; Bailey et al., 2017). Understanding genetic factors that may affect the security of an infant's attachment with the mother may help identify those at risk for attachment disorganization by adding predictive possibility (Bakermans-Kranenburg and van Ijzendoorn, 2007). Failure to consider a child's genotype and differential susceptibility (or diathesis-stress) to experiences (e.g., caregiver sensitivity, responsiveness, and controlling) may pose a barrier to understanding the broader set of predictors of secure attachment pattern and undermine interventions aimed at changing a child's socioenvironmental exposures.

Limitations and Strengths

This study has many strengths, including the prospective design and observational assessments of maternal-child relationship quality (i.e., sensitivity) and attachment patterns; however, there are several limitations to note. First, the sample that we employed for this secondary data analysis is highly educated (69.72% of mothers having a university degree) as compared with the provincial (28.2%) and national (28.5%) averages, which may limit generalizability (Letourneau et al., 2019; Statistics Canada, 2020). Further, the majority of women were married (98.6%) and had household income $\geq$\$70,000 (81.69%). Finally, parity or the presence of siblings for each child was not factored into the analysis, potentially affecting the maternal perception of infant cues, thereby affecting maternal sensitivity (Rutherford et al., 2017). In addition, only "maternal" caregiving quality was assessed; however, rather than seeking to reinforce gender stereotypes, we recognize that primary caregivers may be mothers, fathers, or others. We also recognize that in Canada (Findlay and Kohen, 2012) and in our study (Kaplan et al., 2014), the majority of primary caregivers of infants are mothers.

CONCLUSION

To the best of our knowledge, this is the first study that examines the interaction between maternal parental caregiving qualities (i.e., sensitivity, controlling, and unresponsiveness) and children's ePRS for the CNR1 gene networks in the prefrontal cortex, striatum, and hippocampus in predicting the probability of secure and disorganized attachment patterns in young children. This research provides a foundation to explore genetic susceptibilities to varying caregiving environments in predicting attachment patterns and other outcomes. This research also provides a starting point for exploring other gene networks and influences on children's differential susceptibility to their environments. Promoting secure attachment patterns is a public health goal, as it is associated with lifelong health and a reduced likelihood of all-cause morbidity, chronic inflammation, coronary artery disease, and an array of mental disorders. Further research in the area may allow practitioners to target interventions to support those most at risk for insecure or disorganized attachment, thereby reducing the risk for negative life-long sequelae.

DATA AVAILABILITY STATEMENT

The data generated for this study is subject to the following licenses/restrictions: Privacy and Confidentiality of Participants. Requests to access these datasets should be directed to NL, nicole.letourneau@ucalgary.ca.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Calgary Conjoint Health Research Ethics Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AP-D contributed to data analysis and graphing of genes, wrote and edited the drafts. NL devised the project, conceived the research questions, oversaw data collection, analysis and graphing of figures, and organized, wrote, and edited the drafts. PS contributed by generating the co-expression gene networks, genotyping data QC and polygenic scores calculation. HN conducted the data analysis, described the analysis and results, and prepared all tables. AK and SD reviewed drafts and offered substantive guidance. MH collected data essential to this project and described the measurement. GG contributed to data collection, reviewed drafts, and offered substantive guidance. All authors contributed to the article and approved the submitted version.

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The Interplay Between Prenatal Adversity, Offspring Dopaminergic Genes, and Early Parenting on Toddler Attentional Function

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Background: Few studies have explored the complex gene-by-prenatal environment-by-early postnatal environment interactions that underlie the development of attentional competence. Here, we examined if variation in dopamine-related genes interacts with prenatal adversity to influence toddler attentional competence and whether this influence is buffered by early positive maternal behavior.

Methods: From the Maternal Adversity, Vulnerability and Neurodevelopment cohort, 134 participants (197 when imputing missing data) had information on prenatal adversity (prenatal stressful life events, prenatal maternal depressive symptoms, and birth weight), five dopamine-related genes (*DAT1, DRD4, DRD2, COMT, BDNF*), observed maternal parenting behavior at 6 months and parent-rated toddler attentional competence at 18 and 24 months. The Latent Environmental and Genetic Interaction (LEGIT) approach was used to examine genes-by-prenatal environment-by-postnatal environment interactions while controlling for sociodemographic factors and postnatal depression.

Results: Our hypothesis of a three-way interaction between prenatal adversity, dopamine-related genes, and early maternal parenting behavior was not confirmed. However, consistent two-way interactions emerged between prenatal adversity and dopamine-related genes; prenatal adversity and maternal parenting behavior, and dopamine-related genes and maternal parenting behavior in relation to toddler attentional competence. Significant interaction effects were driven by the *DAT1, COMT*, and *BDNF* genotypes; prenatal stressful life events; maternal sensitivity, tactile stimulation, vocalization, and infant-related activities.

Conclusions: Multiple dopamine-related genes affected toddler attentional competence and they did so in interaction with prenatal adversity and the early rearing environment, separately. Effects were already visible in young children. Several aspects of early maternal parenting have been identified as potential targets for intervention.

Keywords: attention, child, prenatal adversity, dopamine, genes, maternal sensitivity to infant cues, parenting (MeSH)

INTRODUCTION

There is increasing evidence that an adverse prenatal environment contributes to the risk of developing attention-deficit/hyperactivity disorder (ADHD; Banerjee et al., 2007; Thapar and Rutter, 2009). The most commonly studied prenatal risks of ADHD are maternal lifestyle factors, such as smoking, alcohol consumption, substance use, and severe stress/anxiety experienced during pregnancy (Fleming et al., 1988; Banerjee et al., 2007; Li et al., 2010). Further, low birth weight and prematurity at birth—as indicators of a suboptimal intrauterine environment—have also been implicated in the risk for ADHD, particularly inattention symptoms (Bhutta et al., 2002; Strang-Karlsson et al., 2008). However, much less is known about the role of prenatal maternal depression in the development of offspring ADHD symptoms. This is important given that approximately 40% of mothers of children with ADHD have a history of major depression, making them 2–3 times more likely to be depressed than women in the general population (Chronis-Tuscano et al., 2003; Kessler et al., 2006). Furthermore, prenatal depression is consistently linked to shorter gestation and lower birth weight, which are both common risk factors of ADHD (Field et al., 2006; Field, 2011). The available literature suggests that maternal depressive symptoms during pregnancy can negatively shape the offspring's attention system and increase the risk of comorbidity in those children who already have a diagnosis of ADHD (Chronis-Tuscano et al., 2010; Van Batenburg Eddes et al., 2013). Based on the above, in the present study we capture prenatal adversity in three important ways: through the number of stressful life events experienced by women during pregnancy, maternal depressive symptoms during pregnancy, and birth weight of children.

The considerable variability in neurodevelopmental outcomes among children who experience prenatal adversity indicates potential differences in children's vulnerability to the environment. Previous research has highlighted the importance of genetic factors in conferring such vulnerability (Caspi et al., 2002; Rutter, 2006; Laucht et al., 2007). Indeed, gene-environment interactions (G × E) are increasingly recognized as important contributors to the emergence of psychopathology (Caspi and Moffitt, 2006; Rutter, 2006; Belsky et al., 2009). Yet, there have been few published studies examining the contribution of G × E effects to ADHD (Thapar et al., 2007; Nigg, 2012) and even fewer that have specifically focused on the prenatal environment (for a review, see Franke and Buitelaar, 2018). Not surprisingly, these studies have mainly focused on dopaminergic genes, as both pharmacological and genetic research suggest a critical role for dopamine in attentional, motivational, and exploratory neurobehavioral processes (Faraone et al., 2005; Thapar et al., 2007). Regarding attention in particular, animal studies have suggested a direct link between selective lesions of dopaminergic neurons and altered attentional processes in rodents and primates (Nieoullon, 2002; Thiele and Bellgrove, 2018). Based on these studies, the specific attention components that were most affected included selective attention, spatial attention, detection of novelty, and sustained attention (for a review, see Nieoullon, 2002). Notably, the exact result of lesioning dopaminergic neurons in different brain regions depended on the nature of the brain area concerned. As dopamine is mainly present in the frontal cortex and basal ganglia in the brain, it is hypothesized that attention deficits might confer alterations in these subcortical brain structures closely linked to cortical regions rather than simple alterations in dopaminergic transmission (Nieoullon, 2002). Thus, behavioral changes following cortical dopamine depletion have to be interpreted in light of any associated changes in dopaminergic transmission at a subcortical level (Nieoullon, 2002). For instance, methylphenidate, a drug that is most commonly used in the treatment of ADHD by modulating dopaminergic transmission, was found to equally increase frontal cortical activity in both healthy controls and children with ADHD during a response inhibition task, whereas it increased striatal activity only in children with ADHD and decreased it in healthy controls (Vaidya et al., 1998). More directly relevant to our study, genetic variation linked to dopaminergic transmission in both the frontal cortex and related subcortical regions impacted infant attention at age 9 months (Holmboe et al., 2010).

Dopaminergic Genes by Prenatal Adversity Interaction Effects on ADHD

The dopamine transporter *DAT1* gene has been a prime candidate for research in this context. The gene codes for a solute carrier protein responsible for the reuptake of dopamine from the synaptic cleft to the presynaptic neuron. This protein is densely present in the striatum and nucleus accumbens and constitutes the primary mechanism of dopamine regulation in these brain regions (Ciliax et al., 1999). The most widely studied *DAT1* polymorphism is a variable number tandem repeat (VNTR) sequence in the 3′ untranslated region that is 40 base pairs (bp) in length (Vandenbergh et al., 1992). The most common alleles are the 10 (480-bp; 71.9%) and 9 (440-bp; 23.4%) repeats (Doucette Stamm et al., 1995). This polymorphism is believed to be functional, influencing dopamine transporter availability and binding potential (Gizer et al., 2009) and is associated with sustained attention (Loo et al., 2003). *DAT1* has been found to interact with prenatal maternal smoking

(Brookes et al., 2006; Neuman et al., 2007), alcohol consumption (Kahn et al., 2003), and family adversity (Laucht et al., 2007) to increase the risk for ADHD. Some studies reported that the DAT1-prenatal maternal smoking interactions were significant only in boys homozygous for the 10-repeat allele and only for hyperactive-impulsive symptoms (Altink et al., 2008; Becker et al., 2008), while another, smaller study found no interaction effect for *DAT1* and prenatal maternal smoking on ADHD (Langley et al., 2008).

Another popular candidate for G × E studies on ADHD is the dopamine receptor D4 gene (*DRD4*), specifically a 48bp VNTR on exon 3. *DRD4* is predominantly expressed in the frontal lobe, such as the orbitofrontal cortex and anterior cingulate (Floresco and Maric, 2007). The most common alleles of this polymorphism are the 2-, 4-, and 7-repeat alleles, although this varies significantly across ethnic groups (Chang et al., 1996). This VNTR is likely functional in that the 7-repeat allele slightly differs from the 2- and 4-repeat alleles in secondary messenger activity and in response to clozapine, an antipsychotic medication (Asghari et al., 1994, 1995). The VNTR has further been found to influence sustained attention and information processing from an early age (Auerbach et al., 2001; Fan et al., 2003). In terms of its interaction with the environment, results suggest that the 7-repeat allele of *DRD4* exacerbates the effects of prenatal adversity, as reflected in increased risk for ADHD and more severe ADHD symptoms (Grizenko et al., 2012). One study found similar relations but only in the case of teacher-reported inattention symptoms rather than parent-reported ADHD symptoms (Altink et al., 2008). Another, smaller study reported a lack of significant G × E between *DRD4* and any measures of prenatal adversity (i.e., maternal smoking, alcohol use, or child's birth weight (Langley et al., 2008).

An additional dopaminergic gene that has been examined in relation to environmental adversity and ADHD is the dopamine receptor D2 (*DRD2*) gene (Ficks and Waldman, 2009). *DRD2* is expressed in the basal ganglia and prefrontal cortex and is key in regulating the mesolimbic reward system (Usiello et al., 2000). Studies of *DRD2* have tended to focus on a TaqIA restriction site (rs1800497), downstream from *DRD2* located in an exon of a neighboring gene, *ANKK1* (Neville et al., 2004; Grizenko et al., 2012). Nonetheless, this polymorphism is known to influence *DRD2* expression levels (Gluskin and Mickey, 2016). DRD2 has been implicated in affecting selective attention in patients with schizophrenia (Nkam et al., 2017). In terms of G × E interactions involving *DRD2*, ADHD was more prevalent among children whose mothers experienced less stable marital environments (i.e., having had no or multiple marriages) only if they were homozygous for the TaqI-A2 allele (Waldman, 2007).

Another important gene that has been studied in a G × E framework in ADHD is the catechol-O-methyltransferase (*COMT*), which is involved in the degradation catecholamines, such as dopamine. *COMT* has a particularly important role in the frontal cortex, where it accounts for approximately 50–60% of the metabolic degradation of dopamine (Karoum et al., 1994). The gene includes a common functional polymorphism with a methionine ("*met*") to valine ("*val*") substitution at codon 158. The *met* allele is associated with low enzyme activity, while the *val* allele is associated with high enzyme activity (Chen et al., 2004). This polymorphism has been implicated in relation to distractibility (Holmboe et al., 2010) and attentional control (Goldberg and Weinberger, 2004; Blasi et al., 2005; Ciampoli et al., 2017). Regarding its interaction with prenatal adversity among children with ADHD, one study found that those who carried the *COMT* val/val genotype (for rs4680) were more susceptible to the adverse effects of prenatal risks as indexed by lower birth weight to develop early-onset antisocial behavior (Thapar et al., 2005). Furthermore, in a combined analysis of two large cohorts (ALSPAC and PREDO) there was a robust interaction effect of child *COMT* (val/val rs4680) genotype with maternal prenatal anxiety to predict ADHD symptoms assessed at multiple time points (O'Donnell et al., 2017).

Finally, significant G × E effects have also been reported for inattention symptoms involving the brain-derived neurotrophic factor (*BDNF*) gene, which, besides being a regulator of neuronal development and function, plays a role in dopamine neurotransmission (Guillin et al., 2001; Narita et al., 2003). *BDNF* exerts influence on the brain's mesolimbic and corticolimbic reward pathways by modulating their response to dopamine (Guillin et al., 2001). A common polymorphism on the *BDNF* gene in which a valine is replaced by a methionine at codon 66 (Val66Met; rs6265) has been shown to influence the intracellular trafficking and activity-dependent secretion of *BDNF* in brain (Chen et al., 2004). The BDNF gene has been associated with general cognitive performance (Dincheva et al., 2012). Within a G × E context, Lasky-Su et al. (2007) found that in lower SES environments children (6–18-year-old) carrying the risk alleles of rs1013442, rs1387144, or Val66Met was associated with having more inattention symptoms.

Parent-Child Interactions and Their Influence on ADHD

Notably, and perhaps more importantly for clinicians, certain environmental factors have the potential to modify the impact of prenatal adversity in genetically susceptible children (Thomas et al., 2015). Parenting, for instance, is a robust environmental predictor of developmental outcomes in children with ADHD (Deault, 2010). While positive parenting can protect against developing comorbidity in children with ADHD—even when exposed to maternal depression (Chronis-Tuscano et al., 2007)—negative parenting has been associated with elevated ADHD symptomatology above and beyond shared genetic effects (Harold et al., 2013). High levels of negativity in parent-child interactions or reciprocal coercive communication are common in families of children with ADHD (Danforth et al., 1991; Pfiffner et al., 2005; Romirowsky and Chronis-Tuscano, 2014). Sensitive parenting may be particularly effective at buffering the negative effects of prenatal adversity on child cognition and behavior (Laucht et al., 2001; Plamondon et al., 2015; Pickles et al., 2017). Randomized clinical control trials found that parent training promoting positive parent-child interactions was effective in ADHD (Young and Amarasinghe, 2010). Although the exact mechanisms are currently unknown, there is an indication that the positive effects of a more sensitive/less intrusive parenting style on ADHD may be indirect, by supporting

the development of protective mechanisms, such as inhibitory control mechanisms in children (Miller et al., 2019). Importantly, the general recommendation is that for preschool children showing signs of ADHD parent training should be the first line of treatment, and medication introduced only in case when parent training is not effective (Daley et al., 2009; Pelham et al., 2016). Thus, the literature suggests that parents have a key role in the development of their children's attention skills (Gauvain, 2001; Davis and Williams, 2011). Attentional competencies develop through dynamic and continuous interactions between the child and their physical and social surroundings (Vygotsky, 1978; Landry et al., 2002). In this process, parents initially regulate their child's attention through supportive parenting or "scaffolding" until children are able to regulate their own attentional processes (Conner et al., 1997; Gauvain et al., 2001). Failure to develop appropriate attention regulation skills in early childhood can have lasting effects on later development and academic success (Blair, 2002). Although during the preschool years it may be challenging to differentiate delayed regulatory skills from true ADHD, research suggests that, in both cases, parent-child interactions may be key to minimizing later adverse outcomes (Davis and Williams, 2011).

Statistical Issues in Modeling Gene-by-Environment Interaction Effects

To date, most G × E studies on ADHD (and other psychiatric disorders) have considered a single genetic variant and a single environmental exposure at a time, which significantly limits the explanatory value of G × E models for complex phenotypes, such as ADHD. These G × E models often have very small effect sizes and low replication rates (Risch et al., 2009; Lee et al., 2012). One recommended strategy to overcome this limitation is to simultaneously examine multiple candidate genes affecting the same biological pathway (e.g., dopaminergic transmission) as well as multiple relevant environmental factors (Pennington and Bishop, 2009). In a review, Pennington and Bishop (2009) suggested computing composite G and E risk scores across candidate genes and environmental factors and test for G and E main effects and G × E interactions in one omnibus analysis. Then, in case of a significant interaction effect, follow-up analyses should be performed to specify which risk alleles and which environments contribute to the overall effect. However, until now there has been a lack of appropriate statistical methodology to perform such multi-G × E analyses. We recently developed a method for the analysis of complex interactions between multiple genes and environments (Jolicoeur-Martineau et al., 2019, 2020). The Latent Environmental and Genetic InTeraction (LEGIT) approach can be used to construct complex multi-interaction models without the need to estimate an additional parameter for each interaction term, thus improving scalability, especially with higher order interactions. An important limitation of previous G × E models is the lack of information concerning the specific form of the interaction effect (Widaman et al., 2012). For instance, the *diathesis-stress* model assumes that the differences between individuals with and without the "risk" allele of a given genetic variant will manifest only under adverse circumstances, such that individuals carrying the "risk" allele are affected negatively, while those without the "risk" allele remain relatively unaffected by the environment (Belsky et al., 2009). In comparison, the *differential-susceptibility* model posits that individuals carrying the "risk" allele are generally more sensitive to the effects of the environment than those without the "risk" allele (Belsky, 1997; Boyce and Ellis, 2005). Accordingly, compared to those with the non-risk allele, individuals with the "risk" allele exhibit poorer outcomes in negative environments, similar outcomes in average environments, and superior outcomes in positive environments. The LEGIT approach allows us to distinguish between these two theoretical frameworks, which may have important consequences for prevention and intervention strategies.

Here, we use a rich longitudinal dataset to examine how dopaminergic candidate genes simultaneously interact with prenatal adversity, and early parenting to influence toddlers' attentional competence measured longitudinally at two time points. We apply LEGIT—with a $G \times E_1 \times E_2$ design to address this question. Our findings may advance the literature in three important ways. First, we examine the effect of prenatal adversity by including a number of well-established measures of prenatal adversity in one model. Second, we simultaneously consider the modifying effect of multiple dopamine-related genes known to affect the developing human attention system. Third, we complement this by additionally examining important aspects of the early rearing environment that may buffer the negative effects of prenatal adversity in genetically susceptible children. We address these questions using an approach that was specifically designed to deal with the complexity of simultaneously testing multiple interaction effects in relation to an outcome. Due to methodological limitations, few studies to date have attempted to look at the joint contribution of multiple genetic risk variants and multiple environmental exposures (both adverse and protective) to early attention development. This, however, seriously limits our understanding of complex human behavior, which is underlined by the interplay of numerous biological and environmental factors. One novelty of this study is thus the use of LEGIT that enabled the simultaneous testing of a large number of G × E interactions by using latent genetic and environmental features and an alternating optimization algorithm. Another novelty of our study is the inclusion of both macro- and micro-level analytic observations of early maternal behavior. Maternal behaviors included here were analyzed on a second-by-second level within the context of a 20-min mother-infant interaction. Given the time- and labor-intensive nature of collecting such fine-grained data, we are not aware of many G × E studies on early child attention that have used observational measures of early parenting, furthermore both at a macro- and micro-analytic level.

MATERIALS AND METHODS

Participants

The participants were mother-child dyads from the Maternal Adversity, Vulnerability and Neurodevelopment (MAVAN) project, a Canadian community-based prenatal cohort of 590 women and their children in Montreal (Quebec) and

Hamilton (Ontario). Women were recruited in maternity hospitals from 2003 to 2009 during their routine ultrasound examinations. A detailed description of the cohort has been presented elsewhere (O'Donnell et al., 2014). Informed consent was obtained at the time of recruitment and at each time point of data acquisition. Ethics Review Board approval was obtained from the institution of each study site. Retention rates for the MAVAN subjects were 97.4% at 6 months, 84.0% at 18 months, and 80.6% at 36 months. The present study included 134 mother-child dyads with complete data either at 18 or 24 months of child age. The reduction of sample size from 577 to 134 participants is explained by the following: 240 participants had missing genotype data (due to partial funding), 61 participants had missing information on prenatal adversity; seven participants had missing data regarding early parenting; 86 participants had missing information on postnatal maternal depression; 49 participants had missing outcome data at both 18 and 24 months. Thus, the final sample for the complete case analysis included 134 women and their children.

Measures

Genotyping

Child genotype was obtained from buccal swabs. using the TaqMan methods on the ABI-7000 for single nucleotide polymorphism (SNP) markers and ABI-3100 for repeat polymorphisms. To ensure a clear result, any ambiguous genotypes were discarded and the subjects were re-genotyped until the results were unambiguous. Each 20th marker was re-genotyped to check for error rates (0.5%). For the present study, we were interested specifically in genes directly or indirectly related to the dopaminergic system. The five candidate genes included dopamine receptors *DRD2, DRD4*, dopamine transporter *DAT1*, the catechol-o-methyltransferase (*COMT*), and the brain-derived neurotrophic factor (*BDNF*). *DRD2* was captured using SNP rs1800497 (also known as TaqIA) with A as the risk allele (Nyman et al., 2007; Moro et al., 2019). *COMT* was captured using SNP rs4680 with Met as the risk allele (Holmboe et al., 2010; Soeiro-De-Souza et al., 2013). *BDNF* was captured using SNP rs6265 with Val as the risk allele. These SNPs were coded as the number of "risk" alleles divided by two (i.e., 0 for no risk allele, 0.5 for one risk allele, 1 for two risk alleles). *DAT1* was captured using the 40bp VN TR located in exon 15 coded dichotomously as 1 (10R/10R) and 0 when 9R/9R or 9R/10R (Cornish et al., 2005; Holmboe et al., 2010). *DRD4* was captured using the 48bp (VN TR) polymorphism in exon 3 coded dichotomously as 1 (6-8R) and 0 (2-5R), as per Schmidt et al. (2001). Genotype distributions did not deviate from Hardy-Weinberg equilibrium ($p > 0.05$).

Prenatal Life Events

An adapted version of the Prenatal Life Events Scale (Lobel, 1997; Lobel et al., 2000) was used to assess the occurrence of 17 life events (e.g., being robbed, being involved in a serious accident, having someone close die) that women may have experienced during the pregnancy (24–36 weeks). This adapted version did not include those items from the original version of the scale that had an especially low frequency of occurrence. For each event endorsed, participants reported how undesirable or negative the event was on a scale from 0 (not at all) to 3 (very much). Life events that were evaluated as strongly undesirable (i.e., score of 2 or 3) were coded as 1, everything else was coded as 0. Scores were summed to quantify the number of stressful life events. Total scores ranged from 0 to 17, with higher scores indicating the presence of more stressful life events during pregnancy. Not surprisingly, the internal reliability of this scale was low ($\alpha = 0.42$) due to the wide range of possibly unrelated life events.

Prenatal Depressive Symptoms

Women rated their depressive symptoms at 24–36 weeks of pregnancy using the Center for Epidemiologic Studies Depression Scale (CES-D; Radloff, 1977) The CES-D includes 20 items capturing mood-, appetite- and sleep-related symptoms in community-based populations. Each item was rated on a scale from 0 (rarely or none of the time) to 3 (most or all of the time) and the items were summed. Total scores ranged from 0 to 60, with higher scores indicating more severe depressive symptoms. Internal reliability of the CES-D in the present sample was high ($\alpha = 0.92$).

Birth Weight

Children's weight at birth was assessed at the time of delivery (in grams).

Maternal Sensitivity and Parenting Behaviors

When children were 6 months old, maternal sensitivity and maternal parenting behaviors were observed during a 20-min free-play session, which took place in the participant's home and was videotaped for coding purposes. We assessed maternal sensitivity using the Ainsworth Maternal Sensitivity Scales (Ainsworth et al., 1978). This is a validated gold standard, macro-analytic-level measure of maternal sensitivity, focusing on four aspects of early care: sensitivity to infant signals, cooperation vs. interference with ongoing behavior, psychological and physical availability, and acceptance vs. rejection of infant's needs. Scores ranged from 1 to 9, with higher scores indicating more highly involved mothers. Mean inter-rater reliability (intra-class correlation) for the Ainsworth scale ratings was 0.88 ($n = 28$). The four scales were very highly correlated ($r > 0.94$). As such, we used only the sensitivity scale. Maternal parenting behaviors were assessed using the Behavioral Evaluation Strategies and Taxonomies (BEST; Educational Consulting, Inc. Florida, USA; S and K NorPark Computer Design, Toronto). The BEST consists of second-by-second micro-analytic-level frequency ratings and duration measures of maternal and child behaviors (Fleming et al., 1988). Two trained raters scored the duration and frequency of specific behaviors. Inter-rater reliabilities (intra-class correlation) ranged 0.74–0.90 ($n = 18$). Maternal sensitivity and BEST behaviors were coded independently, with coders of one scheme blind to codes on the other. Parental behaviors included maternal attention towards the child, tactile contact between mother and child, maternal vocalization, and mother-child activities. These measures have been used in our past research (Krpan et al., 2005; Giardino et al., 2008; Wazana et al., 2015; Graffi et al., 2018). For the purpose of this study, the duration of the respective maternal behaviors was first

transformed into percentages of the total duration the mother spent interacting with her child, which excluded the time spent feeding, talking to someone else, or where the dyad was obscured. Percentages were subsequently z-standardized and averaged to form a score on the following dimensions:

1. Attention: focused (i.e., concentrated) looking at the infant, unfocused (i.e., unconcentrated) looking at infant or focused looking at an infant-related object (i.e., joint attention), "mother and infant are focusing on the same object"
2. Tactile stimulation: kissing, poking/tickling, mouthing/raspberries, stroking/patting
3. Vocalization: humming/singing, talking, laughing/smiling
4. Infant-related activities: social games, showing toy, play with a toy, play without a toy, rocking/jiggling, grooming the infant

Child Attentional Competency

Attentional competency was assessed using the Attention subscale from the Competence domain of the Infant-Toddler Social and Emotional Assessment (ITSEA) at 18 and 24 months (Briggs-Gowan and Carter, 1998, 2007). The ITSEA is a developmentally and clinically sensitive parent-rated questionnaire of social-emotional problems and competencies in 1–3 year-olds (Briggs-Gowan and Carter, 1998). The Attention subscale is formed by summing five items assessing attentional function, such as "plays with toys for 5 min or more," "looks at things for a minute or longer." Internal consistency of the Attention subscale in the present sample was good (Cronbach's alpha = 0.76 at 18 months and 0.74 at 24 months). Scores were distributed evenly across the range of possible values (0–2) at both time points, values were higher at 24 months (M = 1.43, SD = 0.45) than 18 months (M = 1.29, SD = 0.51), with moderate consistency over time ($ICC_{(3,1)}$ = 0.57, $ICC_{(3,k)}$ = 0.73). Outcome scores were divided by 2 to rescale them between 0 and 1. Using a linear model (LM) with a constrained outcome variable is problematic as model predictions could go beyond the observed range. Therefore, we used a generalized linear model (GLM) with a Quasi-binomial family, which ensures that the outcome is constrained to the range [0, 1] instead of being unconstrained, such as when using a Gaussian family.

Covariates

Covariates included child sex, maternal age at delivery, and maternal education ("high school or less," "some college, completed college, or some university," and "university graduate or more"). We additionally included a covariate that indicated whether the child had available data on attentional competency at 24 months to adjust for the fact that baseline attentional competency was significantly better at 24 months (β = 0.18, S = 9,862.5, $p < 0.0001$). The intercept β_0 of the model represents attentional competency at 18 months, while $\beta_0 + \beta_{24M}$ together represents attention competency at 24 months. All continuous variables were standardized except for maternal age.

Statistical Analysis

Descriptives

Hardy-Weinberg equilibrium of genotype distribution was tested using exact tests (Engels, 2009). Since most continuous variables used in this study are non-normally distributed, we used non-parametric tests to describe the characteristics of our sample. We used chi-square tests for categorical-by-categorical, Wilcoxon rank-sum tests for binary-by-continuous, and Wilcoxon signed-rank tests for paired comparisons. We examined correlations between variables used in analyses using Kendall's tau coefficients.

Main Analyses

Data were analyzed using the LEGIT R (Jolicoeur-Martineau et al., 2019) with a repeated measures design to predict attentional competency at 18 and 24 months. We fitted a 3-way $G \times E_1 \times E_2$ interaction model where G is a weighted sum (i.e., latent score) of the five dopamine-related candidate genes (i.e., *DRD2, DRD4, BNDF, COMT, DAT1*), E_1 is a weighted sum of our three prenatal adversity variables (i.e., prenatal maternal stressful life events, prenatal maternal depressive symptoms, and birth weight), and E_2 is a weighted sum of all early maternal parenting behaviors (i.e., Ainsworth sensitivity, maternal attention, tactile stimulation, vocalization, and infant-related activities). A schematic representation of the proposed three-way interaction model is shown in the **Supplementary Figure 1**. Further information on how the latent sum of G, E_1, E_2, and their interactions were calculated is provided as **Supplemental Material**.

Treatment of Missing Data

Missing information was imputed for participants that had at least one measure available for each latent score (i.e., G, E_1, and E_2), and the outcome variable at either 18 or 24 months (N = 197). All analyses were performed on both the complete cases (N = 134) and the imputed dataset (N = 197). Given that our model included interaction terms, traditional imputation methods which do not account for non-linearities are bound to be biased (Seaman et al., 2012). Thus, we used missForest (Stekhoven and Buhlmann, 2012), which has been shown to outperform the popular multiple imputation method by chained equations (mice) with predictive mean matching (pmm; Buuren and Groothuis-Oudshoorn, 2010). Furthermore, the imputation accuracy of MissForest has been shown to approach state-of-the-art modern imputation techniques (Yoon et al., 2018; Payrovnaziri et al., 2020).

Similar to many complex, non-linear methods, it is not possible to pool estimates from multiple imputations using LEGIT. As the signs of the parameters inside the latent scores may differ randomly (models with the same parameters, but with different signs can be equivalent), pooling across multiple LEGIT models would lead to a regression of the parameters towards zero. Moreover, given the various parameters involved, it is difficult to know which sign is the correct one. All these can make pooling highly inconsistent, if not impossible. In addition, performing variable selection is unfeasible using multiple imputations. For the above reasons, we used a single imputation method called missForest. Contrary to other methods, such as mice, MissForest produces similar imputations when using different random seeds.

Variable Selection

To be more parsimonious, we can apply variable selection to retain only the most important elements in each latent score (G, E_1, E_2). Unfortunately, quality-of-fit measures like the Akaike information criterion (AIC; Akaike, 1998), corrected Akaike information criterion (AICc; Hurvich and Tsai, 1989), and Bayesian information criterion (BIC; Schwarz, 1978) are not defined in GLMs of quasi-binomial family. This means that we cannot use variable selection with these fit measures. Consequently, the variable selection was performed in the LM models, and the retained variables were included in the GLM models of the quasi-binomial family. Variables selected in the LM models generally remained significant in the GLM models, and their relative contribution did not change meaningfully. Variable selection was performed using "parallel natural evolutionary variable selection" available within LEGIT. Models with the lowest AICc value were considered as best fitting the data. Results from the models both with and without variable selection are presented.

In-Sample and Out-of-Sample Effect Sizes

To further assess model fit, we also examined the in-sample effect size and out-of-sample effect size. In-sample effect size was estimated using the regular R^2, out-of-sample effect size (which measures how well the model generalizes to new observations) was estimated using the leave-one-out cross-validated (LOOCV) R^2. The LOOCV was calculated in the same way as the R^2 with the exception that the predictions for a given participant were obtained from a model that did not include the participant in question.

Data analysis was carried out in version 9.4 of the SAS System for Windows (Copyright © 2002–2012, SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA). Graphical outputs and imputations were generated using R version 3.2.5 (R Development Core Team., 2016).

RESULTS

Descriptive Analyses

Sample characteristics are shown in **Table 1**; correlations between the predictors, outcomes and covariates are shown in **Table 2**. Attentional competence at 18 and 24 months were highly correlated ($r_{(103)} = 0.50$, $p < 0.0001$). Attentional competence at 18 months was positively associated with maternal sensitivity ($r_{(120)} = 0.14$, $p = 0.04$) and negatively associated with postnatal depressive symptoms ($r_{(120)} = -0.13$, $p = 0.04$). Attentional competence at 24 months was negatively associated with birth weight ($r_{(117)} = -0.14$, $p < 0.05$), prenatal depressive symptoms ($r_{(117)} = -0.22$, $p = 0.01$), and postnatal depressive symptoms ($r_{(117)} = -0.28$, $p = 0.0002$). Prenatal depressive symptoms were positively associated with prenatal life events ($r_{(134)} = 0.24$, $p < 0.0001$) and postnatal depressive symptoms ($r_{(134)} = 0.41$ $p < 0.0001$). Maternal parenting measures were not significantly associated with each other, except for vocalization, which was positively related to infant-related activities ($r_{(134)} = 0.16$, $p = 0.006$) and maternal sensitivity ($r_{(134)} = 0.19$, $p = 0.003$). Significant gene-environment correlations were observed between *DRD2* and birth weight ($r_{(134)} = -0.16$, $p = 0.02$) and between *DRD4* and prenatal depressive symptoms ($r_{(134)} = -0.15$, $p = 0.04$) and vocalization ($r_{(134)} = 0.15$, $p = 0.03$).

Three-Way Interaction Models

In the complete-case analysis, the G × E_1 × E_2 interaction effect emerged significant ($\beta = -17.17$, SE = 3.50, $p < 0.0001$). However, this was not replicated in the imputed analysis ($\beta = -0.87$, SE = 1.31, $p = 0.51$). Although both the complete-case and imputed data analyses had relatively large in-sample effect sizes ($R^2 = 0.31$ and 0.26, respectively), their out-of-sample effect sizes were very low (LOOCV $R^2 = -0.16$ and 0.07, respectively), indicating poor generalization. The negative LOOCV R^2 and the fact that the three-way interaction effect was only significant in the non-imputed analysis are strongly suggestive of model overfitting. For these reasons, we reran all analyses without the three-way interaction term but retaining all two-way (i.e., G × E^1, G × E_2, E_1 × E_2) interaction terms. Results of the three-way interaction models are shown in **Table 3**.

Two-Way Interaction Models

Results of the two-way interactions models are shown in **Table 4**. All two-way interaction effects were significant in both the complete-case and imputed analyses ($p < 0.0001$). In the complete-case full model, *DAT1* ($\beta = 0.15$, SE = 0.05, $p = 0.002$), *BDNF* ($\beta = 0.25$, SE = 0.12, $p = 0.04$), and *COMT* ($\beta = -0.52$, SE = 0.09, $p < 0.0001$) seemed to be the most important genetic drivers of the observed interaction effects. Among the prenatal adversity factors, maternal stressful life events emerged as most important for the interaction ($\beta = 0.87$, SE = 0.15, $p < 0.0001$). Regarding early maternal parenting, tactile stimulation ($\beta = 0.22$, SE = 0.08, $p = 0.01$), vocalization ($\beta = 0.32$, SE = 0.09, $p = 0.0003$), and infant-related activities ($\beta = 0.40$, SE = 0.11, $p = 0.0003$) seemed to be the most relevant in interacting with dopamine-related genes or with prenatal adversity. The model had moderate effect size (in-sample $R^2 = 0.32$, LOOCV $R^2 = 0.03$), interaction effects are visualized in **Figure 1**.

In the variable selection model, *DAT*1 ($\beta = 0.30$, SE = 0.07, $p < 0.0001$), and *COMT* ($\beta = -0.70$, SE = 0.11, $p < 0.0001$) were retained for the genetic component; prenatal stressful life events ($\beta = 1$) for the adversity component; and maternal vocalization ($\beta = 0.40$, SE = 0.12, $p = 0.0008$), maternal infant-related activities ($\beta = 0.50$, SE = 0.15, $p = 0.001$), and maternal sensitivity ($\beta = 0.09$, SE = 0.05, $p = 0.10$) for the early maternal parenting component. The effect size of the model with variable selection was moderate (in-sample $R^2 = 0.41$ and LOOCV $R^2 = 0.17$).

A very similar picture emerged in the imputed models. In the full model without variable selection, maternal sensitivity emerged as an additional important early parenting behavior for the observed interactions ($\beta = 0.07$, SE = 0.03, $p = 0.03$). In the variable selection model, *DAT1* ($\beta = 0.12$, SE = 0.04 $p = 0.005$), *BNDF* ($\beta = 0.37$, SE = 0.10, $p = 0.0002$) and *COMT* ($\beta = 0.50$, SE = 0.07, $p < 0.0001$) were retained for the genetic component; maternal stressful life events for the

TABLE 1 | Demographic characteristics of MAVAN participants.

***N* = 134 (*n* = 103 at both time points, *n* = 17 at 18 months only, *n* = 14 at 24 months only)**	**M (SD) or N (%)**
Maternal characteristics	
Age	30.46 (4.75)
Education	
High school or less and partial college	21 (15.67%)
Completed college or some university	
University graduate or higher	45 (33.58%)
Income (CAD)	
<15,000	68 (50.75%)
15,000–<30,000	
30,000–<50,000	4 (3.15%)
50,000–<80,000	16 (12.60%)
>80,000	30 (23.62%)
Prenatal depressive symptoms	36 (28.35%)
Postnatal depressive symptoms	41 (32.28%)
Maternal infant-related attention	11.94 (9.43)
Maternal tactile stimulation	11.06 (8.84)
Maternal vocalization	0.04 (0.29)
Maternal infant-related activities	−0.01 (0.51)
Maternal sensitivity	0.05 (0.47)
	0.03 (0.42)
	5.64 (1.92)
Child characteristics	
Gender (boys)	60 (44.78%)
Birth weight (g)	3329.02 (442.37)
DRD2 (Number of A1 alleles)	0: 85(63.43%), 1: 43(32.09%), 2: 6(4.48%)
DRD4	2–5 Repeat: 90 (67.16%), 6–8 Repeat: 44(32.84%)
DAT1	9R/9R or 9R/10R: 59(44.03%), 10R/10R: 75(55.97%)
BDNF (Number of Val Allele)	0: 90(67.16%) / 1: 38(28.36%) / 2: 6(4.48%)
COMT (Number of Met allele)	0: 35(26.12%) / 1: 71(52.99%) / 2: 28(20.90%)
Attentional competence at 18 months	1.29 (0.51)
Attentional competence at 24 months	1.43 (0.45)

TABLE 2 | Kendall-Tau correlation matrix of all variables included in analyses.

Variables	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1. Attention at 18 months	1	–	–	–	–	–	–	–	–	–	–	–
2. Attention at 24 months	0.50***	1	–	–	–	–	–	–	–	–	–	–
3. Prenatal depression	−0.05	−0.22***	1	–	–	–	–	–	–	–	–	–
4. Postnatal depression	−0.13*	−0.28***	0.41***	1	–	–	–	–	–	–	–	–
5. Prenatal life events	0.04	0.06	0.24***	0.14*	1	–	–	–	–	–	–	–
6. Birth weight (g)	−0.12	−0.14*	0.01	0.01	−0.06	1	–	–	–	–	–	–
7. Maternal age	0.08	0.09	−0.05	−0.06	−0.01	0.02	1	–	–	–	–	–
8. Maternal infant-related attention	0.05	−0.04	0.04	0.06	−0.03	−0.10	0.02	1	–	–	–	–
9. Maternal tactile stimulation	0.09	0.08	0.00	0.03	0.02	−0.08	−0.02	−0.02	1	–	–	–
10. Maternal Vocalization	0.05	0.10	−0.11	−0.01	0.01	−0.04	0.09	0.07	0.07	1	–	–
11. Maternal infant-related activities	0.08	−0.03	−0.02	0.02	−0.10	−0.04	−0.11	−0.04	0.06	0.16	1	–
12. Maternal sensitivity	0.14*	0.07	−0.07	−0.09	−0.06	0.09	0.09	0.08	0.07	0.19**	0.05	1
13. *DRD2* genotype	0.02	0.04	0.06	0.07	−0.07	−0.16*	0.11	0.11	−0.02	0.02	0.05	0.06
14. *DRD4* genotype	0.10	0.02	−0.15*	−0.10	−0.10	0.05	0.13	−0.06	−0.10	−0.10	0.16*	0.11
15. *DAT1* genotype	−0.04	−0.02	0.01	−0.01	−0.07	−0.09	−0.00	−0.07	0.00	0.02	0.07	0.01
16. *BDNF* genotype	−0.04	−0.14	−0.06	−0.05	−0.05	−0.01	−0.04	−0.08	−0.04	−0.04	−0.04	0.06
17. *COMT* genotype	0.07	0.11	−0.07	−0.07	−0.05	0.04	0.05	−0.05	−0.05	−0.05	−0.07	−0.05

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

adversity component ($\beta = 1$); and maternal sensitivity ($\beta = 0.07$, SE = 0.03, $p = 0.03$), tactile stimulation ($\beta = 0.22$, SE = 0.06, $p = 0.0006$), vocalization ($\beta = 0.30$, SE = 0.07, $p < 0.0001$), and infant-related activities ($\beta = 0.41$, SE = 0.10, $p < 0.0001$) for early maternal parenting. Effect sizes of both models were moderate (in-sample $R^2 = 0.25$, LOOCV $R^2 = 0.10$ for the full

TABLE 3 | Predicting toddler attentional competence at 18 and 24 months based on the three-way interaction of prenatal adversity, dopamine-related genes, and early maternal parenting behaviors with/without imputation.

	Without Imputation Nobs = 134, *N* = 237	With Imputation Nobs = 197, *N* = 394
Predictors		
Intercept	−0.05	−0.01
24 months present	0.33*	0.40***
Maternal age	0.04*	0.03*
Postnatal depression	−0.21**	−0.28***
Boys	−0.14	−0.23*
Maternal education (college)	−0.18	−0.30
Maternal education (university)	−0.15	−0.37*
Prenatal adversity (E_1)	0.60**	0.03
Dopamine-related genes (G)	−1.19**	−0.79**
Early maternal parenting (E_2)	1.91***	0.45*
$E_1 \times G$	−1.25	−1.26***
$E_1 \times E_2$	3.00***	1.01***
$G \times E_2$	−7.23***	−5.07***
$G \times E_1 \times E_2$	−17.17***	0.87
G		
DRD2	0.21***	0.00
DRD4	0.02	0.05
DAT1	0.05	0.11*
BDNF	0.57***	0.35***
COMT	0.16***	−0.49***
E_1		
Prenatal depressive symptoms	0.16	0.00
Prenatal stressful life events	0.40***	0.85***
Birth weight (g)	−0.44***	−0.14
E_2	0.19	0.00
Infant-related attention	0.28***	0.22***
Tactile stimulation	−0.02	0.30***
Vocalization	0.23	0.41***
Infant-related activities	0.28***	0.08*
Maternal sensitivity		
R^2	0.31	0.26
LOOCV R^2	−0.16	0.07

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

model; in-sample $R^2 = 0.25$, LOOCV $R^2 = 0.14$ for the model with variable selection).

DISCUSSION

In a prospectively followed prenatal cohort, we examined the complex interplay between three important forces of attention development: 1) genetic variations in the dopaminergic pathway (using a genetic score composed of five dopamine-related genes), prenatal adversity (captured through children's birth weight, the presence of prenatal maternal depressive symptoms and stressful life events), and the earliest rearing environment (captured through a range of observed maternal parenting behaviors). Our study benefitted from a sample with rich measures on prenatal adversity, dopamine-related gene variants, observational measures of maternal parenting behavior, and repeated assessments of toddlers' attentional competence. A further strength of our study was the use of a statistical approach (LEGIT) to simultaneously analyze complex G × E interactions, which provides greatly enhanced power over traditional models that analyze a single G × E effect at a time.

Our hypothesis of finding a three-way interaction effect for prenatal adversity, dopamine-related genes, and early maternal behavior on toddlers' attentional competency was not confirmed. Although the complete case analysis indicated the presence of such an interaction effect, the model did not generalize, and when imputing missing observations, the interaction effect was not significant anymore. These observations point to possible model overfitting, especially in smaller samples. In line with this, when we reran the analysis without the three-way interaction term, a more consistent picture emerged. Significant two-way interaction effects emerged for prenatal adversity by dopamine-related genes; prenatal adversity by early maternal behavior; and dopamine-related genes by early maternal behavior on toddler attentional competence in both the complete case analysis and analysis with imputation for missing data. Furthermore, the in- and out-of-sample effect sizes also indicated that the model generalizes.

Our findings suggest that multiple dopamine-related genes interact with prenatal adversity to predict toddler attentional competence. Based on our models, *DAT1*, *COMT*, and *BDNF* emerged as the most significant among the genes tested. Previously, *DAT1* has been one of the most consistently implicated candidate genes in relation to ADHD by linkage, association, and meta-analytic studies (Sharp et al., 2009). Importantly, *DAT1* genotype has been linked to variation

TABLE 4 | Predicting toddler attentional competence at 18 and 24 months based on two-way interactions between prenatal adversity, dopamine-related genes, and early maternal parenting behaviors with/without imputation and with/without variable selection.

	Without Imputation Nobs = 134, *N* = 237		With Imputation Nobs = 197, *N* = 394	
Predictors	**All**	**Best choice**	**All**	**Best choice**
Intercept	0.19	0.52	−0.10	−0.02
24 months present	0.31*	0.30*	0.40***	0.40***
Maternal age	0.03	0.02	0.04**	0.03*
Postnatal depression	−0.35***	−0.31***	−0.28***	−0.28***
Boys	−0.31*	−0.37**	−0.22*	−0.24*
Maternal education (college)	−0.34	−0.28	−0.29	−0.31
Maternal education (university)	−0.25	−0.25	−0.36*	−0.37*
Prenatal adversity (E_1)	0.11	0.06	0.06	0.03
Dopamine-related genes (G)	−0.91*	−0.44	−0.85**	−0.83**
Early maternal parenting (E_2)	0.69*	0.55*	0.51**	0.40*
$E_1 \times G$	−2.02***	−1.44***	−1.31*	−1.09***
$E_1 \times E_2$	1.48***	0.85**	1.00***	0.83***
$G \times E_2$	−6.34***	−2.99***	−5.67***	−4.88***
G				
DRD2	−0.04		0.02	
DRD4	0.03		0.06	
DAT1	0.15**	0.30**	0.10*	0.12**
BDNF	0.25*		0.35***	0.37***
COMT	−0.52***	−0.70***	−0.47*	−0.50***
E_1				
Prenatal depressive symptoms	0.01		−0.00	
Prenatal stressful life events	0.87***	1***	0.84***	1***
Birth weight (g)	−0.12		−0.16	
E_2				
Infant-related attention	−0.02		−0.00	
Tactile stimulation	0.22*		0.24***	0.22***
Vocalization	0.32***	0.40***	0.30***	0.30***
Infant-related activities	0.40*	0.50**	0.40***	0.41***
Maternal sensitivity	0.04	0.09	0.07*	0.07*
R^2	0.32	0.41	0.25	0.25
LOOCV R^2	0.03	0.17	0.10	0.14

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

in both cognitive and neurobiological measures of attention (Gizer et al., 2009; Sharp et al., 2009). Moreover, a number of environmental factors have been hypothesized to moderate the effect of *DAT1* on ADHD-related phenotypes (Franke and Buitelaar, 2018). Some of these include prenatal factors, such as maternal smoking and alcohol use during pregnancy, prenatal maternal stress, and birth weight (for a review, see Franke and Buitelaar, 2018). Contrary to our findings, the single study that looked at *DAT1* by prenatal stress interaction effect on ADHD reported a lack of such effect (Grizenko et al., 2012). However, that study used a retrospective design to collect information on prenatal maternal stress when children were 6–12 years old. The two studies that assessed interactions of *DAT1* with birth weight reported nominally significant effects on the occurrence of conduct problems in children with ADHD in a case-only study (Langley et al., 2008) and significant G × E effects for a genetic index including *DAT1, DRD4*, and *DRD2* and birth weight on ADHD symptoms in a sibling sample (Jackson and Beaver, 2015). Importantly, certain aspects of parenting were also shown to interact with *DAT1* in relation to ADHD-related phenotypes. These include parental expressed emotions, negative and positive parenting practices, and maternal warmth (for a review, see Franke and Buitelaar, 2018). In summary, our study supports prior evidence for the involvement of *DAT1* in ADHD-related phenotypes in interaction with either the prenatal or postnatal environment.

Although a recent meta-analysis did not confirm the main effect of *COMT* gene variants on ADHD, it could not rule out the importance of *COMT* in combination with other factors (Sun et al., 2014). Indeed, in a combined analysis of two large cohorts (ALSPAC and PREDO), prenatal anxiety and child *COMT* genotype predicted ADHD symptoms at multiple time points (O'Donnell et al., 2017). In addition, *COMT* genotype also seemed to interact with prenatal maternal smoking to predict aggressive behavior and autistic symptoms in children with ADHD (Nijmeijer et al., 2010; Brennan et al., 2011) and interact with birth weight to predict antisocial behavior in children with ADHD (Thapar et al., 2005). Interactions of the *COMT* gene with parenting behavior have not been investigated to our knowledge in relation to ADHD. In summary, our findings are in line with previous literature suggesting an interplay between COMT and the prenatal environment to shape ADHD-related phenotypes. Furthermore, we add to the existing literature by showing that a

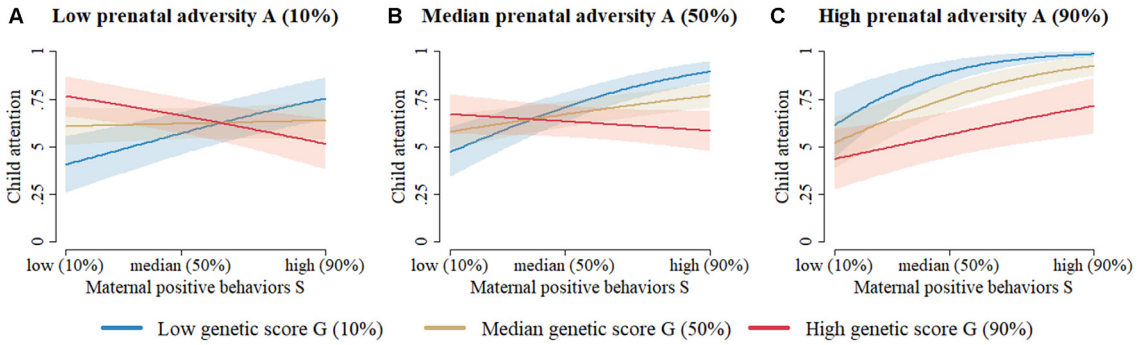

FIGURE 1 | The prediction of toddler attentional competence at 18–24 months based on the two-way interaction model (**Table 4**) without missing data imputation and without variable selection (column 1). **(A)** When prenatal adversity is low, attentional competence of young children with low dopaminergic genetic scores increases from low to high with increasingly positive early maternal parenting behavior. Meanwhile, children with moderate or high dopaminergic genetic scores start at a relatively high level of attentional competence, which seems to be unaffected by an increase in early positive maternal parenting behaviors. **(B)** When prenatal adversity is moderate, young children with low dopaminergic genetic scores start at moderate levels of attentional competence, which rapidly increases as positive early maternal parenting behavior increases. Meanwhile, children with moderate or high dopaminergic genetic scores start at a high level of attentional competence, which seems to be unaffected by an increase in early positive maternal parenting behaviors. **(C)** When prenatal adversity is high, young children with low dopaminergic genetic scores start at a high level of attentional competence, which seems relatively unaffected by increasingly positive early maternal parenting behaviors. Children with moderate dopaminergic genetic scores initially have moderate levels of attentional competence, which increases linearly as positive early maternal parenting behavior increases. Children with high dopaminergic genetic scores initially have low levels of attentional competence, which also increases linearly as early positive maternal parenting behaviors increase. *Note*. Figures for the two-way interaction models with/without imputation and with/without variable selection all look very similar. *Note 2*. Despite the absence of a three-way interaction effect, results must be graphically represented similarly to a three-way interaction model, since all three main components (i.e., G, E_1, and E_2) interact with one another in two-way interactions within the same model.

genetic index including *COMT* interacts with maternal parenting behavior to affect the attentional competence of young children.

Variants in the *BDNF* gene have also been implicated in ADHD-related phenotypes both as exerting a main effect (Langley et al., 2008; Li et al., 2014; Luo et al., 2020) and in interaction with environmental stressors, such as early deprivation or family SES (Lasky-Su et al., 2007; Gunnar et al., 2012) in both European and Asian populations. G × E studies of ADHD-related phenotypes involving the *BDNF* gene, however, are still rare. One interesting study examined the interaction of the *BDNF* Val66Met polymorphism and parenting in children (aged 6–15 years) diagnosed with ADHD and found a significant interaction effect for child *BDNF* by mothers' positive feelings about caring in relation to the development of internalizing comorbidities (Park et al., 2014).

We further found that prenatal adversity interacted with both dopamine-related genes and maternal parenting behavior in affecting toddler attentional functioning. There is growing evidence for the involvement of prenatal adversity in the risk for developing ADHD-related phenotypes (Glover, 2011; Graignic-Philippe et al., 2014), although there is currently insufficient support for a causal relationship (Sciberras et al., 2017). The most commonly researched adversities in relation to ADHD include maternal prenatal smoking, alcohol and substance use, maternal stress, and offspring birth weight (Morgan et al., 2018). Unfortunately, we were unable to investigate the effects of prenatal smoking, alcohol, and substance use as these variables had an extremely large proportion of missing data in our cohort. Nevertheless, we did examine the effects of prenatal maternal stress and birth weight, as well as maternal prenatal depressive symptoms, which have also been consistently implicated in the development of maladaptive child outcomes (Madigan et al., 2018). Of all prenatal adversities considered here, maternal prenatal stress seemed to be the most relevant component when considering offspring dopamine-related genes. This finding is partly in line with a recent study that reported significant G × E effects for prenatal maternal stress and children's *DRD4* genotype but not *DAT1* on ADHD symptoms (Grizenko et al., 2012). However, as both this and our study show, not all children exposed to prenatal adversity will experience later difficulties. Constitutional characteristics, such as genetic variation may be key in determining who will be more susceptible to the deleterious effects of the environment, as is contended by the diathesis-stress or differential susceptibility hypotheses (Belsky, 1997, 2005; Ingram and Luxton, 2005).

Another noteworthy finding of this study is that early positive maternal behavior seemed to buffer the effect of both prenatal adversity and genetic susceptibility although not their joint effect on toddler attentional competence. The observation that positive maternal behavior may attenuate both environmental and genetic risks is in line with previous literature (Sonuga-Barke and Harold, 2018) and has important consequences for guiding interventions such as behavioral parent training programs for families with ADHD. Despite the strong evidence in support of a biological basis for ADHD symptoms, researchers have speculated that the child's environment may play a particularly salient role in determining outcomes for children with ADHD, even if environmental factors may not be the primary cause of their core symptoms (Barkley, 2006). During infancy, the caregiver provides much of the child's attention regulation through orienting (Posner et al., 2014). This external control eventually becomes internalized as toddlers gradually gain

control over their own emotional and cognitive states through self-regulation (Posner et al., 2014). Therefore, understanding the ways in which parents can help their children better regulate their attention, emotions, and behavior is going to be invaluable for the success of behavioral parent training programs, for parents typically play a major role in changing their child's behavioral symptoms (e.g., through parent training and behavior therapy programs; Johnston and Mash, 2001; Deault, 2010). A newly emerging field of "therapy genetics" has produced some promising results to this end. In one study among a large group of toddlers with externalizing problems, the largest effect for a video-feedback-based intervention promoting positive parenting and sensitive discipline was found in children carrying the *DRD4* 7R allele (Bakermans-Kranenburg et al., 2008). In another smaller pilot study of children with ADHD, the largest effects of a behavioral parent training program were seen in children not homozygous for the *DAT1* 10R allele (van den Hoofdakker et al., 2012).

Our study also pinpointed a number of specific maternal behaviors that were linked with improved attentional competence in toddlers, such as maternal sensitivity, tactile stimulation, vocalization, and activities including play and grooming. These behaviors emerged from coded observations of mother-child interactions rather than maternal self-reports. The over-reliance on self-report questionnaires for assessing parenting behavior may limit both the validity and reliability of the parenting behaviors being assessed. In addition, most prior studies tended to isolate one or two parenting behaviors, rather than examining several parenting measures simultaneously to explore if more robust associations exist that go beyond specific measures of parenting (Deault, 2010).

ADHD has classically been viewed as a primarily fixed cognitive "deficit," mainly underlined by genetic and neurobiological mechanisms (Barkley, 1990; Weiss and Hechtman, 1993; Hinshaw, 1994). However, this view falls short in accounting for the way environmental and biological risk factors seem to interact to produce the diverse developmental pathways, clinical outcomes, and frequent comorbidities observed in ADHD (Mannuzza et al., 2004; Castellanos et al., 2005; Halperin et al., 2008). As a result, researchers have recently turned to the biopsychosocial framework to better explain the complex developmental processes underlying the pathophysiology of ADHD (Singh, 2008). Contrary to the fixed deficit model, the biopsychosocial theory posits that ADHD is caused by the interplay of genetic and environmental influences that occur throughout development in underlying neurobiological systems (Sonuga-Barke, 1998, 2009). Accordingly, the original risk for developing the disorder can be moderated by later factors that alter the trajectory of development for better or worse (Taylor, 1999; Singh, 2008). Understanding these moderating influences—both protective and harmful—is essential for predicting key features of the disorder, such as its emergence, persistence, offset, and the frequent development of comorbidities (Sonuga-Barke, 2009). In line with this thinking, here we reported that certain positive aspects of the early maternal behavior moderated the negative impact of both prenatal adversity and genetic susceptibility on toddlers' attentional competence, albeit not their joint effect.

Although our findings were mainly interpreted in relation to the pathophysiology of ADHD, it was done so, since the overwhelming majority of available G × E studies that examined interactions between the very environmental exposures and genetic variants we considered in this study, focused on ADHD-related deficits in attention. However, it is important to note that attention deficits are present in numerous other psychiatric disorders, such as schizophrenia, bipolar disorder, mood disorders, and autism spectrum disorder to name a few (Burack et al., 2017). Furthermore, dysfunctions in the dopamine system that are related to the gene variants we considered here are also implicated, amongst others, in schizophrenia, bipolar disorder, Parkinson's disease, phenylketonuria, and autism spectrum disorder (Diamond et al., 1997; DiCarlo et al., 2019; Nieoullon, 2002; Hayden and Nurnberger Jr, 2006; Scheggia et al., 2012; Mandolini et al., 2019; Pigoni et al., 2019). We plan on following our participants to see if lower attentional competence early in life will evolve into cognitive and psychopathological problems later on.

Inevitably, we were faced with a number of limitations. First, obtaining rich measures and detailed coding of maternal behavior meant that we had to compromise regarding the sample size. However, as Jolicoeur-Martineau et al. (2019) previously demonstrated, LEGIT performs well with sample sizes similar to that of the current study. Second, we assessed maternal prenatal depressive symptoms at a single time point. Consequently, this prevented us from examining the effect of timing and chronicity, the latter of which is a known modifier of the effect of maternal depressive symptoms on child outcomes (Brennan et al., 2000; Hammen and Brennan, 2003; Lahti et al., 2017; Tuovinen et al., 2018). Third, toddlers' attentional competency was rated by the mothers. This can be problematic when mothers are also reporting on their own mood symptoms. Nevertheless, our study benefitted from using observational measures of early maternal parenting behaviors, which were rated by trained coders blind to the mothers' prenatal depressive symptoms and offspring attentional competency. Furthermore, the ITSEA used to assess toddlers' attentional competency is a valid parent-report measure (Carter et al., 2003), which is less prone to measurement error. Parents are asked to report on what is present, i.e., their child's everyday activities that are indicative of the level of their attentional functioning (e.g., *"Plays with toys for 5 min or more."*), rather than what is absent, i.e., deficits in their children's attentional functioning. The assessment of ADHD can be challenging in the early years, thus recognition of important developmental processes, such as attentional competence can be a useful guide to the types of processes that are likely precursors to the disorder (Deault, 2010). As our young participants become older and increasingly capable of understanding verbal task instructions, we aim to repeat these analyses using laboratory-based assessments of child attention.

Implications

As, we have seen here, prenatal adversity can render genetically susceptible children to exhibit lower attentional competence

already in toddlerhood, while a positive early rearing environment facilitates the development of children's attentional competence. Therefore, standard prenatal care should include components that target women's psychological well-being during pregnancy. At the same time, interventions for children with a high susceptibility for developing attentional problems might benefit from promoting positive parenting practices. In addition, these findings underscore the importance of including measurement of the psychosocial environment of the child in line with the biopsychosocial formulation of mental disorders, even when studying neurodevelopmental disorders or related processes (White et al., in press). Furthermore, future research should combine longitudinal developmental cohorts with similar available measures to investigate the complex interplay between the various genetic and environmental components that act to produce complex phenotypes. The computational tools necessary to investigate such complex interactions are now readily available to researchers.

DATA AVAILABILITY STATEMENT

The data for this study will be shared upon request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by REB, Douglas Institute of Health, Montreal, Canada. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

LA, RL, MS, JL, AF, and JK were responsible for study design, data collection, and revision of manuscript. ES, AJ-M, and AW were responsible for study design, analysis, drafting, and revision of manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2021.701971/full#supplementary-material.

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CASE REPORT
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Case Report: Second Report of Joubert Syndrome Caused by Biallelic Variants in IFT74

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Joubert syndrome (JBTS) is a rare ciliopathy characterized by developmental delay, hypotonia, and distinctive cerebellar and brain stem malformation called the molar tooth sign (MTS). We reported a 15-month-old female with dysmorphic features (flat nasal bridge, almond-shaped eye, and a minor midline notch in the upper lips), hypotonia, polydactyly, development delay, and MTS. Whole exome sequencing revealed biallelic heterozygous mutations c.535C>G(p.Q179E/c.853G>T) (p.E285*) in *IFT74*, which were inherited from the parents. So far, only one article reported JBTS associated with *IFT74* gene mutation, and this is the second report of the fifth patient with JBTS due to variants in *IFT74*. All five patients had developmental delay, postaxial polydactyly, subtle cleft of the upper lip, hypotonia, and MTS, but notably without renal and retinal anomalies or significant obesity, and they shared the same mutation c.535C>G(p.Q179E) in *IFT74*, and c.853G>T(p.E285*) that we found was a new mutation in *IFT74* that related with Joubert syndrome. Those findings highlight the need for the inclusion of *IFT74* in gene panels for JBST testing.

Keywords: Joubert syndrome, ciliopathy, IFT74, developmental delay, polydactyly, cleft lip

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INTRODUCTION

Joubert syndrome (JBTS, OMIM: P213300) is a rare, autosomal recessive ciliopathy characterized by three primary findings: a distinctive cerebellar and brain stem malformation called the molar tooth sign (MTS), hypotonia, and developmental delay (Parisi et al., 2017). About 35 ciliopathy-related genes are known to cause JBTS (Radha Rama Devi et al., 2020); those genes encode proteins localized to the pericilia, whose dysfunction would alter cilia composition or signaling (Parisi, 2019). The Intraflagellar transport(IFT) complex is the main module for regulating cilia composition, which consists of IFT-A and IFT-B, and *IFT74* is required for the stabilization of IFT-B (Brown Jason et al., 2015). However, the relationship between JBST and IFT is rarely studied; thus far, only three studies have researched the correlation between JBST and IFT (Halbritter et al., 2013; Bachmann-Gagescu et al., 2015; Luo et al., 2021). In February this year, Luo et al. (2021) reported for the first time that JBST could be caused by *IFT74* mutation. This paper was the second report of the fifth case of *IFT74*-associated JBTS; we found a new mutation in *IFT74*-associated JBTS and present new craniofacial dysmorphisms, which helped to expand the clinical phenotype and genotype of this syndrome.

CASE REPORT

The proband was a 15-month-old female who was referred to our department because of developmental delay. She was the first child of non-consanguineous Chinese parents.

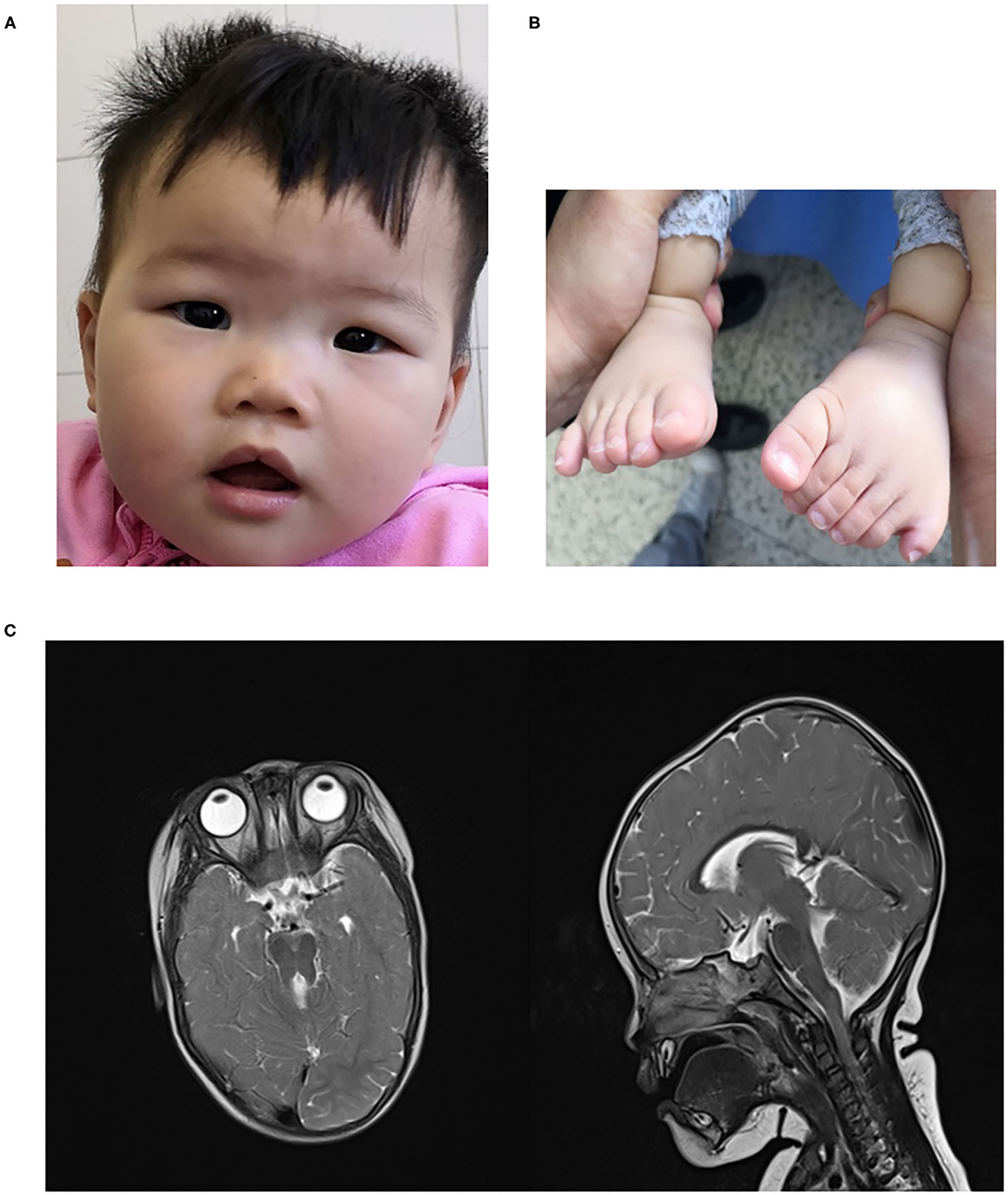

FIGURE 1 | (A) craniofacial dysmorphism **(B)**. polydactyly **(C)** MTS in brain MRI.

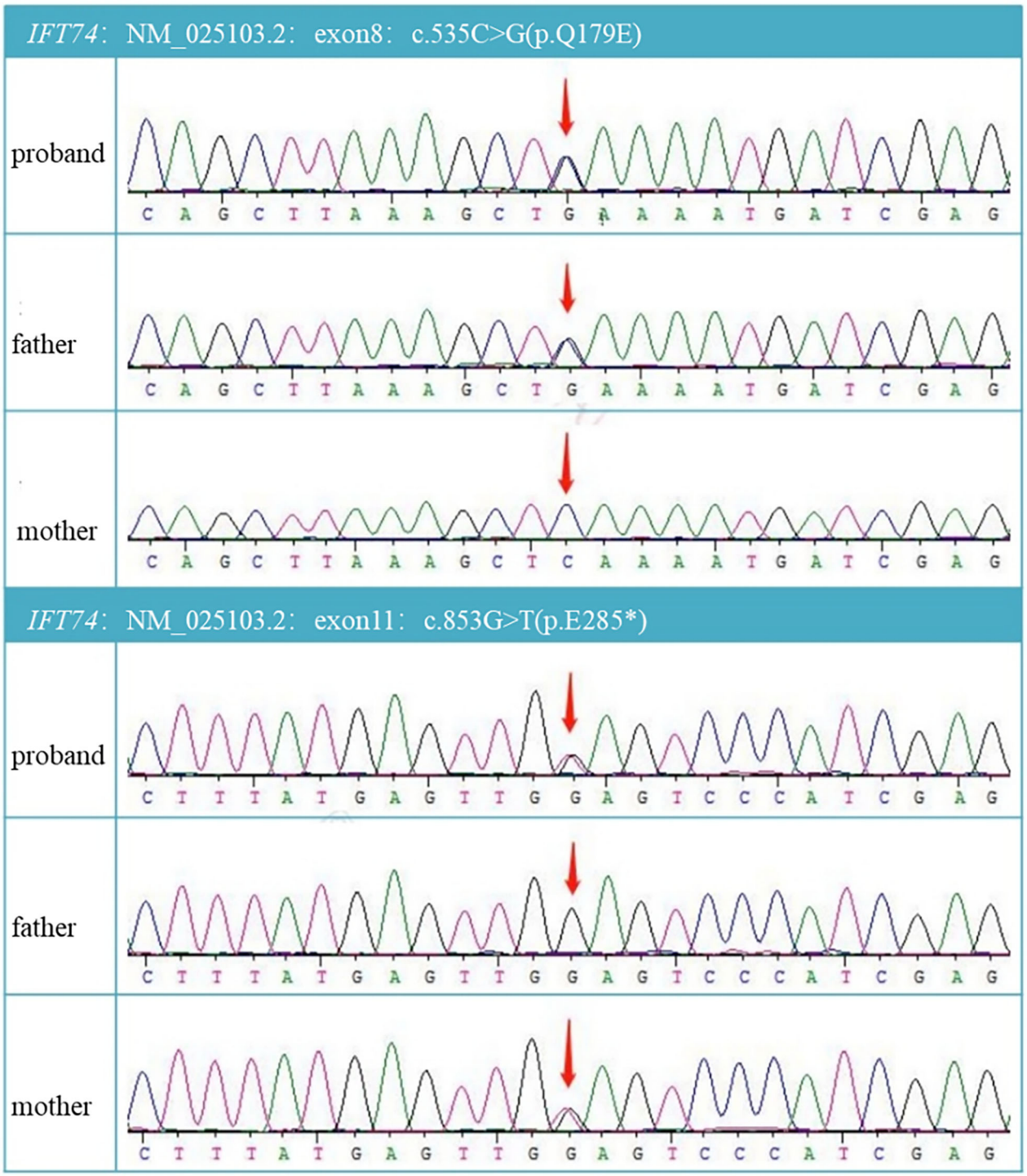

FIGURE 2 | DNA electrophoregram with the c.535C >G in exon8 and c.853G>T in exon11.

She was delivered at term via spontaneous vaginal delivery to a 35-year-old mother after an uncomplicated pregnancy. Birth weight was 3,400 g. Immediately after birth, craniofacial dysmorphisms with a flat nasal bridge, almond-shaped eye, and a minor midline notch in the upper lips and postaxial polydactyly of the hands and feet were noticed (**Figures 1A,B**). She achieved rising head and sitting at 3 and 8 months old, respectively. Delayed motor development was noticed when she couldn't crawl at 1 year, and slight hypotonia of lower limbs was found. She began speaking at 1 year with slowly progressive. At the age of 9 months, she underwent a hand polydactyly excision.

There were no other family members with the presence of birth defects, developmental delay, and/or any other neurological disorders.

On examination at 15 months of age, she can stand with the assistant and speak several words. Her weight was 13 kg, height was 82 cm, body mass index (BMI) was 19.3 kg/m^2. Head circumference was 46 cm. Investigations showed that blood count, urine routine test, biochemical test, thyroid hormones, 25 hydroxyvitamin D3 -were normal. No significant abnormalities were seen in cardiac and abdominal ultrasound examinations. An audiology evaluation was normal. Fundoscopic examination showed no retinal dystrophy, and cranial MRI showed MTS (**Figure 1C**). Neuropsychological development assessment was performed at the age of 16 months; the development quotient (DQ) assessment was as follows: total (64)- gross motor (52)—fine motor (64)—adaptability (64)—language ability (55)—social ability (67).

With parental consent, blood was collected from the child and parents, and whole-exome sequencing was performed. Genomic DNA was extracted from the blood sample with the Blood Genomic DNA Mini Kit following manufacturer's guidelines (CWBIO). The whole-exome library was prepared using the SureSelect Human All Exon V6 (Agilent) and KAPA Hyper Prep Kit (KAPA) following the manufacturer's protocol. All sequencing was performed on the Nova seq 6,000 platform (Illumina) (Testing Service Company: The medical laboratory of Nantong Zhongke, China). Alignment and variant calling were performed with an in-house bioinformatics pipeline. Variants with a minor allele frequency of <0.05 in population databases and expected to affect coding/splicing of the protein or were present in the Human Gene Mutation Database (HGMD) (Stenson et al., 2003) were included in the analysis. The American College of Medical Genetics and Genomics (ACMG) Standards and Guidelines for the interpretation of sequence variants were followed in this study.Two heterozygous variants in *IFT74*, NM_025103.4,c.535C>G(p.Q179E)/c.853G>T(p.E285*) were identified. Sanger sequencing showed the variants were inherited from the parents, confirming that the variants were indeed biallelic (**Figures 2**, **3**). c.535C>G(p.Q179E) was predicted to be "disease causing" with a score of 0.99 (MutationTaster), "benign" with a score of 0.126 (PolyPhen-2), and "tolerated" with a score of 0.14 (SIFT), and c.853G>T(p.E285*) was predicted to be "disease causing" with a score of 1 (MutationTaster).

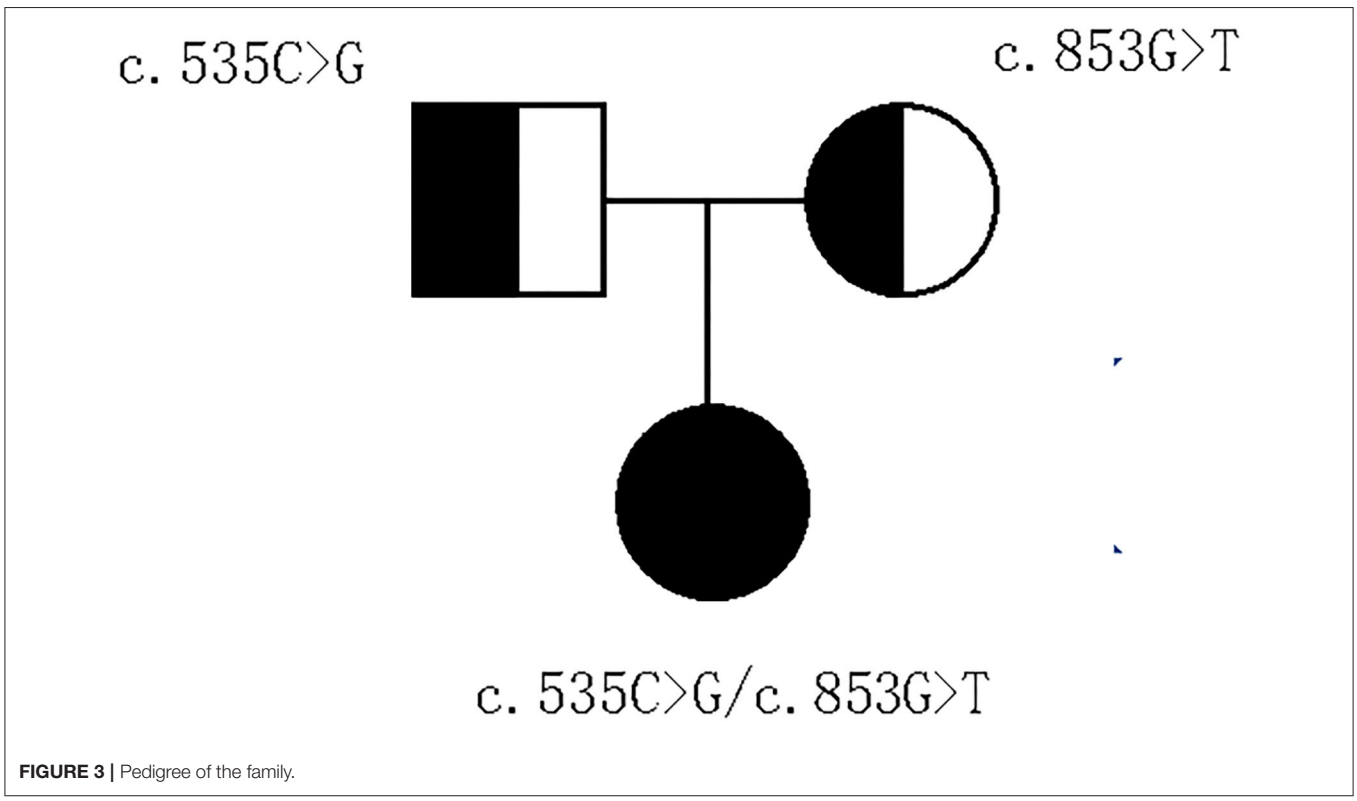

FIGURE 3 | Pedigree of the family.

TABLE 1 | Clinical features of patients with biallelic *IFT74* variants.

Featrue	*IFT74*-JBST case 5 (our case)	*IFT74*-JBST Case 1 (Luo)	*IFT74*-JBST Case 2 (Luo)	*IFT74*-JBST Case 3 (Luo)	*IFT74*-JBST Case 4 (Luo)	*IFT74*-BBS case 1 (Lindstrand)	*IFT74*-BBS case 2 (Kleinendorst)	*IFT74*-BBS case 3 (Mardy)
Age	1 year 3 months	13 years 5 months	1 year 11 months	4 years 6 months	7 years 2 months	36 years	11 years	6 years
Gender	Female	Female	Female	Male	Male	Male	Female	male
Ethnicity	Chinese	Chinese	Chinese	Chinese	Chinese	NA	Dutch	mixed race
Variant 1	c.853G>T (p.E285*)	c.92delT (p.L31Hfs*25)	c.92delT (p.L31Hfs*25)	c.306-24A>G (p.103_135del)	c.85C>T (p.R29*)	Deletion of exon 14–19	c.371_372del (p.Q124Rfs*9)	c.1685-1G > T
Variant 2	c.535C>G (p.Q179E)	c.535C>G (p.Q179E)	c.535C>G (p.Q179E)	c.535C>G (p.Q179E)	c.535C>G (p.Q179E)	c.1685-G>T	c.1685-1G>T	c.1685-1G > T
Height (cm)	13	148.5	78	104	116	NA	162.7	126.7
Weight (kg)	82	40	8.9	14	17.8	NA	70.94	36.9
BMI (kg/m2)	19.33	18.14	14.63	12.94	13.23	NA	26.80	22.99
MTS	+	+	+	+	+	–	–	–
Oculomotor apraxia	–	+	+	+	+	NA	–	–
Respiratory abnormality	–	–	+	+	–	NA	–	–
Hypotonia	+[a]	+	+	+	+	NA	–	–
Retinal involvement	–	–	–	–	–	Retinitis pigmentosa	Rod–cone dystrophy	Early retinal dystrophy
Optic nerve hypoplasia/RNFL defect		+/+	+/+	–/–	–/+	NA/NA	–/–	+
Renal involvement	–	–	–	–	–	–	–	–
Liver involvement	–	–	–	–	–	NA	–	–
Postaxial polydactyly	+	+	+	+	+	+	+	+
Developmental delay	+	+	+	+	+	–	–[b]	+
Intellectual disability	Mild	–	Moderate	Mild	Mild	+	–	–
Hypogonadism (in males) or genital abnormalities (in females)	–	–	–	–	–	Hypogonadism	–	–
Craniofacial dysmorphisms	Midline notch in the upper lip Fat, nasal bridge, almond-shaped eye	Midline cleft lip	Midline notch in the upper lip	Midline cleft lip	Midline notch in the upper lip	Microcephaly	Macrocephaly	Normal
Truncal obesity	–	–	–	–	–	+	+	+
Diabetes mellitus	–	–	–	–	–	–	–	–
Behavioral problem	–	–	Self-mutilation	–	–	–	–	–

JBTS, Joubert syndrome; BBS, Bardet–Biedl syndrome; BMI, body mass index; MTS, molar tooth sign; NA, not available or not mentioned; RNFL, retinal nerve fiber layer; –, not present; +, present.

[a] *slight hypotonia of lower limbs.*

[b] *Only speech delay in childhood.*

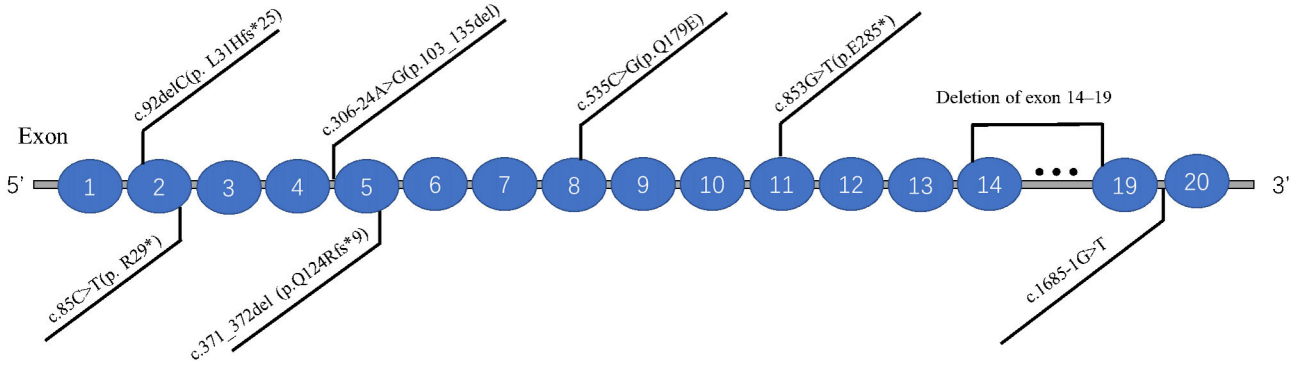

FIGURE 4 | Distribution of eight *IFT74* gene mutations (transcript: NM_025103.4).

Accroding to ACMG, c.535C>G(p.Q179E) was pathogenic(PS1+PS3),while c.853G>T(p.E285*) was uncertain significant(PM2).

LITERATURE REVIEW

The databases of Pubmed, EMCC, EBSCO, and Cochrane Library were searched with the keywords of "Joubert syndrome" and "*IFT74*," and only one related study was found (Luo et al., 2021). And there were three articles (Lindstrand et al., 2016; Kleinendorst et al., 2020; Mardy et al., 2021) about *IFT74* gene mutation related to Bardet-Biedl syndrome (BBS). The clinical features and mutations of all these cases were summary in **Table 1**, **Figure 4**.

DISCUSSION

This is the second report of *IFT74* variants causing a JBTS, validating *IFT74* as a JBTS gene. So far, only one previous report of four cases with JBTS caused by biallelic *IFT74* variants has been published by Luo et al. (2021) this year. Luo et al. described four affected individuals from three non-consanguineous families presenting with MTS on brain MRI, delay in global developmental milestones, postaxial polydactyly, and subtle cleft of the upper lip, all the affected individuals shared one missense variant (p.Q179E), and the pathogenic effects of the variant were evaluated by animal model, the *IFT74* was identified as a JBTS-associated gene. Our patient's phenotype was consistent with Luo et al.'s report and also shared the missense variant (p.Q179E); however, the other mutation, c.853G>T(p.E285*), found in our patient had not been reported in Joubert syndrome. The patient also had craniofacial dysmorphisms with a flat nasal bridge and almond-shaped eye, and these features have not been previously described in patients with a mutation of *IFT74*.

IFT74 gene is located on chr9: 26956412-27066134, with 20 exons (ensembl, 2021). *IFT74* encodes a core intraflagellar transporter protein (IFT) that belongs to a multiprotein complex involved in the transport of ciliary proteins along axonemal microtubules. This protein binds to intraflagellar transporter protein 81 and transports microtubule proteins within the cilium, which is required for ciliogenesis (Ncbi.nlm.nih.gov., 2021). Although JBTS is known to be a recessive and heterogeneous ciliopathy (Bachmann-Gagescu et al., 2020), very little attention was paid to the relationship between *IFT74* and JBTS. Until this year, Luo et al. (2021) comprehensively analyzed the cohort of 4 patients, all of which carried pathogenic mutations in the *IFT74* and showed similar phenotypes, and identified *IFT74* as a JBTS-associated gene.

Previous studies have suggested that *IFT74* gene mutation is mainly associated with BBS, which is characterized by obesity and related complications, retinal cone-rod dystrophy, postaxial polydactyly, cognitive impairment, and renal malformations and/or renal parenchymal disease (Forsythe et al., 2018). Combined with previous reports and our findings, we found that the patients with biallelic variants in *IFT74* all suffered from postaxial polydactyly and none of them had renal involvement, and the cleft lip was a special craniofacial dysmorphism in *IFT74* related JBTS while retinal dystrophy and truncal obesity could be seen in all *IFT74* related BBS. Intellectual disability was common in *IFT74* related JBTS but with a mild or moderate degree. The mutations of *IFT74* with JBTS include 2 non-sense mutations,2 frameshift mutations, 1 splice mutations, 1 missense mutation, and were distributed in exons 2, 5, 8, 11, and intron 4. *IFT74* mutations with BBS include 1 complete gene deletions and 1 splice mutations, distributed in exons 14–19 and intron 19. Those showed that the *IFT74* mutation before exon 11 were associated with JBST, while mutations after it was associated with BBS. Although the mutation was in the same gene, mutations in different locus regions may result in various protein changes, leading to these two distinct syndromes. The most common mutation type is c.535C>G (p. Q179E), which was present in all the *IFT74* related JBST; mechanistic studies suggested that pathogenic variants in *IFT74* lead to defects in cilia length, ciliogenesis, cilia composition, and Hh signaling (Luo et al., 2021), whether c.535C>G (p. Q179E) is hot spot mutations or not need to be confirmed by future data. The mutation c.853G>T(p.E285*) found in this patient had not been reported in Joubert syndrome, and there was no research on the pathogenesis of this mutation, but according to our clinical

finding, it play a vital part in the JBTS, suggesting the loss of function due to this mutation may be pathogenic, further studies on the alter function of this mutation are needed.

In conclusion, our study reported a compound heterozygous mutation in the *IFT74* gene [c.535C>G(p.Q179E)/c.853G>T(p.E285*)] in a Chinese family with JBTS.As far as we know, this is the second report of the fifth case in *IFT74* mutation-related JBST. This patient helps to expand and clarify the clinical spectrum of *IFT74*-related JBST, and the cases with *IFT74* mutation are summarized in **Table 1**. The *IFT74* mutation-related JBST manifested developmental delay, postaxial polydactyly, subtle cleft of the upper lip, hypotonia, and MTS on brain MRI, but notably without renal and retinal anomalies or significant obesity. Our case also presents new craniofacial dysmorphisms of the flat nasal bridge and almond-shaped eye and the new mutation c.853G>T(p.E285*) in JBST.All our findings highlight the need for the inclusion of *IFT74* in gene panels for JBST testing.

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 Fujian medical university union hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

KZ contributed to the acquisition, analysis of data, and writing the first draft. CY contributed to the conception of the work and revised the paper. LG contributed to collect the data and communicated with the pateints family. CX contributed to search information of this disease.

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Early Life Adversity and Polygenic Risk for High Fasting Insulin Are Associated With Childhood Impulsivity

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While the co-morbidity between metabolic and psychiatric behaviors is well-established, the mechanisms are poorly understood, and exposure to early life adversity (ELA) is a common developmental risk factor. ELA is associated with altered insulin sensitivity and poor behavioral inhibition throughout life, which seems to contribute to the development of metabolic and psychiatric disturbances in the long term. We hypothesize that a genetic background associated with higher fasting insulin interacts with ELA to influence the development of executive functions (e.g., impulsivity in young children). We calculated the polygenic risk scores (PRSs) from the genome-wide association study (GWAS) of fasting insulin at different thresholds and identified the subset of single nucleotide polymorphisms (SNPs) that best predicted peripheral insulin levels in children from the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort [N = 467; $p_{t\text{-initial}}$ = 0.24 (10,296 SNPs), $p_{t\text{-refined}}$ = 0.05 (57 SNPs)]. We then calculated the refined PRS (rPRS) for fasting insulin at this specific threshold in the children from the Maternal Adversity, Vulnerability and Neurodevelopment (MAVAN) cohort and investigated its interaction effect with adversity on an impulsivity task applied at 36 months. We found a significant effect of interaction between fasting insulin rPRS and adversity exposure predicting impulsivity measured by the Snack Delay Task at 36 months [$\beta = -0.329$, $p = 0.024$], such that higher PRS [$\beta = -0.551$, $p = 0.009$] was linked to more impulsivity in individuals exposed to more adversity. Enrichment analysis (MetaCore™) of the SNPs that compose the fasting insulin rPRS at this threshold was significant for certain nervous system development processes including dopamine D2 receptor signaling. Additional enrichment analysis (FUMA) of the genes mapped from the SNPs in the fasting insulin rPRS showed enrichment with the accelerated cognitive decline GWAS. Therefore, the genetic background associated with risk for adult higher fasting insulin moderates the impact of early adversity on childhood impulsivity.

Keywords: ALSPAC, MAVAN, fasting insulin, impulsivity, early life adversity

INTRODUCTION

Early life adversity (ELA) increases the risk for adult chronic disease, including psychopathology, metabolic, endocrine, and cardio-metabolic conditions (Malaspina et al., 2008; Rice et al., 2010; O'Donnell and Meaney, 2017; Van den Bergh et al., 2017; McQuaid et al., 2019; Monk et al., 2019). Neuroimaging studies have associated certain prenatal adversities with altered structural and functional trajectories in brain development (Bock et al., 2015; Egeland et al., 2015; Kim et al., 2015; O'Donnell and Meaney, 2017; Osborne et al., 2018; Monk et al., 2019). Since certain areas of the brain continue developing until late adolescence, the brain is also highly sensitive to postnatal adversity. Childhood adversity has been linked to long term behavioral outcomes and neurobiological consequences: emotional problems (Danese et al., 2020), aggressive behaviors (El-Khodary and Samara, 2020), changes in brain electrical activity (Marshall et al., 2004; Vanderwert et al., 2010), cognitive functions (Burneo-Garcés et al., 2019), and executive functions (Ursache et al., 2016). The mechanisms contributing to the development of these phenotypes involve gene by environment interactions resulting in behavioral differences (e.g., attention, impulsivity, and food preferences). However, not all individuals exposed to adversity develop these alterations. Responses to early adversity exposure have individual differences that are mostly driven by the genetic background.

At the neuroendocrine level, ELA is linked to alterations in responsivity to stress while also altering insulin sensitivity at different ages. Stressful conditions or adversities happening early in life, either pre- or postnatally, can affect glucose homeostasis and insulin function in the short and long terms. Some adversities are associated with a higher risk for insulin resistance and diabetes, such as: maternal/paternal history of diabetes (Beck-Nielsen and Groop, 1994; Groop et al., 1996), exposure to gestational diabetes (Krishnaveni et al., 2005), socioeconomic status (Everson et al., 2002), placental insufficiency (Camacho et al., 2017), cigarette smoking (Mouhamed et al., 2016), maternal malnutrition (Reusens et al., 2011), and chronic stress (Han et al., 2016; Yan et al., 2016). Such adverse events associate with both growth and metabolism (Wijlaars et al., 2011; Bhopal et al., 2019; Salmela et al., 2019). These events also alter responses to subsequent stressors (McGowan et al., 2009; Labonte et al., 2012; Peckins et al., 2020) and induce chronic inflammation (Flouri et al., 2020; Kokosi et al., 2020), both of which modify glucose homeostasis and insulin sensitivity (Facchi et al., 2020; Zannas et al., 2020). Beyond acute effects on brain development and child behavior (Lim et al., 2018; Chen et al., 2019; Lambert et al., 2019), long-term effects of adversity increase the risk for both metabolic diseases (Thomas et al., 2008; Fuller-Rowell et al., 2019) as well as psychopathologies later in life (Vaughn-Coaxum et al., 2019; Lund et al., 2020; Vogel et al., 2020).

Insulin is one of the primary hormonal regulators of metabolism in animals with several different functional roles (Brockman and Laarveld, 1986). Although most peripheral tissues depend on insulin signaling to acquire glucose, such is not the case with the brain as insulin is not needed for glucose transport into neurons (Kullmann et al., 2016). However, brain insulin does play a role as a neuroregulatory peptide (Woods et al., 1979; Bruning et al., 2000; Hallschmid et al., 2012) acting in different brain areas such as the ventral tegmental area, striatum, hypothalamus, hippocampus, olfactory bulb, and prefrontal cortex (Ghasemi et al., 2013). Insulin within these areas modulates the development and expression of different executive function behaviors (Kullmann et al., 2016), such as attention, inhibitory control, and working memory. Insulin has also been shown to reduce activity in the prefrontal areas that control behaviors such as inhibitory control of eating (Heni et al., 2015). Furthermore, abnormal insulin levels and function are seen in Alzheimer's patients where insulin impairments have been linked to learning deficits and memory formation impairments (Zhao and Alkon, 2001).

Considering that the genetic background represents variations in biological function, our objective was to develop a model that predicts an executive function behavior, impulsivity, as a function of the interaction between biological markers of elevated fasting insulin levels and ELA in children. To do so, we first assessed the relationship between a polygenic risk score (PRS) derived from genome-wide associations with high fasting insulin (Scott et al., 2012) and the actual peripheral insulin levels measured in children from the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort (Boyd et al., 2013; Fraser et al., 2013). The genome-wide association study (GWAS) of fasting insulin (fasting insulin GWAS) was performed in adults where the insulin measured was collected from individuals following a fasting period (Scott et al., 2012). Since our study inspects the role of insulin in children, we used the ALSPAC cohort's data on peripheral insulin levels to identify the polygenic markers most highly associated with peripheral insulin levels in children. We further refined these markers to only include single nucleotide polymorphisms (SNPs) that significantly predicted peripheral insulin levels in ALSPAC. Because brain insulin levels are not readily measured or available, a genetic marker reflecting peripheral insulin levels in children was used to inspect insulin's role in neurodevelopmental behaviors. Using the SNPs identified in the discovery cohort ALSPAC, we calculated a refined PRS (rPRS) in an independent cohort [Maternal Adversity, Vulnerability and Neurodevelopment (MAVAN)] to investigate the interaction between the genetic background associated with fasting insulin in children and ELA to predict childhood impulsivity. Our methodology allowed us to create a PRS that is highly associated with fasting insulin in children (ALSPAC) and then investigate behavior outcomes in an independent cohort of children (MAVAN), thus refining a GWAS obtained by an adult cohort (Scott et al., 2012).

MATERIALS AND METHODS

Participants

We used data from two prospective birth cohorts, one based in England (ALSPAC) (Northstone et al., 2019) and the other in Canada (MAVAN) (O'Donnell et al., 2014) to analyze the gene by environment interaction effects on cognitive neurodevelopment outcomes.

Avon Longitudinal Study of Parents and Children

The ALSPAC cohort included pregnant women from the county of Avon, United Kingdom (Boyd et al., 2013; Fraser et al., 2013; Northstone et al., 2019) ($N = 14{,}541$) with expected delivery dates between April 1991 and December 1992. Additional recruitment ($N = 913$) was done during later phases, bringing the total sample size to 15,454. Participants provided informed written consent to participate in the study. Consent for biological samples had been collected in accordance with the Human Tissue Act (2004). Ethics approval for the study was obtained from the ALSPAC Ethics and Law Committee and the local research ethics committees (a full list of the ethics committees that approved different aspects of the ALSPAC studies is available at http://www.bristol.ac.uk/alspac/researchers/research-ethics/). Data were collected during clinic visits or with postal questionnaires. Please note that the study website contains details of all the data that is available through a fully searchable data dictionary and variable search tool at http://www.bristol.ac.uk/alspac/researchers/our-data/. For the purpose of our analysis, we included children of 8.5 years old (an age closer to the outcome measure in the MAVAN cohort), whose mothers had a pregnancy duration between 37 and 42 weeks, a maternal age at delivery greater than 18 years, a child birthweight greater than 2 kg, child alive at 1 year of age, and we only included singleton pregnancies in the analysis. **Figure 1** describes the subset of the sample for the purpose of the analyses in the ALSPAC cohort. There were 467 subjects with complete data available for the analyses.

Maternal Adversity, Vulnerability and Neurodevelopment Project

The study MAVAN is a birth cohort that followed up children from birth up to 6 years of age in Montreal (Quebec) and Hamilton (Ontario), Canada, and has 630 recruited participants (O'Donnell et al., 2014). Mothers aged 18 years or above, with singleton pregnancies, and fluent in French or English were included in the study. Several maternal chronic illnesses, including placenta previa and history of incompetent cervix, impending delivery, a fetus/infant affected by a major anomaly, or gestational age < 37 weeks composed the exclusion criteria. Approval for the MAVAN project was obtained by the ethics committees and university affiliates (McGill University and Université de Montréal, the Royal Victoria Hospital, Jewish General Hospital, Centre hospitalier de l'Université de Montréal and Hôpital Maisonneuve-Rosemount) and St. Joseph's Hospital and McMaster University, Hamilton, QC, Canada. Informed consent was obtained from all participants. **Figure 2** describes the criteria and selection of MAVAN sample for the purpose of our research. There were 101 subjects with complete data available for the analyses.

Genotyping

Avon Longitudinal Study of Parents and Children

Children in the ALSPAC cohort were genotyped using the Illumina HumanHap550 quad chip genotyping platform by the Wellcome Trust Sanger Institute, Cambridge, United Kingdom and the Laboratory Corporation of America, Burlington, NC, United States (Richmond et al., 2017). Standard quality control (QC) procedure was applied: participants with inconsistent self-reported and genotyped sex, minimal or excessive heterozygosity, high levels of individual missingness ($>3\%$), and insufficient sample replication (IBD < 0.8) were excluded. Also, SNPs with call rate $< 95\%$, MAF $< 1\%$, or not in Hardy-Weinberg Equilibrium (HWE; $p < 5 \times 10^{-7}$) were removed. Following the QC, the genotyping data was imputed using Impute v3 and Haplotype Reference Consortium (HRC) imputation reference panel (release 1.1), which resulted in 38,898,739 SNPs available for analysis.

The population structure of ALSPAC cohort was described using principal component (PC) analysis (Patterson et al., 2006; Price et al., 2006), which was conducted on the genotyped SNPs with MAF $> 5\%$ with the following pruning parameters for linkage disequilibrium: 100-kilobase sliding window, an increment of 5 SNPs, and variance inflation factor (VIF) threshold of 1.01. To account for population stratification, the first ten PCs were included in the analysis.

Maternal Adversity, Vulnerability and Neurodevelopment

Genome-wide platforms (the Infinium PsychArray v1 or the PsychChip v1.1/v1.2, Illumina, Inc.) were used to genotype 229,456 autosomal SNPs of buccal epithelial cells of children in MAVAN, according to the manufacturer's guidelines. SNPs with call rate $< 95\%$, MAF $< 5\%$, or not in HWE ($p < 1 \times 10^{-30}$) were removed. Afterward, imputation using the Sanger Imputation Service (McCarthy et al., 2016) and HRC as the reference panel (release 1.1) was performed and SNPs with an info score > 0.80 were retained for the analysis, resulting in 16,249,769 autosomal SNPs.

Similar to the ALSPAC cohort, the population structure of the MAVAN cohort was evaluated using PC analysis of all autosomal SNPs that passed the QC and not in high linkage disequilibrium ($r^2 > 0.2$) across 50-kilobase region and an increment of 5 SNPs (Price et al., 2006). Based on the inspection of the scree plot, the first three PCs were the most informative of population structure and were included in all subsequent analyses.

Polygenic Risk Scores

The rPRS procedure was administered in this study to inspect the interaction between genetic markers for fasting insulin and ELA to predict impulsivity in children using a GWAS constructed from adult data.

Avon Longitudinal Study of Parents and Children

The fasting insulin PRS was calculated using the fasting insulin GWAS, shown in **Figure 3** ($N = 108{,}557$) from the Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC) (Scott et al., 2012). Prior to any PRS calculation, the GWAS was subjected to LD clumping with r^2 of 0.2 and ALSPAC cohort as a reference dataset. PRS at 100 different GWAS p-value thresholds were calculated for each subject in the ALSPAC cohort as a sum of the risk alleles count weighted by the effect size described in the GWAS for each SNP (Dudbridge, 2013; Wray et al., 2014). Using ALSPAC as a discovery cohort,

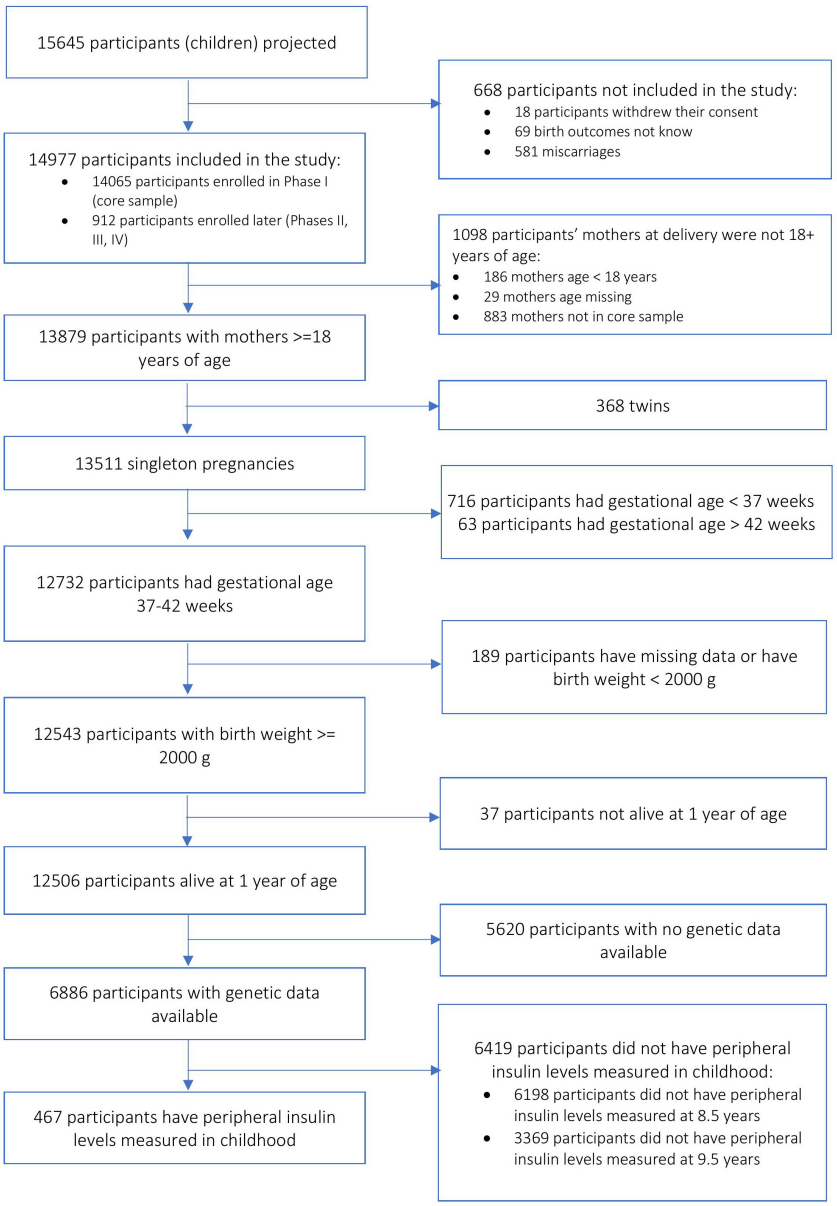

FIGURE 1 | Block scheme describing sample selection in the ALSPAC cohort.

we identified the threshold at which the PRS had the best prediction of peripheral insulin levels in children at age 8.5 years. The strongest (R^2 = 0.039) and most significant (p = 0.071) association in children at age 8.5 years was identified to be with a PRS at $p_{t\text{-initial}}$ = 0.24 threshold (consisting of 10,296 SNPs) as shown in **Figure 4**. To further refine the PRS, a process explained through **Figure 5**, we ran a linear regression analysis for each SNP within the 0.24 threshold PRS to find which SNPs were significantly associated ($p_{t\text{-refined}} < 0.05$) with the peripheral insulin levels. There were 57 SNPs significantly associated with peripheral insulin levels within the SNPs included in the 0.24 threshold. The list of these SNPs can be found in **Table 1** with their corresponding p-values from the fasting insulin GWAS (Scott et al., 2012). These 57 SNPs included in the rPRS were ranging in p-values from 0.000123 to 0.238 in the original GWAS (Scott et al., 2012), however, they were all significantly associated with the peripheral insulin levels in children (all p-values < 0.05). This finding confirms that SNPs associated with adult risk for high fasting insulin may not be the same as SNPs associated with children risk for high fasting insulin.

Maternal Adversity, Vulnerability and Neurodevelopment

The 57 SNPs within the PRS that we discovered to be associated with peripheral insulin levels in the ALSPAC cohort were used

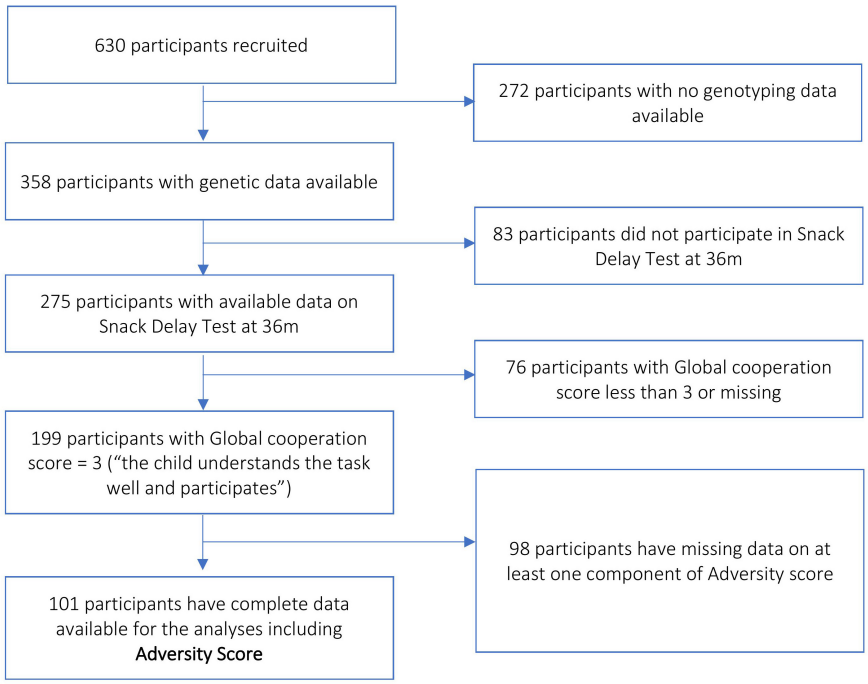

FIGURE 2 | Block scheme describing sample selection in the MAVAN cohort.

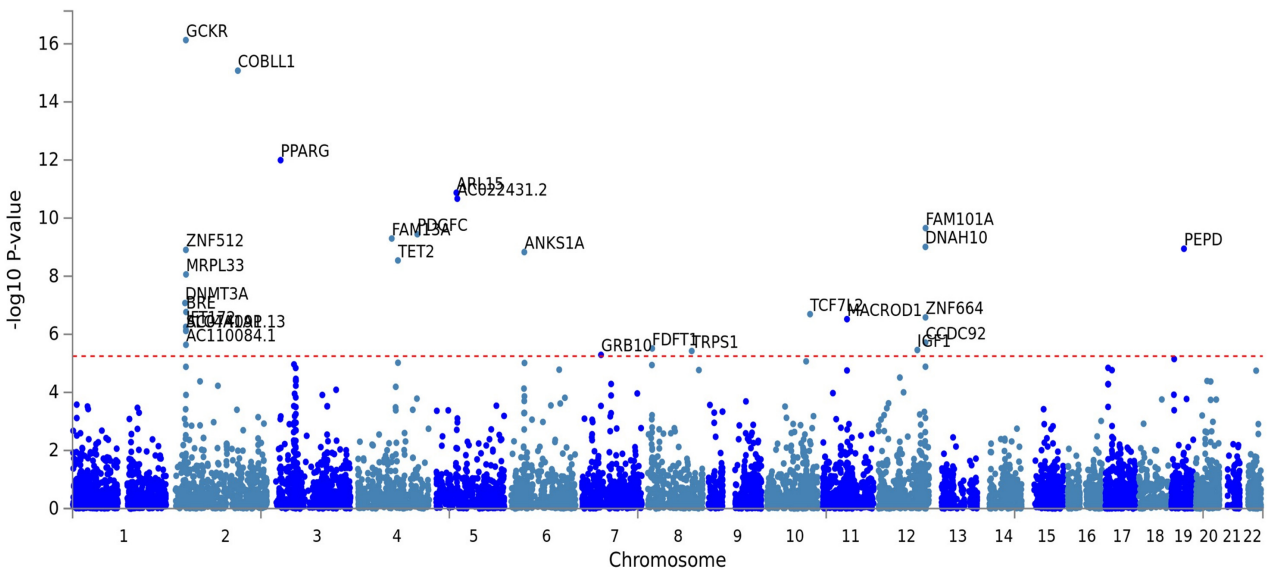

FIGURE 3 | Genome-wide association study (GWAS) for fasting insulin from the Meta-Analyses of Glucose and Insulin-related traits Consortium (MAGIC). Each dot represents a single nucleotide polymorphism (SNP), with the *x*-axis showing genomic location and the *y*-axis showing the association level of each respective loci to fasting insulin. The gene names for all significant SNPs are displayed in the plot. This plot was obtained from FUMA.

to construct a PRS in MAVAN. The PRS was standardized. Since the SNPs were selected through a refinement process of a PRS that was created through conventional means, we henceforth refer to this PRS in the MAVAN cohort as the rPRS. The rPRS was calculated similarly to the PRS scores in ALSPAC, as a weighted sum of 57 SNPs.

Early Life Characterization

To investigate the interaction between the PRS and ELA, we estimated adversity exposure using a cumulative score involving different environmental variables (Silveira et al., 2017) for each individual in the MAVAN cohort as described by Silveira et al. (2017) and de Lima et al. (2020). The adversity

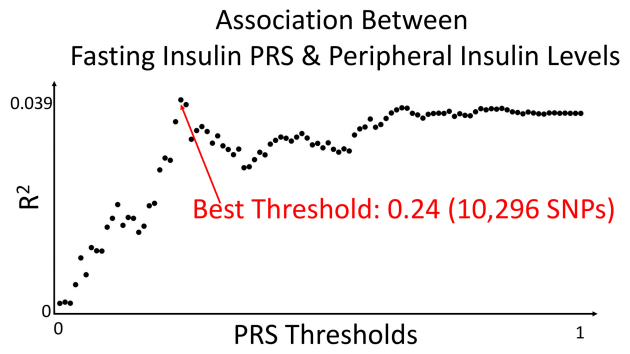

FIGURE 4 | Association between fasting insulin PRS and peripheral insulin levels. We calculated polygenic risk scores from the fasting insulin GWAS at 100 different thresholds. Using ALSPAC as a discovery cohort, we identified the PRS threshold with the strongest correlation in predicting peripheral insulin levels in children at age 8.5 years. The strongest (R^2 = 0.039) and most significant (p = 0.071) association was identified to be 0.24 in children at age 8.5 years in the ALSPAC cohort [N = 467; $p_{t-initial}$ = 0.24 (10,296 SNPs)].

score was created by combining several markers of adversity. The following instruments were included as markers in the score: (1) *The Health and Well-Being Questionnaire* (Kramer et al., 2001) to obtain information on how often and to what degree the woman lacked money for basic needs (Kanner et al., 1981) using the *Daily Hassles Scale*, on chronic stress with the romantic partner (Pearlin and Schooler, 1978), on conjugal violence (Newberger et al., 1992; Parker et al., 1993), on anxiety during pregnancy (Dunkel-Schetter, 1998), on birth size percentile, and on gestational age; (2) *Smoking during pregnancy*; (3) *Household gross income* (Daveluy et al., 1998); (4) *Child Health Questionnaire* (Plante et al., 2002) to assess acute, chronic conditions and hospitalizations; (5) *Maternal mental health* through the Beck Depression Inventory (BDI) (Beck and Ward, 1961), Edinburgh Postnatal Depression Scale (EPDS) to screen for postpartum depression (Cox et al., 1987), and State-Trait Anxiety Inventory (STAI) to measure psychological components of state and trait anxiety (Spielberger, 2010); (6) *Attachment* through the Preschool Separation-Reunion Procedure (PSRP) applied at 36 months (Cassidy et al., 1992) (Solomon and George, 2008); (7) *Family Assessment Device* to assess family functioning based on the McMaster Model of Family Functioning (Moss et al., 2004). For every item with a continuous score, we used either the 15th or the 85th percentile as the cut-off to add a point to the adversity score. Presence of each component yields one point, and the adversity score represents the summation of the points where the higher the score, the more adversity has been experienced by the individual.

Behavioral Outcomes

In the Snack Delay Task at 36 months (Golden et al., 1977; Campbell et al., 1982; Vaughn et al., 1984; Kochanska et al., 1996), the children placed their hands flat on a table in front of them and restrained themselves from eating a single M&M candy from under a glass cup placed on the table. The children were told to delay eating until the research assistant rang a bell. The test was conducted over four distinct trials (using delays of 10, 20, 15, and 30 seconds). Halfway through each trial delay, the experimenter lifted the bell but did not ring it. The children received a behavioral score for each trial ("behavior code") based on attempts to eat the candy before the bell rang. Coding ranged from 1 to 7, as displayed in **Table 2**. For each trial, the ability of the children to wait for the M&M was also recorded (snack delay latency to eat: 1 = child keeps hands on the table during the entire time either before OR after the bell is lifted and 2 = child keeps hands on the table during the entire time before AND after the bell is lifted). This latter score (1 or 2) and the behavioral code score (ranging from 1 to 7) culminated to provide a total performance score (ranging from 2 to 9) for each of the four trials. A "global cooperation score" rated the ability of the child to engage and complete the task (0 = the child is unwilling or unable to engage in the task; 1 = the child is unwilling or unable to complete the task because of feeling tired, angry, irritable, or sick, or does not have the capacity to understand the instructions; 2 = the child does all the trials but has comprehensive or motivational difficulties, or is passive or inhibited, and 3 = the child understands the task well and participates). Children with a global score of 3 were included in the analysis. The Snack Delay Task was applied to children in the MAVAN cohort at the age of 36 months.

Statistical Analysis

Data analyses were carried out using R (R Core Team, 2019). Baseline comparisons between low and high PRS groups were done in the MAVAN cohort. Mean differences of the main confounding variables were assessed using the Student's t-test for independent samples if they were continuous variables or the chi-square test if they were categorical variables. Significance levels for all measures were set at $\alpha < 0.05$.

We used linear regression models to test the association between the outcome of impulsivity, evaluated using the Snack Delay Task in this case, and the predictors including adversity exposure, rPRS, and their interaction term with sex and the first three genomic PCs as covariates. In summary, we ran the following three linear regression models:

1. Outcome ~ sex + PC1 + PC2 + PC3 + Adversity Score
2. Outcome ~ sex + PC1 + PC2 + PC3 + rPRS
3. Outcome ~ sex + PC1 + PC2 + PC3 + Adversity Score × rPRS

To identify the form of interaction between the rPRS and the adversity score, we used Roisman's method (Roisman et al., 2012; Belsky et al., 2015) of simple slopes analysis and examined the regions of significance (RoS) to determine the range of values of the predictor for which regression of the outcomes on the moderator (rPRS) is statistically significant. To explore the form of interaction, Roisman also recommends the use of two metrics designed to help identify between diathesis-stress and differential susceptibility models: the proportion of interaction (PoI) index and the proportion affected (PA) or the percentage above index. Both metrics show a preponderance of differential susceptibility when greater than a certain threshold. As a sub-analysis to handle

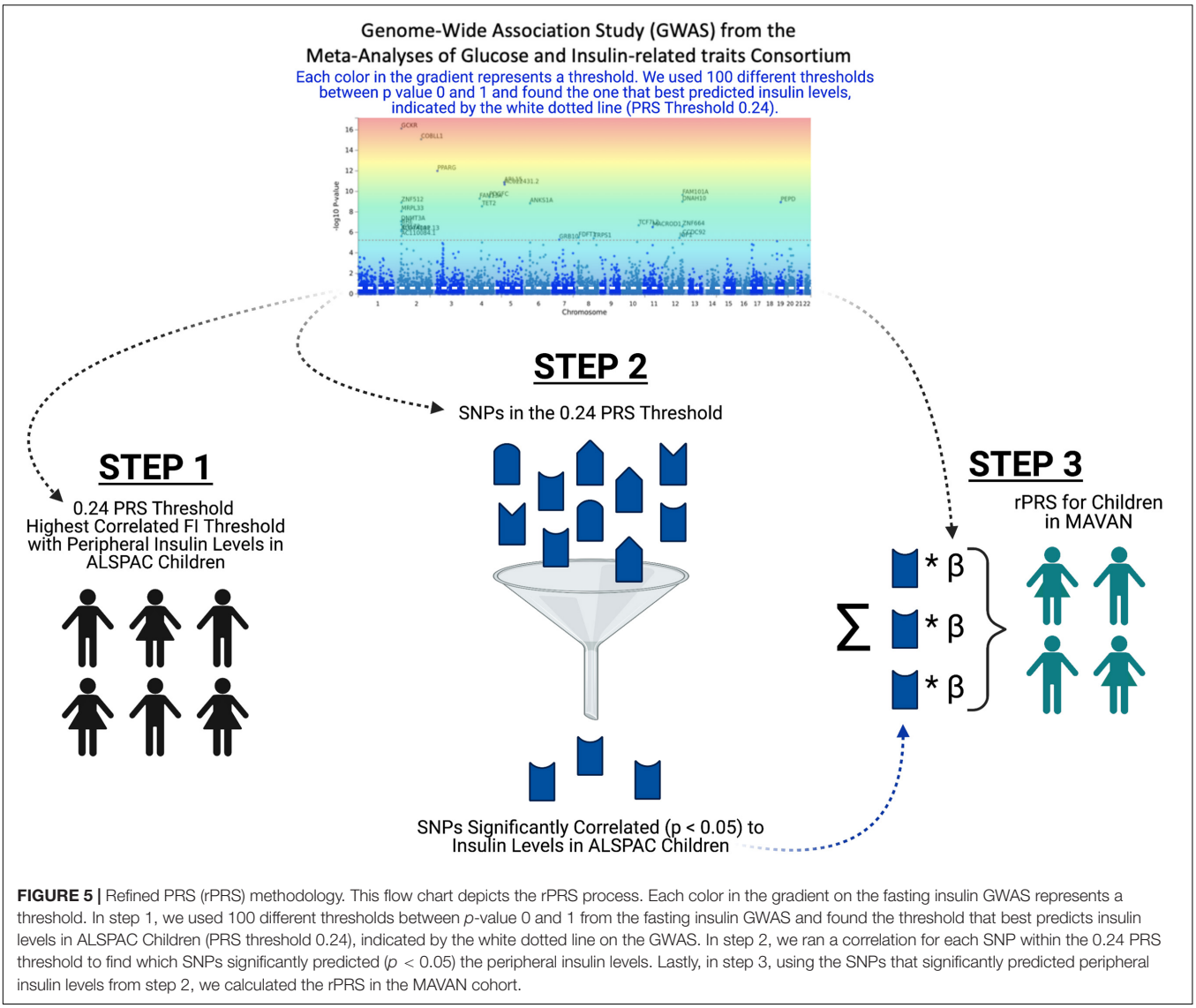

FIGURE 5 | Refined PRS (rPRS) methodology. This flow chart depicts the rPRS process. Each color in the gradient on the fasting insulin GWAS represents a threshold. In step 1, we used 100 different thresholds between *p*-value 0 and 1 from the fasting insulin GWAS and found the threshold that best predicts insulin levels in ALSPAC Children (PRS threshold 0.24), indicated by the white dotted line on the GWAS. In step 2, we ran a correlation for each SNP within the 0.24 PRS threshold to find which SNPs significantly predicted ($p < 0.05$) the peripheral insulin levels. Lastly, in step 3, using the SNPs that significantly predicted peripheral insulin levels from step 2, we calculated the rPRS in the MAVAN cohort.

the missing cases for the adversity score, we imputed the data with hot-deck imputation (hot.deck package in R) (Cranmer et al., 2020), assuming missing at random mechanisms. We imputed all adversity score components, calculated the extended adversity score on an additional 98 subjects, and repeated the linear regression analysis on the imputed datasets of 199 subjects each, reporting the pooled estimates from 30 imputed sets.

Enrichment Analysis

Enrichment analyses for gene ontologies were performed using MetaCore™ (Clarivate Analytics) on the SNPs that compose the fasting insulin rPRS. Furthermore, gene-based enrichment analyses were performed in FUMA[1] (MacArthur et al., 2017; Watanabe et al., 2017; Aguet et al., 2019) after mapping the SNPs composing the fasting insulin rPRS to genes with the biomaRt package in R (Durinck et al., 2005, Durinck et al., 2009). We also used GeneMANIA (Warde-Farley et al., 2010) to determine if the genes were part of a network. Specifically, the gene list derived from the fasting insulin rPRS is entered in GeneMANIA. GeneMANIA then extracts linked mRNA expression data from the Gene Expression Omnibus (GEO) and connects co-expressed data to form functional association networks. The node sizes represent gene scores indicating the number of paths that start at a given gene node and end up in one of the query genes.

[1]https://fuma.ctglab.nl/

RESULTS

Baseline Characteristics

Baseline comparisons between low and high rPRS groups were performed in the MAVAN cohort. No differences were found for the main confounding variables in the MAVAN cohort, as shown in **Table 3**. Participants' characteristics for ALSPAC cohort are

TABLE 1 | Single nucleotide polymorphisms (SNPs) included in the refined PRS for MAVAN.

SNP	*P*-value (Fasting Insulin GWAS)
rs7574670	0.000123
rs13225097	0.000846
rs196808	0.005017
rs6885750	0.010573
rs4841679	0.011172
rs870870	0.018874
rs2665316	0.019226
rs4405319	0.031478
rs11724118	0.045920
rs10804992	0.046243
rs6552502	0.058479
rs397234	0.058594
rs11898925	0.061942
rs1866816	0.061986
rs7807790	0.062096
rs2965106	0.062112
rs275146	0.062816
rs7155790	0.065848
rs9840453	0.067170
rs11693862	0.073821
rs1492377	0.074815
rs1377315	0.083513
rs4686837	0.088538
rs4803789	0.089959
rs10520768	0.090182
rs2295308	0.093524
rs7598551	0.094168
rs7332334	0.101271
rs728586	0.103540
rs4779876	0.103930
rs1935492	0.103989
rs7983099	0.112130
rs923554	0.118880
rs1885414	0.123295
rs9863801	0.126196
rs11891202	0.135653
rs4382157	0.136635
rs5753103	0.150747
rs17035960	0.151128
rs884972	0.151753
rs10002944	0.154237
rs10145606	0.156256
rs892114	0.159405
rs6543408	0.178625
rs4766912	0.184907
rs7138803	0.185122
rs2544164	0.197849
rs12219445	0.198058
rs1664256	0.199221
rs11603179	0.201429
rs11820303	0.211331
rs2341647	0.217823
rs6888754	0.219617
rs9808140	0.221567
rs1058065	0.223190
rs12731669	0.231006
rs7243066	0.238047

TABLE 2 | The Snack Delay Task was performed at 36 months in the children from the MAVAN cohort.

Snack Delay – Test Scoring	
1	Eats before bell is lifted
2	Eats after bell is lifted
3	Touches candy before bell is lifted
4	Touches candy after bell is lifted
5	Touches cup or candy before bell is lifted
6	Touches cup or candy after bell is lifted
7	Waits for bell to ring before touching cup or candy

Each child was asked to place their hands flat on a table and to restrain themselves from eating a single M&M candy from under a glass cup placed on the table in front of them. The children were instructed to delay eating until the research assistant rang a bell. The test was conducted over four distinct trials (using delays of 10, 20, 15, and 30 seconds) and the scores of each trial were added together for the final cumulative score.

reported in **Table 4**. **Table 5** details the degree of missing data for each component of the adversity score.

Interaction Between Fasting Insulin PRS and the Adversity Score Associates With Impulsivity in MAVAN

We performed a linear regression analysis to investigate the interaction effect between the refined genetic score (rPRS) and adversity exposure on the Snack Delay Task in the MAVAN cohort applied at 36 months, adjusted by population stratification PCs and sex. A significant interaction effect was observed, as displayed in **Figure 6**, between fasting insulin rPRS and adversity exposure on impulsivity measured by the Snack Delay Task [$\beta = -0.329$, $p = 0.024$]. Simple slope analysis at $\pm$ 1 SD rPRS showed that higher ELA is linked to more impulsivity in children with higher rPRS [$\beta = -0.551$, $p = 0.009$]; there was no effect of adversity on impulsivity in the low rPRS group [$\beta = 0.139$, $p = 0.348$]. The region of significance is to the right side of the red line in **Figure 6**, which suggests that the association between impulsivity and the rPRS is significant in children highly exposed to adversity. We also analyzed the form of the interaction according to Roisman (Roisman et al., 2012). The RoS, as well as the PoI (0.984) and PA (0.782) are consistent with the diathesis-stress model.

To obtain a distribution of the statistics of interest (interaction coefficient) and its confidence interval, we applied a non-parametric bootstrap, which resulted in estimated beta = −0.329 (SE = 0.1764) and 95% confidence interval (−0.6729, −0.0202) for the effect of interaction between rPRS and adversity on Snack Delay Task in MAVAN.

The main effect of the refined genetic score (rPRS) on the Snack Delay Task in MAVAN [$N = 101$] applied at 36 months, adjusted by PCs and sex, was not significant [$\beta = -0.190$, $p = 0.126$]. The main effect of adversity on the Snack Delay Task in MAVAN [$N = 101$] applied at 36 months, adjusted by PCs and sex, was also not significant [$\beta = -0.131$, $p = 0.171$]. When performing imputations on the missing cases of the adversity score (final $N = 199$), the effect of the interaction between rPRS

TABLE 3 | Participants' characteristics in MAVAN.

Sample descriptive	Total (*n* = 101)	Low PRS (*n* = 50)	High PRS (*n* = 51)	*p*
Sex – male	44.6% (45)	42.0% (21)	47.1% (24)	0.756
Maternal age at birth (years)	30.51 (4.65)	30.17 (4.36)	30.84 (4.94)	0.470
Gestational age (weeks)	39.32 (1.17)	39.34 (1.27)	39.29 (1.08)	0.846
Birth weight (g)	3,326 (458)	3,371 (472)	3,281 (443)	0.325
Duration of breastfeeding (months)	7.23 (4.82)	6.91 (4.83)	7.54 (4.83)	0.511
Smoking during pregnancy	11.9% (12)	10.0% (5)	13.7% (7)	0.786
Maternal education – university degree or above	62.4% (63)	68.0% (34)	56.9% (29)	0.342
Low income at 36 m	11.2% (11)	12.2% (6)	10.2% (5)	1.000
Self-reported ethnicity (Caucasian)	78.1% (75)	80.9% (38)	77.1% (37)	0.2

Numbers are presented as mean (SD) or percentage (number of participants). Comparison between low/high PRS groups were carried out using Student t-test for continuous variables and chi-square test for categorical variables.

and adversity score on Snack Delay outcome was no longer statistically significant [$\beta = 0.082$, $p = 0.168$].

TABLE 4 | Participants' characteristics in ALSPAC.

Sample descriptive	Total (*n* = 467)
Sex – male	52.5% (245)
Maternal age at birth (years)	29.89 (4.44)
Gestational age (weeks)	39.76 (1.18)
Birth weight (g)	3,532 (482)
Breastfeeding at 3m (yes)	51.5% (240)
Smoking during pregnancy (yes)	19.1% (89)
Maternal education – university degree or above	18.7% (84)
Low Socioeconomic Status (SES) measured at 2 years, 9 months	37.3% (174)
Self-reported ethnicity (White)	99.8% (457)

Numbers are presented as mean (SD) or percentage (number of participants).

TABLE 5 | Details of missing data for specific components of the adversity score in Maternal Adversity, Vulnerability and Neurodevelopment (MAVAN).

Instruments included in the Adversity score	Number of items	*N* available	Missing
Birth information	2	199	0
Household gross income	1	199	0
Child hospitalization	1	199	0
Family Assessment Device	12	136	63
Attachment (scoring 28 min video of parent-child interaction)	1	165	34
Beck Depression Inventory	21	170	29
Edinburgh Postnatal Depression Scale	10	182	17
State-Trait Anxiety Inventory	40	195	4
Pregnancy anxiety	1	172	27
Smoking during pregnancy	1	172	27
Marital strain	9	193	6
Daily Hassles Scale	5	199	0
Physical/sexual abuse	2	199	0
Adversity score	Composite	101	98

For the main analysis, only complete cases were considered. A sub-analysis after imputing the missing variables using hot deck imputation was also performed.

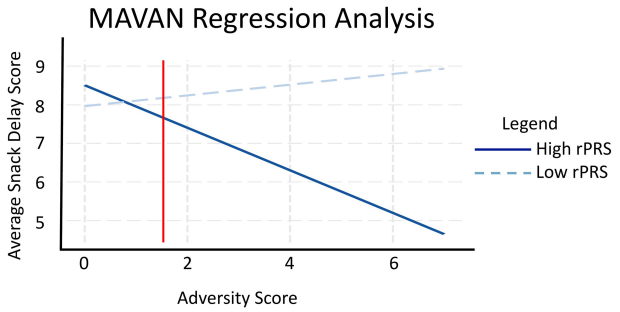

FIGURE 6 | Maternal Adversity, Vulnerability and Neurodevelopment regression analysis. rPRS was calculated for children in MAVAN using the fasting insulin GWAS with significant SNPs identified in ALSPAC through the rPRS methodology. Linear regression analysis showed a significant interaction between fasting insulin rPRS and adversity exposure on impulsivity [$N = 101$, $\beta = -0.329$, $p = 0.024$], tested by the Snack Delay Task applied at 36 months: higher adversity is linked to higher impulsivity in children with higher rPRS [rPRS = mean ± 1 SD, $\beta = -0.551$, $p = 0.009$], represented by the solid blue line. The dashed light blue line represents the low rPRS group, where the effect of adversity on impulsivity using the Snack Delay Task was not significant [$\beta = 0.139$, $p = 0.348$]. We also analyzed the form of these interactions according to Roisman. The regions of significance, as well as the proportion of interaction (PoI = 0.984) and proportion affected (PA = 0.782) are consistent with the diathesis stress form of interaction.

Enrichment Analysis on Fasting Insulin PRS

Enrichment analyses (MetaCore™) of the SNPs that compose the fasting insulin refined genetic score (rPRS) show that this subset of SNPs was significant for several nervous system development processes, as shown in **Figure 7**. Enrichment analysis (FUMA, see text footnote 1) of the genes mapped by the SNPs that compose the fasting insulin rPRS showed that these genes were significantly differentially upregulated in the following brain specific tissues, as shown in **Figure 8**: hippocampus, frontal cortex Brodmann area 9 (BA9), anterior cingulate cortex Brodmann area 24 (BA24), and the hypothalamus. Furthermore, these genes also had a significant GWAS enrichment for accelerated cognitive decline after conversion of mild cognitive impairment to Alzheimer's

disease (*FDR adjusted p-value* = 0.013). Using GeneMANIA (Franz et al., 2018), we discovered that this set of genes was part of a single co-expression network in *Homo sapiens*, as shown in **Figure 9**, indicating their shared involvement in biological processes (Ma et al., 2018).

DISCUSSION

The purpose of this study was to explore whether the genetic background associated with higher fasting insulin interacts with ELA to predict impulsivity, tested using the Snack Delay Task, in children. We demonstrated that the calculation of a rPRS, consisting of SNPs most associated with peripheral insulin levels in children and representing the risk for high fasting insulin levels early in life, can be derived from the GWAS of fasting insulin in adults. This refined polygenic score interacted with ELA exposure to predict impulsivity in children in the MAVAN cohort. Additionally, we observed that the SNPs composing the fasting insulin rPRS and their mapped genes were significantly correlated with various nervous system development processes.

Instead of using an arbitrary *p*-value threshold to calculate the PRS, we calculated the PRS at one hundred different thresholds in the independent cohort ALSPAC as a training sample. To calculate the PRSs, we applied the PRSoS tool (Chen et al., 2018). For each threshold, we explored the association between PRS and peripheral insulin levels within the ALSPAC cohort. This technique allowed us to identify the threshold of 0.24 to best predict peripheral insulin levels in children (Chen et al., 2020). To further refine this PRS, we associated each SNP within the subset obtained from the 0.24 threshold PRS with peripheral insulin levels in the discovery cohort ALSPAC and selected only the SNPs that significantly predicted the peripheral insulin levels in children to calculate our final rPRS. This refinement was necessary as the genetic markers for fasting insulin levels in adults may not be comparable to the genetic markers for fasting insulin levels in children. Since there is no GWAS available to identify the SNPs most associated with risk for high fasting insulin levels in children, we took an alternative approach, the rPRS, that allowed us to identify a subset of SNPs associated with high fasting insulin levels in children. Usually, analyses identifying which PRS threshold should be used are based on the greatest proportion of variance explained in the outcome, which would be the Snack Delay Task in this study. That does not take into account which PRS threshold is best predicting the phenotype composing the PRS itself, making our approach distinctive. Another strength in our approach is that we used a training sample to identify the best PRS threshold to use in our test sample. Subsetting the list of SNPs further adds to our distinctive methodology because we can be confident that the genetic background of fasting insulin used within the analysis is in fact correlated with actual peripheral insulin levels in children. The rPRS is a better predictor than peripheral insulin levels because the genetic background represents a more stable characteristic than the fluctuant insulin levels, which oscillate diurnally and may not be an accurate representation of a child's fasting insulin levels later in adulthood. By using the rPRS, which was calculated using the GWAS related to adult fasting insulin levels, we obtained a more accurate representation of the risk of a child to develop high fasting insulin. There is no overall effect of either adversity score or rPRS, but there is an interaction effect of adversity and rPRS on impulsivity at 36 months. Specifically, the effect of adversity on impulsivity was seen for individuals with a higher rPRS for fasting insulin.

Although the results of the interaction between rPRS and adversity were no longer significant after imputing the missing cases for the adversity score, we want to emphasize that the adversity score is a composite measure computed based on several different tools and assessments, including total scores of instruments (e.g., BDI) or complex behavioral tasks such as the Attachment profile assessed through detailed coding of filmed interactions (Strange Situation Task) (**Table 5**). Therefore, as much as imputations can be technically performed, we are not convinced that the imputed data can capture the multifaceted feature of our unique composite score, so our main analysis is focused on complete cases. Our missing data was mostly related to unit-level non-response (no information was collected for the respondent on a specific survey/instrument/questionnaire) rather than item non-response (the respondent was missing one or two questions of the survey/instrument/questionnaire) (Dong and Peng, 2013). Unit-level non-response can be more challenging to impute with confidence (Yan and Curtin, 2010). Relaxing the complexity of the adversity score mentioned above and considering the missing components of the score as missing items in the dataset, the hot-deck imputation was applied. While imputation is useful and necessary to support analysis and summarization, the imputation model should be properly specified, which we believe is difficult to achieve in this particular case. Some of the variables that compose the adversity score, for example the Attachment security information, are derived from a laboratory procedure designed to capture the balance of attachment and exploratory behavior under conditions of increasing moderate stress (Solomon and George, 1999), a unique measure that is hardly comparable to any other measure available in the dataset. Finally, we assumed a missing at random mechanism for the missing data, although adversity itself could be associated with the missingness pattern (Howe et al., 2013; Houtepen et al., 2018). Therefore, the results of this sub-analysis should be considered with caution.

The gene ontology enrichment analyses, done through MetaCore^{TM}, showed that the SNPs composing the fasting insulin rPRS are associated with nervous system development. Some of the enriched processes should be highlighted, such as the neurophysiological process of dopamine D2 receptor signaling in the central nervous system. The dopamine system has been linked to impulsive behavior in animal models and human studies (van Gaalen et al., 2006; Dalley and Roiser, 2012). This finding is interesting because it suggests a potential neurodevelopment pathway to support the relationship between the genetic background linked to insulin, ELA, and dopamine. Previous animal models from our laboratory have demonstrated that animals exposed to ELA showed a pronounced aversion to delayed rewards in addition to an increase in the medial prefrontal cortex D2 levels (Alves et al., 2019). Additionally, our

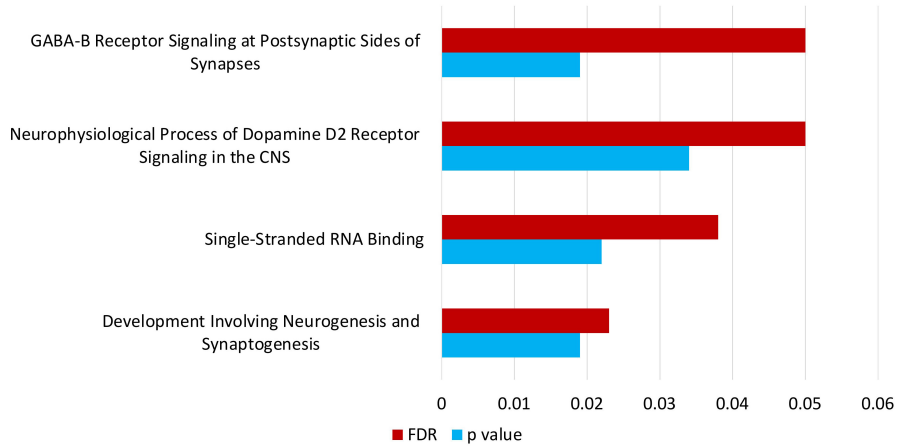

FIGURE 7 | Enrichment analysis through Metacore™. Enrichment analysis of the SNPs that compose the fasting insulin rPRS shows that this subset of SNPs is significant for certain nervous system development processes: neurophysiological process of GABA-B receptor signaling at postsynaptic sides of synapses (p = 0.019, FDR = 0.05), neurophysiological process of dopamine D2 receptor signaling in the CNS (p = 0.034, FDR = 0.05), single-stranded RNA binding (p = 0.022, FDR = 0.038), and development involving neurogenesis and synaptogenesis (p = 0.019, FDR = 0.023).

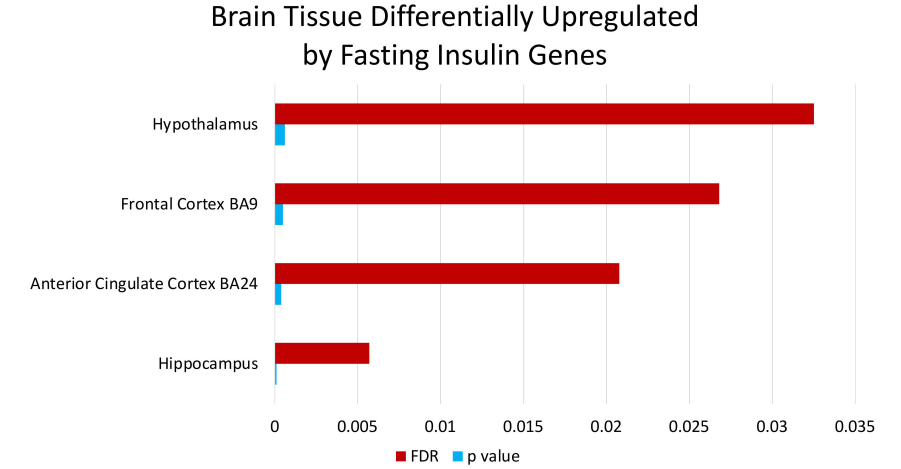

FIGURE 8 | Brain tissue differentially upregulated by fasting insulin genes. Enrichment analysis through FUMA of the genes mapped by the SNPs that compose the fasting insulin rPRS show that these genes are significantly differentially upregulated in brain specific tissues identified in the figure.

laboratory has shown that animals that have experienced ELA have a delay in dopamine release in the nucleus accumbens in response to palatable food, but insulin administration reverts this delayed effect (Laureano et al., 2019). These studies show that the relationship between ELA and dopamine is moderated by insulin. The set of fasting insulin SNPs identified in this present study could lead to further insight on the genetic background linking dopamine to impulsivity. Since the mechanism involving this association is still unknown, our findings bring us a step closer to this understanding.

Enrichment analyses in MetaCore™ revealed that the rPRS is enriched for single-stranded RNA binding. This suggests that the SNPs composing the rPRS play a crucial role in post-transcriptional regulation of gene expression (Guo et al., 2014) and hence are key in gene by environment interaction effects. The genes mapped to the SNPs in the rPRS are also differentially upregulated in the frontal cortex BA9, which is known to be involved in several executive functions such as short-term memory, inductive reasoning, working memory, and planning (Yogev-Seligmann et al., 2008). These findings, in addition to the genes being enriched for the accelerated cognitive decline GWAS, suggest that the genetic background associated with fasting insulin can impact several neurodevelopment executive functions, impulsivity being one of them, as well as risk for cognitive decline later in life. This aligns with studies that identified insulin receptors at hippocampal glutamatergic synapses, suggesting a role of insulin in neurotransmission, synaptic plasticity, and modulation of learning and memory,

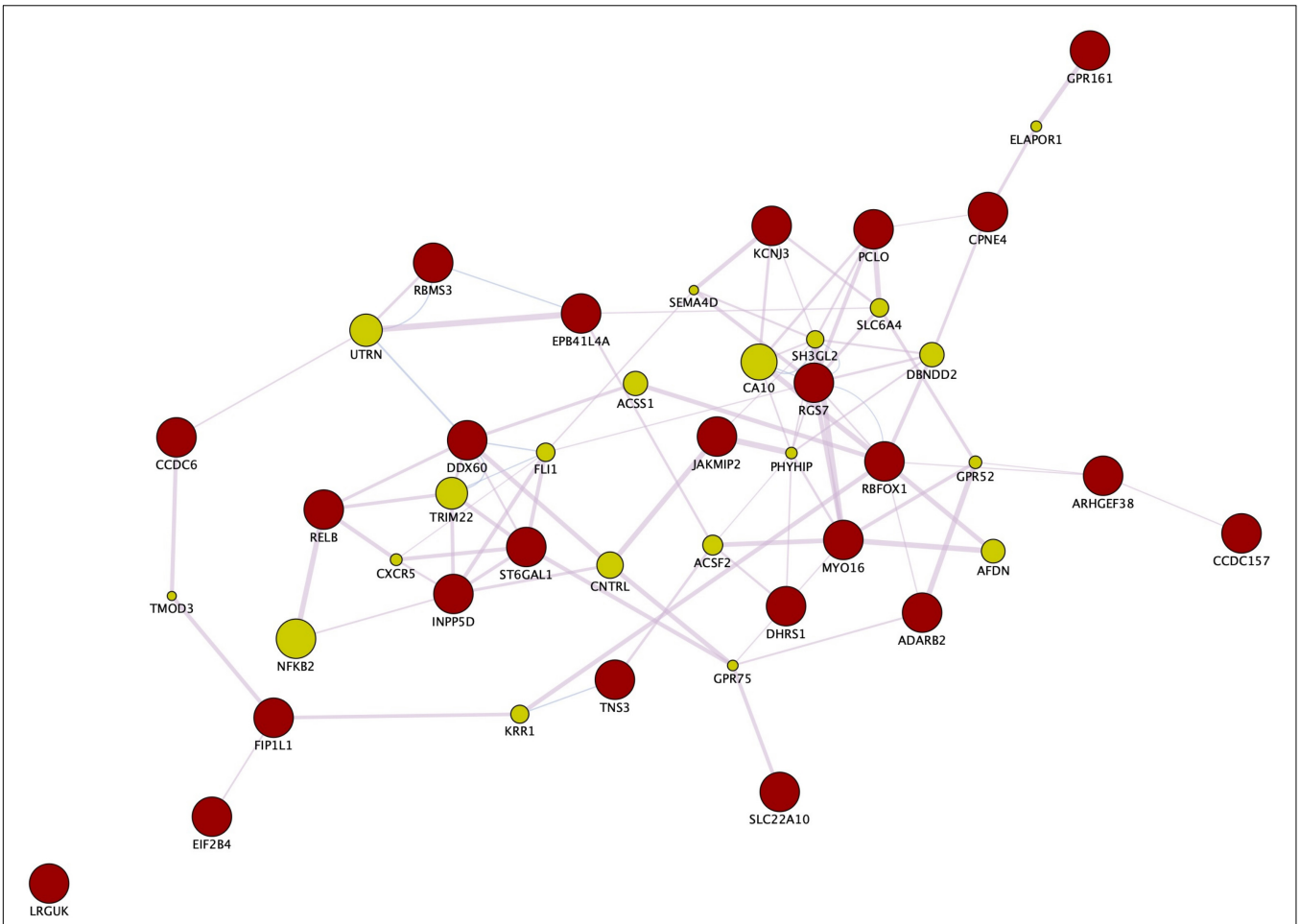

FIGURE 9 | Gene co-expression network. Enrichment analysis through GeneMANIA and Cytoscape of the genes mapped by the SNPs that compose the fasting insulin rPRS show that these genes are part of a co-expression network in *Homo sapiens*. Red circles indicate query genes, whereas green circles indicate related genes added by GeneMANIA. GeneMANIA translates mRNA expression data from Gene Expression Omnibus (GEO) to functional association networks that connect co-expressed genes through the pink lines displayed. The node sizes represent gene scores, indicating the number of paths that start at a given gene node and end up in one of the query genes.

while its inhibition is described in Alzheimer's disease and related animal models (Bomfim et al., 2012).

There are limitations within our study. Our discovery cohort and our testing cohort both largely consist of White/European ancestry, allowing us to identify the SNPs required within one cohort and testing the hypothesis within another cohort that has a similar population structure. Unfortunately, we cannot be certain that this subset of SNPs will be relevant for a different ancestry. Different ancestries have distinct allele frequencies (Frudakis et al., 2003) and this could result in peculiarities in the interaction between the genetic background and the environment. In fact, differential linkage disequilibrium between ancestral populations can produce false-positive SNPs when local ancestry is ignored, meaning that gene expression traits have differences as a function of genetic ancestry (Park et al., 2018). Furthermore, several studies showed genomic differences when investigating multi-ancestry genomic analysis (Bryc et al., 2015; Sung et al., 2019). In addition to ancestry, culture can impact one's behaviors, especially those related to executive functions like impulse control. There have been several examples of gene-culture interactions such as the cultivators in West Africa whose agriculture, which consisted of malaria-carrying mosquitos, showed preference for the hemoglobin S (*HbS*) "sickle-cell" allele to provide protection from malaria (Livingstone, 1958). Similarly, Polynesians being exposed to cold stress and starvation during their long open-ocean voyages may have resulted in positive selection for thrifty metabolism leading to type 2 diabetes susceptibility in present day Polynesians (Houghton, 1990). This gene-culture evolution emphasizes that one's lifestyle and environment have lasting impact and could be responsible for the differences seen in gene-environment interactions. Unfortunately, to the best of our knowledge, there is currently no fasting insulin GWAS available in a different ancestry for us to address this limitation within our

work. Future studies including a discovery cohort with peripheral insulin information in children and testing cohort of similar population structure in children are warranted.

These results together confirm that both ELA and the biological machinery associated with higher insulin levels are important factors influencing impulsivity in children. Our analyses showed that the genetic background associated with high fasting insulin levels moderates the effects of adversity on childhood impulsivity. This reinforces the idea that insulin signaling, which is implicated in metabolism and child growth, also plays a role in neurodevelopment. Previous studies have shown that impulsivity is a core feature of both psychopathology and metabolic diseases (Schachar and Logan, 1990; Silveira et al., 2012; Testa et al., 2019). Therefore, the interaction described here could be the basis to explain the co-morbidity associated with ELA exposure. Our results align with Hari Dass et al. (2019), which used a biologically-informed polygenic score based on insulin-related gene networks to predict both childhood impulsivity and risk for dementia later in life.

In conclusion, our present findings provide support for the impact of exposure to ELA in interaction with the genetic profile associated with high fasting insulin in predicting executive functions such as impulsivity in children. This research can be highly impactful as it provides insights into the vulnerability of executive function disorders early on in an individual's life. The biological mechanisms that we discovered to be involved in these processes can inform the development of early interventions and more efficient management of such health outcomes.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

Ethics approval for the ALSPAC study was obtained from the ALSPAC Ethics and Law Committee and the local research ethics committees (a full list of the ethics committees that approved different aspects of the ALSPAC studies is available at http://www.bristol.ac.uk/alspac/researchers/research-ethics/). Ethics approval for the MAVAN project was obtained from obstetricians performing deliveries at the study hospitals and by the institutional review boards at hospitals and university affiliates: McGill University, l'Université de Montréal, the Royal Victoria Hospital, Jewish General Hospital, Centre Hospitalier de l'Université de Montréal, Hôpital Maisonneuve-Rosemont, St Joseph's Hospital, and McMaster University, Hamilton, ON, Canada. Informed consent was obtained from the parents/guardians of the participants. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AB and PPS designed the experiments. AB, LMC, ZW, IP, and SP performed the analysis. LMC, CP, IP, RDL, MJM, and PPS provided important feedback and support for the data analysis and manuscript writing. AB wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.704785/full#supplementary-material

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Prenatal Maternal Stress From a Natural Disaster and Hippocampal Volumes: Gene-by-Environment Interactions in Young Adolescents From Project Ice Storm

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Gene-by-environment interactions influence brain development from conception to adulthood. In particular, the prenatal period is a window of vulnerability for the interplay between environmental and genetic factors to influence brain development. Rodent and human research demonstrates that prenatal maternal stress (PNMS) alters hippocampal volumes. Although PNMS affects hippocampal size on average, similar degrees of PNMS lead to different effects in different individuals. This differential susceptibility to the effects of PNMS may be due to genetic variants. Hence, we investigated the role of genetic variants of two SNPs that are candidates to moderate the effects of PNMS on hippocampal volume: COMT (rs4680) and BDNF (rs6265). To investigate this, we assessed 53 children who were *in utero* during the January 1998 Quebec ice storm. In June 1998 their mothers responded to questionnaires about their objective, cognitive, and subjective levels of stress from the ice storm. When children were 11 1/2 years old, T1-weighted structural magnetic resonance imaging (MRI) scans were obtained using a 3T scanner and analyzed to determine hippocampal volumes. We collected and genotyped the children's saliva DNA. Moderation analyses were conducted to determine whether either or both of the SNPs moderate the effect of PNMS on hippocampal volumes. We found that objective hardship was associated with right hippocampal volume in girls, and that the BDNF and COMT genotypes were associated with left hippocampal volume in boys and girls. In addition, SNPs located on COMT moderated the effect of maternal objective distress in boys, and subjective distress in girls, on both right hippocampal volume. Thus, we conclude that an individual's genotype alters their susceptibility to the effects of PNMS.

Keywords: prenatal maternal stress (PNMS), hippocampal volumes, COMT, BDNF, gene-by-environment interactions, natural disaster

INTRODUCTION

The hippocampus plays a key role in memory formation and learning (Spalding et al., 2013; Voss et al., 2017) and has been involved in spatial mapping and internalizing behaviors, such as anxiety and depression (Engin and Treit, 2007; Gatt et al., 2009; Gujral et al., 2017). In addition, the hippocampus is involved in control of hypothalamic-pituitary-adrenal (HPA) axis negative feedback (Snyder et al., 2011), and corticosteroid exposure seems to be linked to hippocampal volume and function (Sapolsky et al., 1990; Brown et al., 2004). Stressors occurring between the fetal period and childhood, as well as genetic factors [reviewed in Miguel et al. (2019)], can influence hippocampal development, thereby inducing long-term effects on brain structure and function.

Prenatal maternal stress (PNMS) is one such factor that has been thoroughly researched for its effects on hippocampal development in animals. These studies have provided substantial evidence that PNMS affects hippocampal development (Charil et al., 2010; Ortega-Martinez, 2015; Grigoryan and Segal, 2016). Magnetic resonance imaging (MRI) scans demonstrate that PNMS is associated with reduced hippocampal volume (Uno et al., 1994; Schmitz et al., 2002; Coe et al., 2003). Although these studies consistently demonstrate a reduction in hippocampal volume following PNMS, Schmitz et al. (2002) report a sex-specific effect, with reduced volume only observed in female rats, while other studies find effects in both sexes.

In humans, Qiu et al. (2013) have demonstrated that increased maternal anxiety during pregnancy was associated with reduced hippocampal growth in the offspring's first 6 months of life, suggesting that maternal anxiety during pregnancy predicts differences in hippocampal development; however, it remains unclear whether it is the heritable trait of anxiety or exposure to maternal stress hormones in the intrauterine environment that precipitates this effect. Recent research yields inconsistent results, such that stressful life events during the prenatal or early postnatal period were not associated with hippocampal volume (Marečková et al., 2018). Many studies have also investigated the effect of postnatal stress on hippocampal structure in humans. It has been demonstrated that patients with posttraumatic stress disorder, as a result of adversity in early life or adulthood, exhibit reduced hippocampal volume (O'Doherty et al., 2015; Bromis et al., 2018). However, other research has failed to replicate previous findings (De Bellis et al., 1999; Bonne et al., 2001; Yamasue et al., 2003). For example, a study of Vietnam war veterans with PTSD and their combat-naïve identical twins suggests, in fact, that smaller hippocampal volumes represent a pre-existing risk factor for developing PTSD in the face of trauma (Shin et al., 2006). Taken together, the disparity in findings concerning the effect of stress on hippocampal structure in humans, and the different effects of PNMS in male and female animal models, suggests that individuals may be differentially susceptible to the effects of PNMS. Differential susceptibility can occur as a function of genetic differences in the population that alter an individual's vulnerability to the effects of life events.

Indeed, single-nucleotide polymorphisms (SNPs) that affect brain development were observed to moderate the effect of maternal anxiety during pregnancy on the child's brain structure. For example, a SNP rs6265 converting a valine (Val) to methionine (Met) on the brain-derived neurotrophic factor (BDNF) gene, which promotes the growth, maturation and survival of nerve cells, influences the degree to which maternal anxiety induces DNA methylation in the offspring, and influences the relationship between the offspring's methylation and brain volume (Chen et al., 2015). Specifically, this paper reports that the Met/Met genotype in offspring was associated with a greater impact of maternal anxiety on DNA methylation and with a greater correlation between DNA methylation and right amygdala volume. Meanwhile, the Val/Val genotype was associated with a greater correlation between DNA methylation and left hippocampal volume. Moreover, another study reported that the interaction between the BDNF rs6265 Met allele and low family cohesion is associated with smaller left hippocampal volume in subjects with pediatric bipolar disorder (Zeni et al., 2016). Rabl et al. (2014) explored gene -by- environment effects between SNPs and adverse life events on hippocampal volume in healthy individuals. Among the SNPs studied, catechol-*O*-methyltransferase (COMT) Val158Met and BDNF Val66Met moderated the association between adverse life events and hippocampal volume in a large sample of healthy humans. The rs4680 COMT gene variant induces a Val to Met amino acid transition at codon 158 (Val158Met), resulting in a 4-fold decrease in enzyme activity in Met carriers. Qiu et al. (2015) investigated whether SNPs in the COMT gene of offspring could moderate the effects of maternal anxiety on brain structure, specifically prefrontal and parietal cortical thickness. The authors found that among rs737865-val158met-rs165599 haplotypes, the A-val-G haplotype exhibited a positive relationship between maternal anxiety and the offspring's cortical thickness in the right ventrolateral prefrontal cortex and the right superior parietal cortex. Meanwhile, the G-met-A haplotype exhibited a negative relationship between maternal anxiety and the offspring's cortical thickness in the bilateral precentral gyrus and dorsolateral prefrontal cortex. This demonstrates that particular COMT genotypes confer heightened vulnerability of frontal and parietal cortex regions to the effects of prenatal maternal anxiety. While these studies demonstrate that SNPs moderate the relationship between maternal anxiety and *in utero* neurodevelopment, further research is required to elucidate this relationship. The research to date has investigated only short-term effects of maternal anxiety on brain structure, demonstrating effects on children up to 6 months old.

Unlike animal studies, human research often lacks a randomly assigned stressor. The limitation of stressors from human studies is that they may be associated with an individual's traits, such as impulsivity or neuroticism, which may be transmitted to the offspring genetically. If a pregnant woman experiences stress that she or the father may have induced, in part, by their own temperament (e.g., divorce or job loss), and their child grows up to develop a similarly difficult temperament, it becomes almost impossible to determine the extent to which the association between the stress in pregnancy and the child's difficulties are due to genetic transmission, the intrauterine environment, and the postnatal rearing environment. Natural disasters randomly

affect large populations and have a sudden onset that affects pregnant women in different stages of pregnancy. Project Ice Storm provides a unique opportunity to determine the effects of the mothers' objective hardship, subjective distress and cognitive appraisal of a stressor through a quasi-randomly assigned event. Consequently, we use natural disasters to study the effects of prenatal maternal stress on the development of the offspring.

In the current project, studying PNMS derived a natural disaster, our goal was to (1) determine the effect of three measures of PNMS (objective hardship, subjective distress, and a negative cognitive appraisal) on left and right hippocampal volume in young adolescents; (2) determine the effect of genetic variants of COMT Val158Met and BDNF Val66Met on left and right hippocampal volume; and (3) determine the extent to which selected SNPs moderate the effect of PNMS on hippocampal volume. Since sex is an important determinant for the effects of stress on brain development, we expected that SNPs would differentially affect males and females.

MATERIALS AND METHODS

Participants

Recruitment

Following the ice storm in January 1998, our research group contacted obstetricians in the Montérégie, a region southeast of Montreal, Canada that was highly affected by the crisis. Physicians from four hospitals in the region identified women who met the following criteria: (1) were pregnant during, or within 3 months of the ice storm; (2) French Canadian; and (3) 18 years old or older. The families that responded were significantly better educated and had higher incomes than the regional averages. Families who gave consent have been assessed periodically. The protocols were approved by the Research Ethics Board of the Douglas Hospital Research Centre.

Participants

In this study, 53 children who were *in utero* during the ice storm, or were conceived within 3 months of the ice storm, were assessed at the age of 11 1/2 years. Among the 53 children, 15 (28.3%) had been in their first, 14 (26.4%) in their second, and 10 (18.9%) in their third trimester on January 9, 1998 (the peak of the ice storm). The remaining 14 (26.4%) children were conceived within 3 months of the storm; they were also considered as "exposed" because maternal stress hormones could still be elevated within 3 months of a major stressor. The participants included 27 boys and 26 girls for whom both brain and genotype data were available. We included children who were conceived within 3 months following the ice storm (preconception) because of the potential for long-term effects of the ice storm which continued to affect the population after the reference date of January 9, 1998. All participants were right-handed.

Measures

Prenatal Maternal Stress

Objective hardship, subjective distress, and cognitive appraisal measures were collected through maternal questionnaires mailed to the families on June 1, 1998, 5 months after the beginning of the ice storm.

Objective hardship

To estimate objective hardship of the mother, our group developed a questionnaire with items to evaluate four categories of exposure objectively (threat, loss, scope, and change). Questions in each category quantified experiences such as "number of days without electricity" and "number of displacements from home". Each category has a maximum score of eight points and is summed to create the Storm32 score (Laplante et al., 2007). In our sample, the Storm32 scores ranged from 5 to 24 and averaged 11.55 (SD = 4.53).

Subjective distress

The subjective distress of mothers was evaluated using the 22-item Impact of Event Scale-Revised (IES-R) (Weiss and Marmar, 1997), which includes questions concerning the severity of posttraumatic stress-like symptoms in three categories (hyperarousal, intrusion, and avoidance). The IES-R has good internal consistency (α = 0.93) and satisfactory test-retest reliability (r = 0.76) (Brunet et al., 2003) and was adapted to relate specifically to the ice storm. A cutoff score of 33 is often used to screen for probable PTSD. In our sample, the IES-R scores ranged from 0 to 40 and averaged 9.43 (SD = 9.68).

Cognitive appraisal

The mother's cognitive appraisal of the storm was assessed by asking "If you think about all of the consequences of the ice storm on your household members, would you say they were" and providing five response options on a Likert scale ["Very negative" (1), "Negative" (2), "None" (3), "Positive" (4), and "Very positive" (5)]. As our interest is the effect of negative cognitive appraisal about the ice storm on child outcomes, we compared the "Negative cognitive appraisal group" (recoded as 0), which included participants who had rated the consequences as very negative and negative, with a "Neutral/Positive cognitive appraisal group" (recoded as 1), which included participants who had rated the consequences as none, positive, or very positive.

Hippocampal Volume

MRI acquisition

Anatomical magnetic resonance imaging (MRI) was performed at the *Unité de Neuroimagerie Fonctionnelle (UNF) du Centre de Recherche de l'Institut Universitaire de Gériatrie de Montréal* (CRIUGM) on a 3.0T Siemens MAGNETOM Trio TIM Syngo (Siemens, Erlangen, Germany), with a 12-channel head coil. A total of 65 children underwent a three-dimensional, high-resolution, whole brain, structural T1-weighted magnetization-prepared gradient-echo image (MP-RAGE) sequence; TR = 2,300 ms, TE = 2.98 ms, TI = 900 ms; 256 mm field of view, 1 mm slice thickness, 176 slices, sagittal acquisition, time = 9 min. For this study, only 53 children's data were analyzed, as rest of 12 children had no genetic data.

MRI preprocessing

All MR images were converted from their standard Digital Imaging and Communications in Medicine (DICOM) format to

MINC2 (Medical Image NetCDF). Images were then corrected for intensity non-uniformity and underwent normalization for signal intensity (Talairach and Tournoux, 1988).

Total intracranial volume

For each subject, total intracranial volume (TIV) was automatically obtained using the Brain Extraction based on non-local Segmentation Techniques (BEaST) method (Eskildsen et al., 2012). The resulting skull masks were then manually corrected by an expert rater.

Automatic segmentation of the hippocampus

Bilateral hippocampal volumes, including subfields [(i) cornu ammonis (CA) 1, (ii) CA2/CA3, (iii) CA4/dentate gyrus, (iv) stratum radiatum/stratum lacunosum/stratum moleculare, and (v) subiculum] were automatically segmented using the Multiple Automatically Generated Templates brain segmentation (MAGeT-Brain) algorithm, which includes input from digital atlases by Winterburn et al. (2013), based on five high-resolution (0.3 mm isotropic) T1-weighted images (two males and three females, ages 29–57, avg. 37). The hippocampal atlases described here are available freely online[1] and when used with the MAGeT-Brain[2] segmentation technique produce reliable delineations of the hippocampus, including subfields.

Manual corrections and normalization

Whole left- and right-hippocampal volumes were delineated by merging the automated segmentation outputs of the hippocampal subfields to produce a single label for the whole hippocampus in each hemisphere. To increase the precision and validity of volumetric results, all hippocampal labels underwent manual corrections by an expert rater following the Pruessner segmentation protocol (Pruessner et al., 2000). Finally, to control for interindividual differences in total intracranial volume that may account for differences in hippocampus volume, hippocampal volumes were normalized by calculating a hippocampal volume-total intracranial volume ratio (HCV/TIV).

Table 1 presents descriptive statistics for all of the PNMS, demographic, and brain variables for boys and girls separately.

Genotype Assessment

When Project Ice Storm children were 8 1/2 years old, saliva samples were collected during a laboratory assessment using Oragene DNA self-collection kit (OG-500) (DNA Genotek) and stored at room temperature until further analysis. DNA extraction was performed using PrepIT-L2P kit (DNA Genotek) according to the manufacturer's instructions. DNA yield was measured using NanoDrop 8000 Spectrophotometer V2.1 (Thermo Fisher Scientific). DNA was stored at −80°C until analysis. rs6265 (BDNF) and rs4680 (COMT) were genotyped using Sequenom iPLEX Gold Technology (Ehrich et al., 2005) at McGill University and the Génome Québec Innovation Centre. All participants had call rates >98%, indicating generally good quality DNA and results.

[1] info2.camh.net/kf-tigr/index.php/Hippocampus

[2] cobralab.ca/software/MAGeTbrain

Genotype Frequencies

The major/major homozygote, heterozygote, and minor/minor homozygote genotype frequencies of each SNP are presented in **Table 2**. In addition, we tested each SNP for accordance with the Hardy-Weinberg equilibrium, which indicates whether the genotype frequencies in our sample were representative of the general population. Indeed, the SNP located on COMT met the Hardy-Weinberg equilibrium; however, the SNP located on BDNF could not be tested for the Hardy-Weinberg equilibrium because the minor genotype was not represented. Although studying BDNF using our sample was, therefore, limited, we still assessed the effect of having 1 or 2 major BDNF alleles on hippocampal volume because of the importance of this gene in hippocampal development. SNP genotype coding for COMT for the three different comparisons was as follows: AA (1) vs. AG (2) vs. GG (3); AA (1) vs. AG + GG (2); GG (1) vs. AA + AG (2). Genotype coding for the single BDNF comparison: CC (1) vs. TC (2).

Control Variables

Many factors are known to alter fetal development, and thus, induce long-term effects on hippocampal volume. To control for this, we tested the effect of some of these factors (maternal cigarette smoking during pregnancy, maternal alcohol use during pregnancy, socioeconomic status, birth weight, and timing of exposure during pregnancy) on HCV/TIV ratio in boys and girls. We controlled for factors that were correlated with HCV/TIV. The correlations between these potential control variables and HCV/TIV are presented in **Table 3**.

Statistical Analysis

Correlations and ANOVAs were conducted to determine the associations between PNMS and SNPs on HCV/TIV ratio. Moderation hierarchical regression analyses were conducted to determine whether either of the two SNPs moderate the effect of PNMS on HCV/TIV ratio. More specifically, analyses tested whether the two SNPs of interest moderate the effect of objective hardship, subjective distress, and/or cognitive appraisal on left and right HCV/TIV ratios. First, as mentioned above (**Table 3**), potential covariates that correlated significantly with the outcome were entered in the model. Accordingly, when testing for effects in both left and right HCV/TIV ratio in boys, we controlled for the number of glasses of alcohol the mother drank per week during her pregnancy. In addition, for left HCV/TIV ratio in girls we controlled for socioeconomic status (SES). No potential covariates were added to the right HCV/TIV model in girls. Additionally, for analyses focusing on subjective distress or cognitive appraisal, objective hardship was included as a covariate. Second, one of the components of prenatal stress (objective hardship or subjective distress or cognitive appraisal) was entered. Third, genotypes (COMT or BDNF) were entered: analyses included the number of major or minor alleles, but also dichotomized variables such that genotype groups included either minor allele carriers (minor merged with heterozygote) vs. major/major genotype carriers, or major allele carriers (major merged with heterozygote) vs. minor/minor genotype carriers.

TABLE 1 | Descriptive analysis of variables.

Sex Child	Variables	*N*	Min	Max	Mean	SD
Boys	Objective PNMS	27	5.00	24.00	11.2593	4.26608
	Subjective PNMS	27	0.00	40	10.2593	10.89316
	Maternal cognitive appraisal	27				
	Negative	7 (25.9%)				
	Positive	20 (74.1%)				
	Number of days of pregnancy when ice storm happened	27	−62	274	89.93	101.424
	Gestational age at birth (weeks)	27	32.29	41.29	39.4709	1.99009
	Edinburgh postnatal depression score	26	1.00	16.00	7.7308	4.01555
	Socioeconomic status (SES) Hollingshead scale	27	11.00	44.00	27.5926	10.39696
	Number of cigarettes/day	27	0.00	15.00	1.2593	3.85898
	Number of glasses of alcohol/week	27	0.00	2.00	0.1611	0.45454
	Child birth weight	27	1655.0	4185.0	3405.945	611.0644
	Left HCV	27	2302	3649	3002.48	339.067
	Right HCV	27	2310	3406	2953.37	296.586
	Left HCV_TIV RATIO	27	0.18	0.25	0.2125	0.01974
	Right HCV_TIV RATIO	27	0.18	0.23	0.2092	0.01825
	TIV	27	1243630	1603640	1412972.22	93490.764
Girls	Objective PNMS	26	5.00	24.00	11.8462	4.85545
	Subjective PNMS	26	0.00	24	8.5692	8.36712
	Maternal cognitive appraisal	26				
	Negative	11 (42.3%)				
	Positive	15 (57.7%)				
	Number of days of pregnancy when ice storm happened	26	−73	261	92.38	99.407
	Gestational age at birth (weeks)	26	32.86	41.43	39.4451	1.63997
	Edinburgh postnatal depression score	26	1.00	13.00	5.1538	3.42569
	Socioeconomic Status (SES) Hollingshead scale	26	11.00	65.00	26.9231	12.21449
	Number of cigarettes/day	26	0.00	25.00	2.1538	5.40698
	Number of glasses of alcohol/week	26	0.00	2.00	0.1182	0.43080
	Child birth weight	25	1855.0	4432.0	3426.486	535.0978
	Left HCV	26	2426	3322	2932.38	246.412
	Right HCV	26	2350	3201	2864.00	233.635
	Left HCV_TIV RATIO	26	0.18	0.27	0.2170	0.02025
	Right HCV_TIV RATIO	26	0.18	0.25	0.2120	0.02002
	TIV	26	1177870	1579900	1354365.38	83589.316

HCV, hippocampal volume; TIV, total intracranial volume.

TABLE 2 | Genotype frequencies.

	Boys			Girls		
	Major/Major homozygote	**Heterozygote**	**Minor/Minor homozygote**	**Major/Major homozygote**	**Heterozygote**	**Minor/Minor homozygote**
COMT rs4680	GG; Val/Val 3 (11.1%)	GA; Val/Met 20 (74.1%)	AA; Met/Met 4 (14.8%)	GG; Val/Val 5 (19.2%)	GA; Val/Met 8 (30.8%)	AA; Met/Met 13 (50.0%)
BDNF rs6265	CC; Val/Val 9 (33.3%)	CT; Val/Met 18 (66.7%)	TT not represented	CC; Val/Val 4 (15.4%)	CT; Val/Met 22 (84.6%)	TT not represented

Frequency is represented in number of participants and percent of participants. For BDNF, the minor homozygous Met/Met genotype was not represented in our sample.

TABLE 3 | Associations between risk factors and HCV/TIV ratios in boys and girls.

	Boys		Girls	
	Left HCV/TIV ratio	**Right HCV/TIV ratio**	**Left HCV/TIC ratio**	**Right HCV/TIV ratio**
Mother's cigarettes during pregnancy	$r = -0.179$	$r = -0.202$	$r = -0.151$	$r = -0.314$
Maternal alcohol during pregnancy	$r = -0.389^{*}$	$r = -0.418^{*}$	$r = 0.155$	$r = 0.313$
Socioeconomic status	$r = -0.333^{\#}$	$r = -0.281$	$r = 0.449^{*}$	$r = 0.247$
Birth weight	$r = 0.309$	$r = 0.278$	$r = -0.040$	$r = -0.014$
Timing of exposure during pregnancy	$r = 0.062$	$r = 0.047$	$r = 0.121$	$r = 0.106$

r, Pearson's correlation coefficient; $^{\#}p < 0.10$, $^{*}p < 0.05$.

Finally, the PNMS-by-SNP interaction term was added to the model. All analyses were conducted for boys and girls separately.

RESULTS

Bivariate Correlations Between PNMS or Genotype and HCV/TIV Ratio

As shown in **Table 4**, in boys, there was a marginally significant correlation between greater objective hardship and larger left HCV/TIV ratio ($r = 0.324$, $p = 0.099$). In girls, objective hardship was positively correlated with right HCV/TIV ratio ($r = 0.392$, $p = 0.048$). Neither subjective distress nor cognitive appraisal was significantly associated with HCV/TIV ratio in girls or boys.

In boys, the COMT genotypes were correlated with left HCV/TIV ratio ($r = 0.561$, $p = 0.002$) with a similar trend on the right HCV/TIV ratio ($r = 0.334$, $p = 0.089$) (**Table 4**). An ANOVA ($p = 0.008$, $F = 5.917$, df = 2) and Tukey *post hoc* test demonstrated that in boys the major homozygote genotype (GG) was significantly associated with larger left HCV/TIV ratio compared to the heterozygote (GA) ($p = 0.037$) and that of the minor homozygote (AA) (**Figure 1**). For BDNF genes, there was a trend for the major homozygote (CC) of the BDNF gene to be associated with smaller Left HCV/TIV ratio ($r = 0.369$, $p = 0.058$) in boys (**Table 4**), however, no significant finding for right HCV/TIV ratio was observed.

In girls, there were no associations between COMT genotypes and HCV/TIV. For BDNF genotypes, the major homozygote genotype (CC) associated with larger left HCV/TIV ratio ($r = -0.457$, $p = 0.019$), with a similar trend with right HCV/TIV ($r = -0.348$, $p = 0.082$) (**Figure 2**).

Gene-by-Environment Interaction

Moderation hierarchical regression analyses were conducted to test whether the two SNPs of interest moderate the effect of objective hardship, subjective distress and cognitive appraisal on the ratio of left and right HCV/TIV ratio.

TABLE 4 | Associations between PNMS and genotypes with HCV/TIV ratios in boys and girls.

	Boys		Girls	
	Left HCV/TIV ratio	**Right HCV/TIV ratio**	**Left HCV/TIV ratio**	**Right HCV/TIV ratio**
Objective PNMS	$r = 0.324^{\#}$	$r = 0.280$	$r = 0.305$	$r = 0.392^{*}$
Subjective PNMS	$r = -0.014$	$r = 0.317$	$r = 0.142$	$r = 0.023$
Cognitive appraisal	$r = -0.093$	$r = 0.032$	$r = 0.103$	$r = -0.019$
COMT (rs4680)	$r = 0.561^{**}$	$r = 0.334^{\#}$	$r = -0.099$	$r = -0.259$
BDNF (rs6265)	$r = 0.369^{\#}$	$r = 0.160$	$r = -0.457^{*}$	$r = -0.348^{\#}$

HCV, hippocampal volume; TIV, total intracranial volume.
r, Pearson's correlation coefficient; $^{\#}p < 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$.
Genotype coding:
COMT: AA = 1, AG = 2, GG = 3.
BDNF: CC = 1, TC = 2.

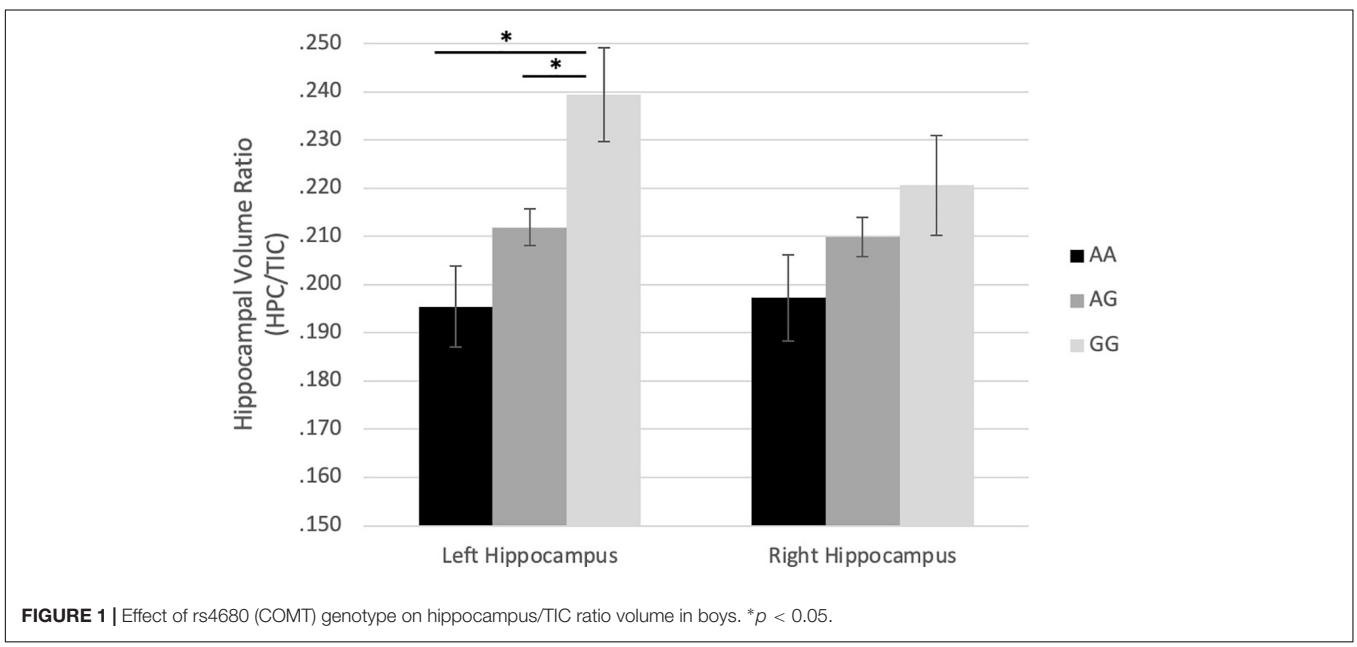

FIGURE 1 | Effect of rs4680 (COMT) genotype on hippocampus/TIC ratio volume in boys. $^{*}p < 0.05$.

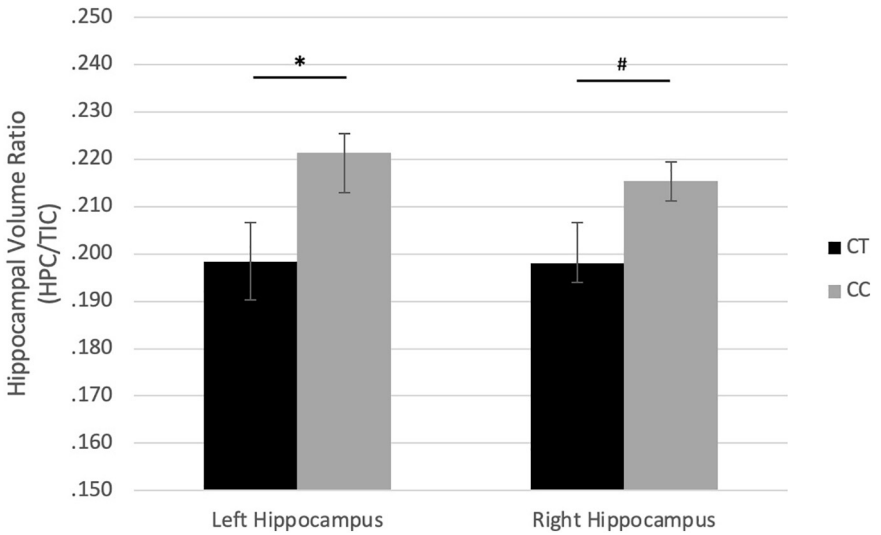

FIGURE 2 | Effect of rs6265 (BDNF) genotype on hippocampus/TIC ratio volume in girls. $^{\#}p < 0.10$, $^{*}p < 0.05$.

Effect of PNMS, Genotype and Gene-by-Environment Interaction on HCV/TIV Volume in Boys

For the left HCV/TIV ratio in boys

In the objective hardship model, we found that the covariate, maternal alcohol consumption during pregnancy, explained 15.1% (p = 0.045) of variance in the left HCV/TIV. Next, a marginally significant 9.3% of additional variance was explained by objective hardship levels (p = 0.099): greater hardship was associated with larger left HCV/TIV (**Table 5**). The addition of the main effects of the genotypes increased variance explained by 16.3% (p = 0.020) with significant effects from COMT genotypes [major homozygotes (GG) vs. heterozygotes (GA) vs. minor homozygotes (AA)], or 14.9% (p = 0.026) additional variance explained when comparing effects from major homozygotes (GG) vs. minor allele carriers (AA + AG). The full models explained 40.6 and 39.2% of variance, respectively. The main effect of minor homozygotes (AA) vs. major allele carriers (AG + GG) and the interactions were not significant.

In the subjective distress model, maternal alcohol consumption and objective hardship explained a significant 24.4% of variance in the outcome (p = 0.035). Subjective distress explained a non-significant additional 1.8% of variance. The main effects of the COMT genotypes [major homozygotes (GG) vs. heterozygotes (GA) vs. minor homozygotes (AA)] significantly increased variance explained by 15.3% (p = 0.025) while a significant additional 13.8% of the variance (p = 0.035) was explained by the major homozygotes (GG) vs. minor-allele carriers (AA + AG) genotypes. The full models explained 41.4 and 40.0% of variance, respectively. The main effect of minor homozygotes (AA) vs. major allele carriers (AG + GG) as well as the interactions were not significant.

In the cognitive appraisal model, maternal alcohol consumption during pregnancy and objective hardship explained a significant 24.4% of variance in the outcome (p = 0.035). Cognitive appraisal explained a non-significant additional 0.7% of variance. The COMT genotype main effect (major homozygote vs. heterozygote vs. minor homozygote) significantly increased variance explained by 17.3% (p = 0.018), while a significant additional 14.5% of the variance was explained by the comparison of the minor homozygotes vs. major-allele carriers (p = 0.031). The full models explained 42.3 and 39.6% of variance, respectively. The main effect of minor homozygotes (AA) vs. major allele carriers (AG + GG) as well as the interactions were not significant.

For the right HCV/TIV ratio in boys

In the objective hardship model, we found that the covariate, maternal alcohol consumption during pregnancy, explained 17.5% (p = 0.03) of the variance. Neither the main effect of objective hardship (6.7%) nor of COMT genotypes (1.3–2.5%) were significantly associated with right HCV/TIV. However, COMT genotypes [major homozygotes (GG) vs. minor-allele carriers (AA + AG)] were found to significantly moderate the effect of objective hardship on right HCV/TIV ratio (R^2-Change = 0.122, p = 0.049), with the full model explaining 37.8% of the variance in right HCV/TIV volume (**Table 5**). As shown in **Figure 3**, for major homozygotes (GG), there was a negative association between objective hardship and right HCV/TIV volume (p = 0.084) with greater objective hardship associated with smaller volumes; however, for minor-allele carriers (GA and AA), there was no association between objective hardship and right HCV/TIV volume (p = 0.106). Below objective hardship levels of 10.86 (slightly below the group average) there was a significant difference in right HCV/TIV volume between genotypes, with major homozygotes having larger volumes than the minor-allele carriers. At low levels of maternal objective hardship, the mean right HCV of GG boys is approximately 4 standard deviations (SD) above the group mean while at moderate levels of hardship the GG and A-allele carriers all

TABLE 5 | Effect of PNMS, genotype and gene-by-environment interaction on HCV/TIV volume in boys, controlling for maternal alcohol consumption during pregnancy with left and right HCV/TIV volume.

	Left HCV/TIV ratio (Boys)										Right HCV/TIV ratio (Boys)									
	Objective PNMS			Genotype			Interaction			Total	Objective PNMS			Genotype			Interaction			Total
	BETA[a]	CH R^2	*p*	BETA	CH R^2	*p*	BETA	CH R^2	*p*	R^2	BETA[a]	CH R^2	*p*	BETA	CH R^2	*p*	BETA	CH R^2	*p*	R^2
COMT																				
AA vs. AG vs. GG				0.437	0.163	**0.020**	1.586	0.032	0.272	0.439				0.170	0.025	0.387	−2.101	0.057	0.188	0.324
AA + AG vs. GG	0.305	0.093	0.099	−0.399	0.149	**0.026**	−1.422	0.014	0.473	0.407	0.259	0.067	0.158	−0.121	0.014	0.521	4.154	0.122	**0.049**	0.378
AA vs. AG + GG				0.233	0.047	0.228	0.626	0.002	0.817	0.293				0.124	0.013	0.527	−1.676	0.013	0.545	0.268
	Subjective PNMS			Genotype			Interaction			Total	Subjective PNMS			Genotype			Interaction			Total
	BETA[b]	CH R^2	*p*	BETA	CH R^2	*p*	BETA	CH R^2	*p*	R^2	BETA[b]	CH R^2	*p*	BETA	CH R^2	*p*	BETA	CH R^2	*p*	R^2
COMT																				
AA vs. AG vs. GG				0.426	0.153	**0.025**	−0.026	0.000	0.970	0.414				0.201	0.034	0.301	0.716	0.032	0.318	0.363
AA + AG vs. GG	−0.142	0.018	0.465	−0.388	0.138	**0.035**	0.293	0.003	0.731	0.403	0.250	0.055	0.192	−0.154	0.022	0.411	−0.958	0.037	0.285	0.356
AA vs. AG + GG				0.227	0.045	0.246	0.428	0.005	0.691	0.312				0.135	0.016	0.484	0.559	0.009	0.602	0.322
	Cognitive appraisal			Genotype			Interaction			Total	Cognitive appraisal			Genotype			Interaction			Total
	BETA[b]	CH R^2	*p*	BETA	CH R^2	*p*	BETA	CH R^2	*p*	R^2	BETA[b]	CH R^2	*p*	BETA	CH R^2	*p*	BETA	CH R^2	*p*	R^2
COMT																				
AA vs. AG vs. GG	0.095	0.007	0.654	0.453	0.173	**0.018**	−1.187	0.043	0.210	0.466	0.235	0.041	0.265	0.199	0.033	0.312	1.617	0.079	0.113	0.395
AA + AG vs. GG				−0.395	0.145	**0.031**	0.897	0.023	0.375	0.418				−0.107	0.011	0.570	−1.847	0.096	0.083	0.390

CH R^2, Change in R-squared (R^2).
a: control for the number of glasses of alcohol the mother drank per week during her pregnancy.
b: control for the number of glasses of alcohol the mother drank per week during her pregnancy, objective PNMS.
COMT genotype coding:
AA (1) vs. AG (2) vs. GG (3);
AA (1) vs. AG + GG (2);
GG (1) vs. AA + AG (2);
Bold means significant.

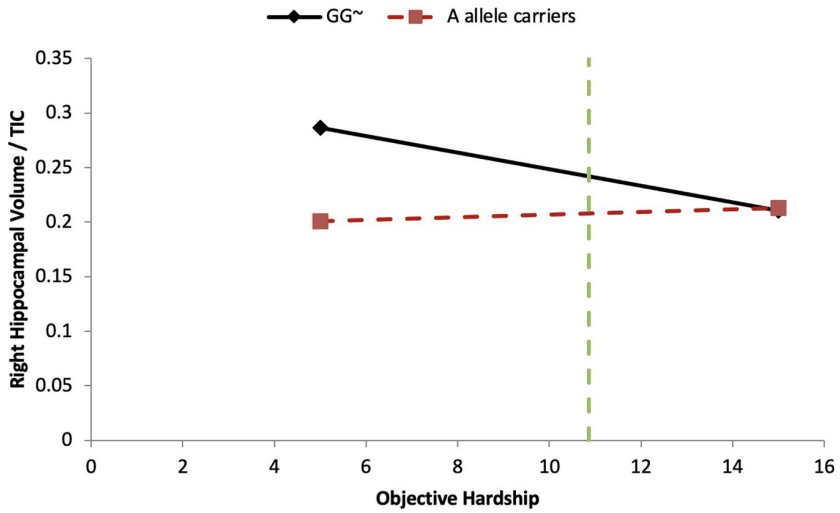

FIGURE 3 | COMT moderates objective hardship on right HCV/TIV ratio in boys. Moderation analyses demonstrate that there is a significant COMT-by-Objective PNMS interaction effect in boys ($p = 0.049$). For the major homozygote (COMT genotype GG, solid line) there is a marginally significant negative association between objective hardship and right HCV/TIV ratio volume ($p = 0.084$); however, for the heterozygote and minor homozygote COMT genotypes (A allele carriers, red dashed line) there is no association between objective hardship and right HCV/TIV ratio. There is a region of significance ($p < 0.05$) when objective hardship levels are below 10.86, such that participants with major genotypes have a larger right HCV/TIV ratio compared to minor allele carriers. The green dashed line indicates the value of 10.83. $\sim p < 0.10$.

averaged right HCV at the group mean. The other interactions with objective hardship were not significant.

The main effects of subjective distress or cognitive appraisal on right HCV/TIV volume, or the interactions including them, were not significant.

Thus, in boys, the only significant interaction effect between any maternal stress components and genotypes was between objective hardship and COMT on right HCV/TIV ratio. The full models, including maternal stress, COMT genotype, G × E interactions (if significant), and the covariate maternal alcohol consumption during pregnancy, explained 25.5–42.3% of the variance.

There were no significant interaction effects between PNMS and BDNF genotypes on hippocampus volumes in boys.

Effect of PNMS, Genotype and Gene-by-Environment Interaction on HCV/TIC Volume in Girls

For the left HCV/TIV ratio in girls

In the objective hardship model the covariate, SES, explained 20.1% ($p = 0.021$) of variance. There were no significant main effects of objective maternal stress (0.8%) nor of COMT genotypes (<0.1–0.7%), and no significant interaction effects.

In the subjective hardship model, SES and objective hardship explained together a marginally significant 21.0% of variance in the outcome ($p = 0.067$). Controlling for these covariates, subjective distress explained a non-significant additional 0.1% of variance, and COMT genotypes main effect were also non-significant (<0.1–0.7%). However, the COMT genotypes [major homozygotes (GG) vs. heterozygotes (GA) vs. minor homozygotes (AA)] were found to significantly moderate the effect of subjective distress on left HCV/TIV ratio (R^2-Change = 0.144, $p = 0.047$), with the full model explaining 35.8% of the variance in the outcome (**Table 6**). As shown in **Figure 4A**, for major homozygotes (GG), there was a marginally significant negative association between subjective distress and left HCV/TIV ratios ($p = 0.092$) with greater subjective distress related to smaller volumes. Left HCV/TIV ratio is approximately 1 SD above the mean at low levels of maternal subjective distress but at high levels of distress (a log-IESR of 3.2 which is equivalent to an IES-R score of 23.5 indicative of possible PTSD) they are about 1 SD below the mean. For minor homozygotes or heterozygotes, however, there was no association between subjective distress and left HCV/TIV ratios. There is no statistical region of significance within the observed range. The COMT comparison of major homozygotes (GG) vs. minor-allele carriers (AA + AG) (**Figure 4B**) was also found to significantly moderate the effect of subjective distress on left HCV/TIV ratio in girls (R^2-Change = 0.330, $p = 0.001$), with the full model explaining 54.1% of the variance in left HCV/TIV volume (**Table 6**). As shown in **Figure 4B**, for major homozygotes (GG), there was a significant negative association between subjective distress and left HCV/TIV ratio ($p = 0.0026$) with greater subjective distress related to smaller volumes; at low maternal subjective distress, the left HCV/TIV ratio for GG homozygotes was about 3 SD above the mean, while at high levels it was about 1.3 SD below the group mean. However, for minor-allele carriers, there was no association between subjective distress and left HCV/TIV ratios which remained at about the group mean level. Below log-transformed subjective distress level of 1.480 (equivalent to a low IES-R score of 3.39) there was a significant difference in left HCV/TIV volume between genotypes, with major homozygotes having larger volumes than the minor-allele carriers. However, above log-transformed subjective distress level of 2.771 (equivalent of a low-moderate IES-R score of 14.97)

TABLE 6 | Effect of PNMS, genotype and gene-by-environment interaction on HCV/TIC volume in girls, controlling for socioeconomic status With left HCV/TIV.

	Left HCV/TIV ratio (Girls)										Right HCV/TIV ratio (Girls)									
	Objective PNMS			Genotype			Interaction			Total	Objective PNMS			Genotype			Interaction			Total
	BETA	CH R^2	*p*	BETA	CH R^2	*p*	BETA	CH R^2	*p*	R^2	BETA	CH R^2	*p*	BETA	CH R^2	*p*	BETA	CH R^2	*p*	R^2
COMT																				
AA vs. AG vs. GG				−0.055	0.003	0.777	−0.467	0.020	0.468	0.233				−0.227	0.051	0.235	−0.623	0.036	0.320	0.241
AA + AG vs. GG	0.106	0.008	0.628	0.002	0.000	0.991	0.653	0.014	0.544	0.224	0.392	0.154	**0.048**	0.122	0.015	0.529	0.791	0.021	0.462	0.189
AA vs. AG + GG				−0.084	0.007	0.664	−0.527	0.020	0.461	0.237				−0.260	0.066	0.177	−0.816	0.049	0.235	0.269
	Subjective PNMS			Genotype			Interaction			Total	Subjective PNMS			Genotype			Interaction			Total
	BETA	CH R^2	*p*	BETA	CH R^2	*p*	BETA	CH R^2	*p*	R^2	BETA	CH R^2	*p*	BETA	CH R^2	*p*	BETA	CH R^2	*p*	R^2
COMT																				
AA vs. AG vs. GG				−0.058	0.003	0.769	−1.211	0.144	**0.047**	0.358				−0.220	0.048	0.261	−1.091	0.117	0.070	0.327
AA + AG vs. GG	0.031	0.001	0.879	0.009	0.000	0.966	3.271	0.330	**0.001**	0.541	−0.098	0.009	0.628	0.108	0.011	0.591	2.785	0.244	**0.007**	0.418
AA vs. AG + GG				−0.084	0.007	0.671	−0.640	0.035	0.347	0.252				−0.260	0.066	0.185	−0.738	0.047	0.257	0.275
	Cognitive appraisal			Genotype			Interaction			Total	Cognitive appraisal			Genotype			Interaction			Total
	BETA	CH R^2	*p*	BETA	CH R^2	*p*	BETA	CH R^2	*p*	R^2	BETA	CH R^2	*p*	BETA	CH R^2	*p*	BETA	CH R^2	*p*	R^2
COMT																				
AA vs. AG vs. GG				−0.090	0.008	0.637	0.335	0.009	0.618	0.286				−0.247	0.059	0.209	0.739	0.045	0.270	0.268
AA + AG vs. GG	0.255	0.059	0.197	0.061	0.003	0.759	−0.120	0.000	0.916	0.272	0.104	0.010	0.608	0.154	0.022	0.444	0.124	0.000	0.915	0.186
AA vs. AG + GG				−0.095	0.009	0.621	0.414	0.012	0.573	0.289				−0.264	0.068	0.178	1.062	0.077	0.141	0.308

CH R^2, Change in R-squared (R^2).
COMT genotype coding:
AA (1) vs. AG (2) vs. GG (3);
AA (1) vs. AG + GG (2);
GG (1) vs. AA + AG (2);
Bold means significant.

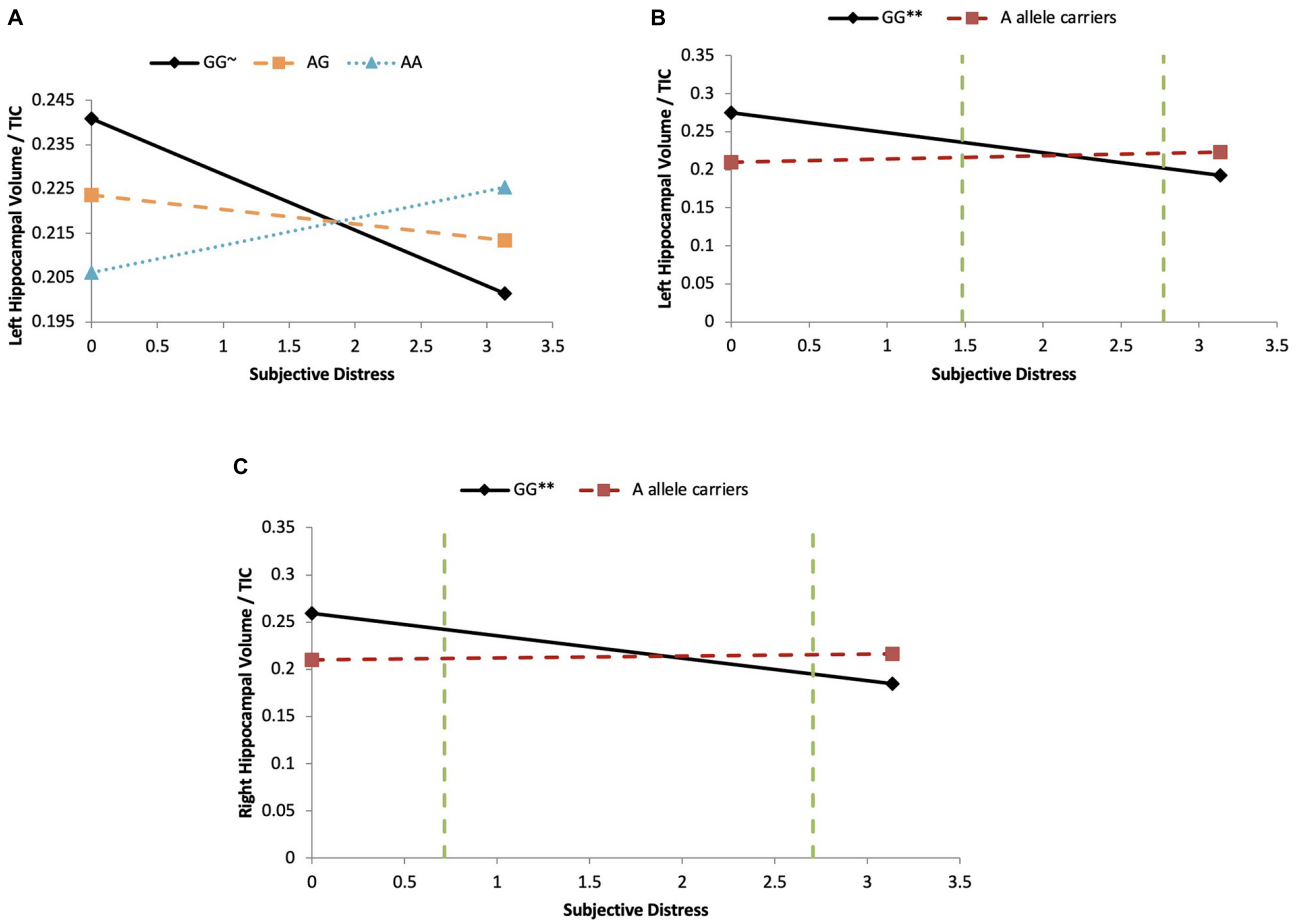

FIGURE 4 | COMT moderates subjective distress on left HCV/TTV ratio in girls. **(A)** *Major homozygotes vs. heterozygotes vs. minor homozygotes:* Moderation analyses demonstrate that there is a significant COMT-by-subjective distress interaction effect on left HCV/TIV ratio in girls ($p = 0.047$). For the minor homozygote (AA genotype, dotted line) and heterozygote (AG genotype, dashed line) there is no association between subjective distress and left HCV/TIV ratio ($p = 0.165$ and $p = 0.409$, respectively). For major homozygote (GG genotype, solid line) there is a marginally significant negative association between subjective distress and left HCV/TIV ratios ($p = 0.092$). There are no statistical significance transition points within the observed range of the predictor. $\sim p < 0.10$. **(B)** *Major homozygotes vs. minor allele carriers on left HCV/TIV ratio:* Moderation analyses demonstrate that there is a significant COMT-by- subjective distress interaction effect on left HCV/TIC ratio in girls ($p = 0.001$). For major homozygotes (GG genotype, solid line), there is a significant negative association between subjective distress and left HCV/TIV ratio ($p = 0.0026$) but for minor homozygotes and heterozygotes (AA and AG genotypes, red dashed line) there is no association between subjective distress and left HPV/TIV ratio ($p = 0.129$). There are regions of significance ($p < 0.05$) such that below log-transformed subjective distress levels of 1.48 and above 2.77 there is a significant difference between left HCV/TIV ratio of major homozygotes and minor-allele carriers. $^{**}p < 0.01$. **(C)** *Major homozygotes vs. minor allele carriers on right HCV/TIV ratio:* Moderation analyses demonstrate that there is a significant COMT-by-subjective distress interaction effect on right HCV/TIC ratio in girls ($p = 0.007$). For major homozygotes (GG genotype, solid line) there is a significant association between subjective distress and right HPV/TIV ratio volume ($p = 0.008$); however, for minor homozygotes and heterozygotes (AA and AG genotypes, red dashed line) there is no significant association between subjective distress and HCV/TIV ratio ($p = 0.272$). There is a region of significance ($p < 0.05$) between genotypes below log-transformed subjective distress levels of 0.717 and above 2.705, such that right HCV/TIC ratio were significantly different between the major homozygotes and the minor-allele carriers. The two green dashed lines indicates the regions of significance. $^{**}p < 0.01$.

major homozygotes had significantly smaller volumes than the minor-allele carriers. The interaction with minor homozygotes vs. major allele carrier was not significant.

Controlling for SES and objective hardship, the main effect of cognitive appraisal and its interactions with COMT genotypes were not significant.

For the right HCV/TIV ratio in girls

In the objective hardship model, objective hardship had a significant main effect on right HCV/TIV ratio, such that higher PNMS was associated with a larger ratio, explaining an additional 15.4% of the variance ($p = 0.048$). However, there was no significant main effect of any genotypes on right HCV/TIV volumes (R^2-change = 1.5–6.6%), or of any interactions involving objective hardship.

Controlling for objective hardship, the effect of subjective distress (R^2-change = 0.9%) and COMT genotypes (R^2-change = 1.1–6.6%) on right HCV/TIV volumes were not significant. However, COMT genotypes [major homozygotes (GG) vs. minor-allele carriers (AA + AG)] moderated

the effect of subjective distress on right HCV/TIV ratios (R^2-change = 0.244, p = 0.007) with the full model explaining 41.8% of the variance in the outcome (**Table 6**). As shown in **Figure 4C**, for major homozygotes (GG), there was a significant negative association between subjective distress and right HCV/TIV ratios (p = 0.008) with greater subjective distress related to smaller volumes; at low subjective distress GG homozygotes had right HCV/TIV ratios about 1.25 SD above the group mean while at high levels their HCV/TIV ratio was about 1.6 SD below the mean. For minor-allele carriers (AA + AG), however, there was no association between subjective distress and right HCV/TIV ratios. Below subjective distress level of 0.717 (equivalent to a very low IES-R score of 1.05) major homozygotes (GG) had significantly larger volumes than the minor-allele carriers (AA + AG), and above subjective distress level of 2.705 (equivalent to a low-moderate IES-R score of 13.95) major homozygotes (GG) had significantly smaller volumes than the minor-allele carriers (AA + AG). The other moderations involving subjective distress were not significant.

There were no significant main effect of cognitive appraisal or interactions involving this variable.

Thus, in girls, the significant interaction effects between maternal stress components and genotypes were between subjective distress and COMT on left and right HCV/TIV ratio. The full models, including maternal stress, COMT genotype, G × E interactions (if significant), and SES for the HCV/TIC ratio, explained 16.8–54.1% of the variance.

Additionally, there were no significant interaction effects between PNMS and BDNF genotypes on hippocampus volume in girls.

DISCUSSION

The main objective of this study was to test whether children's genotype would moderate the effects of maternal stress derived from 1998 Quebec ice storm during pregnancy on their hippocampal volume, thereby resulting in differential effects of PNMS on brain structure.

First, we examined the main effect of each component of PNMS on hippocampal volume. The previous literature reported associations between increased prenatal maternal anxiety and reduced hippocampal growth (Qiu et al., 2013), and the non-association between perinatal stressful life events and hippocampal volume (Marečková et al., 2018). Surprisingly, then, we found that higher maternal objective hardship was associated with larger, not smaller, right hippocampal volume in girls. Therefore, it seems that different types of stressors during pregnancy could result in mixed findings, at least when not taking genotype into account.

Second, zero-order correlations revealed associations between genotypes and hippocampal volume with COMT (rs4680) and BDNF (rs6265). Major homozygotes of COMT (Val/Val) are associated with larger hippocampal volume in boys, while major homozygotes of BDNF (Val/Val) are associated with larger hippocampal volume in girls. Although COMT Val carriers were reported in previous studies to have smaller hippocampal volumes than Met allele carriers in healthy individuals (Giordano et al., 1993; Taylor et al., 2007), our observation is consistent with the recent finding that COMT Val allele was significantly associated with larger hippocampal volume in healthy Chinese college students (ages from 19 to 21 years) (Wang et al., 2013). These correlations do not, of course, take prenatal stress into account.

The only significant gene-by-environment interactions involved the COMT genotype. Under maternal objective hardship conditions that are below the group mean (**Figure 3**), boys with the COMT major homozygotes have more than one standard deviation greater right hippocampal volume than that of minor-allele carriers who have approximately average ratios; however, because the right HCV/TIV ratios of boys with major homozygotes decrease as objective hardship increases (while those of the minor allele carriers are unaffected by PNMS), under high objective hardship there is little difference in HCV/TIV ratios between COMT genotypes. COMT is highly expressed in the hippocampus and the COMT minor allele results in reduced COMT enzymatic activity 3–4 fold which leads to higher dopamine levels. The HPA axis is activated in response to stress, which results in the release of the cortisol. Although Project Ice Storm tends to have very low correlations between objective hardship and maternal cortisol levels at recruitment (5 months after the start of the storm), our finding might suggest that high acute cortisol levels at the time of the storm (that would have been triggered by stress) and lower dopamine availability in Val carriers due to the higher enzymatic activity may collaborate to negatively affect hippocampal volume in boys. Similarly, in girls at low levels of maternal subjective distress, girls with major homozygotes again exhibit about one standard deviation greater left and right hippocampal volumes than those associated with minor allele carriers. However, this group difference is not seen under conditions of low-medium subjective distress, and is reversed in both the left and right hippocampi under conditions of higher subjective distress (**Figures 4B,C**). Our finding is supported by a recent PTSD study in which COMT Val158Met polymorphism moderated the relationship between PTSD symptom severity and hippocampal volume (Hayes et al., 2017): reduced left hippocampal volume was observed with increasing PTSD symptom severity in Val/Val carriers, but there was no association between PTSD symptom severity and left hippocampal volume for either of the Met allele carriers, and there were no significant main effects, nor an interaction effect, for the right hippocampus. Further, the authors suggested that putatively lower dopamine availability in Val carriers may interact with traumatic stress to negatively affect hippocampal structure.

Remarkably, in our study adolescents in the major homozygote group have a significantly larger hippocampal volume in low objective and low subjective PNMS conditions compared to high PNMS conditions, while heterozygotes and minor homozygotes do not seem to be affected by the levels of PNMS. Therefore, our results suggest that as maternal stress increases in severity children who are carriers of the Val/Val genotype are more likely to show reductions in hippocampal volume than Met carriers, indicating that

the major homozygotes may be more susceptible to the effects of PNMS.

In contrast to the sex differences in the effects of the COMT and BDNF genotypes on hippocampal volume at early adolescence, it has previously been reported that the minor homozygote BDNF genotype is associated with reduced hippocampal volume in *both* male and female adults (Bueller et al., 2006). Given that we measured hippocampal volume at 11 1/2 years old, rather than in adulthood, it is possible that the genotype-dependent sex differences in male and female hippocampal volume are specific to this age group, which represents the onset of adolescence. The mechanism underlying the sex-specific effects of COMT and BDNF SNPs on hippocampal volume in early adolescence remains unclear but is likely influenced by sex hormones that are highly expressed in the hippocampus and interact with hippocampal function (Goldstein et al., 2001). Sex hormones may influence the role of BDNF and COMT through various mechanisms. For example, estrogen is known to alter levels of BDNF expression, thereby leading to disproportionate regulation of BDNF protein levels in females compared to males (Sohrabji and Lewis, 2006). These same sex hormone differences found between adolescent males and females may also explain observed sex differences in the moderation effects. While the COMT genotype moderated the effect of prenatal maternal objective hardship in boys, this SNP moderated the effect of maternal subjective distress in girls. These sex-specific results are in line with our original hypothesis, which postulated that results would differ between males and females and indicate sex differences in brain development that are particularly prevalent at the onset of puberty. Our finding was supported by Jahnke et al. (2021) in which maternal stress during pregnancy was reported to influence the functioning of HSD11B2 in placenta in a sex-specific manner, suggesting that maternal chronic stress may exhaust HSD11B2's protective mechanism, exposing the newborn to high amounts of maternal cortisol, which could alter the fetal HPA axis and influence long-term neurobehavioral development.

Limitations and Advantages

Although the results described here further our understanding of the interaction between genetic variants and PNMS on hippocampal structure in children, as our sample included only 53 children, further research with larger cohort is required to validate these findings. Given the importance of the BDNF gene, it is unfortunate that our sample was missing subjects with the minor homozygote genotype for a full investigation of this gene's effects. On the other hand, natural disasters provide unique opportunities to study the effect of an objective, randomly assigned stressor in a human population, thereby overcoming many limitations of previous human gene-by-environment research. The 1998 Quebec ice storm was a powerful enough stressor that high scores on the maternal objective hardship scale predicted a full standard deviation lower IQ in the children at age 2 (Laplante et al., 2004) and again at 5 1/2 years (Laplante et al., 2008), higher body mass index and obesity rates at ages 5 1/2 (Dancause et al., 2012) through age 15 (Liu et al., 2016), and higher insulin secretion (Dancause et al., 2013) and pro-inflammatory cytokines (Veru et al., 2015) at age 13. Consequently, using this population-level acute stressor, we have been able to delineate the influences of PNMS and SNPs on hippocampal volume in early adolescent offspring.

Our findings support the hypothesis that individuals exhibit differential susceptibility to the effects of PNMS, and that genetic variants that alter the function or expression of proteins, underlie these differences. We showed that, in some cases, genetic variants, PNMS, and the gene-by-environment interaction combined explained up to half the variance in hippocampal volume. This is significant as it increases our understanding of how genetic and environmental factors work in combination to affect hippocampal development.

CONCLUSION

This work has increased our understanding of gene-by-environment interactions during prenatal brain development. Specifically, we have found that a SNP located on COMT significantly moderates the effects of PNMS on hippocampal volume, resulting in differential susceptibility between COMT genotypes to the effects of PNMS. In addition, the effect of different aspects of PNMS – objective hardship and subjective distress – on hippocampal volume was differentially moderated by the SNPs of interest. When moderated by SNPs located on COMT, subjective distress exhibited greater effects on hippocampal volume than objective hardship and cognitive appraisal in girls, while objective hardship had the most effect in boys. Overall, in accordance with our hypothesis, these results suggest that a child's genotype can alter their vulnerability to the effects of PNMS; however, these effects are often specific to a particular sex and/or aspect of stress.

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 Research Ethics Board of the Douglas Hospital Research Centre. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SK designed and implemented Project Ice Storm. LC-L, SY, and SK conceived of the current experiment. RD ran the automated segmentation pipelines, did the manual corrections of the amygdala, and total brain volume segmentations derived from the automated segmentations. GE, LC-L, and SY ran the statistical analyses. SY interpreted the data and drafted an

early version of the manuscript. LC-L, SK, and DL provided intellectual contributions for the rationale, interpretation of the data, and prepared the final manuscript for submission. All authors contributed to the article and approved the submitted version.

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Cognitive Development and Brain Gray Matter Susceptibility to Prenatal Adversities: Moderation by the Prefrontal Cortex Brain-Derived Neurotrophic Factor Gene Co-expression Network

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Background: Previous studies focused on the relationship between prenatal conditions and neurodevelopmental outcomes later in life, but few have explored the interplay between gene co-expression networks and prenatal adversity conditions on cognitive development trajectories and gray matter density.

Methods: We analyzed the moderation effects of an expression polygenic score (ePRS) for the Brain-derived Neurotrophic Factor gene network (BDNF ePRS) on the association between prenatal adversity and child cognitive development. A score based on genes co-expressed with the prefrontal cortex (PFC) BDNF was created, using the effect size of the association between the individual single nucleotide polymorphisms (SNP) and the BDNF expression in the PFC. Cognitive development trajectories of 157 young children from the Maternal Adversity, Vulnerability and Neurodevelopment (MAVAN) cohort were assessed longitudinally in 4-time points (6, 12, 18, and 36 months) using the Bayley-II mental scales.

Results: Linear mixed-effects modeling indicated that BDNF ePRS moderates the effects of prenatal adversity on cognitive growth. In children with high BDNF ePRS, higher prenatal adversity was associated with slower cognitive development in comparison with those exposed to lower prenatal adversity. Parallel-Independent Component Analysis (pICA) suggested that associations of expression-based SNPs and gray matter density significantly differed between low and high prenatal adversity groups. The brain IC included areas involved in visual association processes (Brodmann area 19

and 18), reallocation of attention, and integration of information across the supramodal cortex (Brodmann area 10).

Conclusion: Cognitive development trajectories and brain gray matter seem to be influenced by the interplay of prenatal environmental conditions and the expression of an important BDNF gene network that guides the growth and plasticity of neurons and synapses.

Keywords: BDNF, polygenic score, prenatal adversity, cognitive development, gray matter

INTRODUCTION

Brain-derived Neurotrophic Factor (BDNF) is a protein involved in several biological pathways – from neurogenesis, promotion of neuronal survival and differentiation, to modulation of synaptic plasticity – playing a central role in both the developing and adult nervous system (Hempstead, 2014). Acting through its high-affinity tyrosine receptor kinase B (TrkB) receptor, it mediates neurite and spine outgrowth (Binder and Scharfman, 2004; Ji et al., 2005), and this signaling is also important for synaptic plasticity (Ji et al., 2005; Lu et al., 2014), a phenomenon that enables the organism to change according to environmental stimuli, and makes possible learning and memory. Also, it controls short and long-lasting synaptic interactions in the hippocampus, and its expression mediates working memory processes in the prefrontal cortex (Gold et al., 2003; Xing et al., 2012; Kowiański et al., 2018). BDNF is expressed in almost all brain regions, but the highest levels are found in the frontal cortex, hippocampus, and amygdala (West et al., 2014). Several studies indicate altered BDNF expression in brain structures like the prefrontal cortex (PFC), hippocampus, and striatum in post-mortem human brains of patients that suffered psychiatric illnesses. There are decreased levels of BDNF mRNA and protein expression in the hippocampus of suicide victims (Banerjee et al., 2013), and significant differences in BDNF transcripts allow to distinguish schizophrenia, bipolar disorder, and major depressive disorder patients from healthy subjects, suggesting that the BDNF system is implicated in several physiological aspects of brain development (Molendijk et al., 2012; Banerjee et al., 2013; Reinhart et al., 2015).

Prenatal exposure to stress, maternal depression/anxiety, low social support, and poor access to prenatal health services have long-term effects on child cognitive development that are well documented (Monk et al., 2012; O'Donnell et al., 2014a; Silveira et al., 2017). Brain plasticity and maturation are affected by positive and negative environmental exposures during sensitive periods of development (Nelson and Gabard-Durnam, 2020). The brain matures in a hierarchical manner, meaning that the quality of maturation of early-developing regions will affect the subsequent development of other regions (Tottenham, 2019). Gene expression in different brain regions at different developmental stages indicates that timing is an important factor at the transcriptome level (Somel et al., 2009; Haeussler et al., 2017). This makes complex cerebral regions, for instance, the PFC, particularly sensitive to environmental conditions. The PCF receives several inputs from all other cortical areas, playing a key role in planning and performance of higher thinking, cognitive, affective, and social behaviors throughout development (Kolb et al., 2012); such interconnectivity results in a longer period needed for maturation (Fuster, 2015).

Expression of BDNF, and TrkB receptors begins early during brain development, especially in the cortical plate, both in rodents and primates (for a review, see Bartkowska et al., 2010). Therefore, it is not surprising that disturbances in its function early in life have remarkable effects upon neuronal structure and function. For example, transgenic mice with a functional reduction in BDNF or TrkB genes have a curtailment of dendritic arborization in cortical neurons in the prepubertal period (Xu et al., 2000; Gorski et al., 2003), and impairments in memory (Gorski et al., 2003). In this scenario, studies using animal models of prenatal stress have reported altered BDNF signaling during post-natal development (Badihian et al., 2020; Sobolewski et al., 2020). Stressors such as maternal immune activation during gestation, repeated restraint, or variable stress during pregnancy, cause altered BDNF expression in the PFC at different ages during development of the offspring (Matrisciano et al., 2012; Hemmerle et al., 2015; Niu et al., 2020). Accordingly, prefrontal TrkB and glucocorticoid receptor (GR) activities are known to be modulated by exposure to stressors (reviewed in Barfield and Gourley, 2018), and TrkB-GR interaction has been suggested (Numakawa et al., 2009). Therefore, prolonged variations in glucocorticoids could affect both GR and BDNF-TrkB function in the PFC (Barfield and Gourley, 2018), contributing to stress-induced cognitive alterations. In addition, abnormal signaling in the BDNF/TrkB pathway was reported to lead to abnormalities in the GABAergic and glutamatergic activities in the PFC (Sakata et al., 2009; Zhang et al., 2013).

Negative exposures during the prenatal and early postnatal period have been associated with cognitive and brain development in different ways. Behaviorally, the attainment of cognitive skills is understood as a developmental cascade, characterized by a cumulative process in which functioning at a lower level of behavior (e.g., visuomotor integration, fine motor skills, habituation) affects higher-level functions that develop later (e.g., IQ, language and executive functions) (Almas et al., 2016; Choi et al., 2018; Camerota and Willoughby, 2019). In terms of neurodevelopment, experiments with infant rats exposed to caretakers that displayed abusive behaviors show increased levels of methylation of BDNF DNA throughout the life span, and reduced BDNF gene expression in the adult PFC (Roth et al., 2009). Prenatal exposure to stress was also associated with high methylation and lower expression of the BDNF gene

in the PFC and hippocampus (Roth et al., 2011; Badihian et al., 2020). In humans, brain structure is also impacted by early life stressors, resulting in several morphological and functional alterations (Buss et al., 2010; Hair et al., 2015; Noble et al., 2015). The mentioned interrelated pathways affect the developing individual resulting in a predisposition for disease and poorer developmental outcomes later in life. Adolescence is also a sensitive period for PFC development. The PFC is one of the last brain regions to mature (Fuster, 2015; Hoops and Flores, 2017), and it is known to undergo significant structural remodeling, with dendritic and synaptic pruning during adolescence (Bourgeois et al., 1994; Shaw et al., 2020). This period of synaptic remodeling is believed to generate a refinement of connections (Barfield and Gourley, 2018). Therefore, exposure to adversities during this period can impact on PFC circuitry, and on adult behavior (Shaw et al., 2020). However, before this period of pruning, there is an initial phase of neuronal differentiation, dendritic spine and synapse overproduction, that occurs during prenatal and early childhood periods that will influence future development, stressing the relance of this sensitive window (Bourgeois et al., 1994; Lotfipour et al., 2009).

In summary, a large body of evidence indicates that early exposure to environmental adversity affects cognitive development, and some individuals are more susceptible than others to this long-term effect. Individual differences likely affect the impact of environmental exposure on several child developmental outcomes (Belsky, 2013; Silveira et al., 2017). It was shown that genetic variation in the BDNF gene (the Val66Met polymorphism), which decreases BDNF function (Egan et al., 2003), can lead to lower memory levels (Egan et al., 2003), and is associated with impairment in executive functioning (Nagata et al., 2012). This is particularly significant in individuals with high levels of early life adversity (Gabrys et al., 2017), in which this variation was associated with difficulties in attentional flexibility, a PFC-based function. Also, previous studies involving Val66Met polymorphisms suggested a role of the BDNF gene in moderating the effects of early adversity on attention problems and child behavior (Drury et al., 2012; Gunnar et al., 2012; O'Donnell et al., 2014b). However, it is known that the action of a gene is not isolated, but correlated in concert with other genes in functional networks (Gaiteri et al., 2014).

Genome-wide association studies (GWAS) are an important technological advance for the understanding of human health and disease but are still not able to inform the underlying tissue-specific mechanisms that explain phenotypic variation (Silveira et al., 2017; Hari Dass et al., 2019). GWAS considers only the highly significant genetic variants associated with a disease, thus are not enlightening of the several manifestations or endophenotypes that may precede the phenotype (Dalle Molle et al., 2017; Hari Dass et al., 2019). We propose a novel genomics approach, using a biologically informed genetic score based on genes co-expressed with the BDNF gene specifically in the PFC (BDNF ePRS) during the prenatal and early life periods to investigate the association with child cognitive development from 6 to 36 months of age. For a sub-sample of participants that we were able to follow up and collect structural Magnetic resonance images at age 9 we analyzed the multivariate association between the single nucleotide polymorphisms (SNPs) from the BDNF ePRS and gray matter density in order to uncover the mechanism of the interaction between prenatal environment and genotype and its association with brain development.

MATERIALS AND METHODS

Participants and Cohort Characteristics

Participants' data were derived from the Maternal Adversity, Vulnerability and Neurodevelopment prospective community-based cohort MAVAN (O'Donnell et al., 2014). A hundred and fifty-seven children from two sites - Montreal (Québec) and Hamilton (Ontario), Canada - composed the sample of the present study. Pregnant women were recruited around 13 to 20 weeks of gestation from obstetric clinics in hospitals. They were eligible to take part in the study if over 18 years of age, fluent in either English or French, and did not have serious obstetric complications during the pregnancy or delivery of the child, had a child with extremely low birth weight, or had any congenital diseases. Children were monitored from birth up to 12 years of age using several assessments of neurodevelopment. Ethical approval for this study was obtained from the Douglas Mental Health University Institute (Montreal) and St-Joseph's Healthcare (Hamilton Integrated Research Ethics Board). For this work, we considered cognitive neurodevelopmental data from the 6, 12, 18, and 36-months postnatal periods (N = 157), and a magnetic resonance imaging from a follow-up sample of 47 children at age nine (mean age = 9.3, SD = 1.4), the characteristics of the sample are shown in **Table 1**.

Measures

Cumulative Prenatal Adversity Score

The cumulative prenatal adversity score is a measure used to describe prenatal adversity conditions. It is composed of several indicators identified in the literature as being related to negative children's outcomes (Silveira et al., 2017). It surveyed pregnancy conditions, maternal mental health during pregnancy (anxiety, depression), presence of chronic diseases such as

TABLE 1 | Maternal Adversity, Vulnerability and Neurodevelopment (MAVAN) sample characteristics.

Variables	Cognitive development sample (6–36 months)	MRI sample (9 years)
	***N* = 157**	***N* = 47**
Gestational weeks, *M* (SD)	39.0 (1.2)	39.3 (1.2)
Birth weight (grams), *M* (SD)	3326.3 (448.3)	3256.1(458.7)
Income less than $30,000 a year	26 (16.5%)	16 (34.0%)
Maternal education: some community-college or less	14 (8.9%)	6 (12.8%)
Male sex	76 (48.4%)	28 (59.6%)
Smoking during pregnancy	17 (10.8%)	11 (23.4%)
Montreal site	81 (51.5%)	38 (80.8%)
Cumulative prenatal score, *M* (SD)	1.3 (1.2)	1.2 (1.2)

TABLE 2 | Psychometric scales used to compose the Cumulative Prenatal Adversity Score.

Measure	Description	Scoring
Daily Hassles Scale (Lobel and Dunkel-schetter, 1990)	Indicates the level of struggle and frequency in respect to lack of money for basic needs such as food and electricity since the beginning of pregnancy. The mean test-retest reliability of the scale is.79.	Lack of money score above 9.
Center for Epidemiological Studies Depression Scale (CESD) (Radolf, 1977)	Assesses depressive symptomatology in the general population with emphasis on affective and somatic components. 20 items are scored on a 4-point Likert scale and high scores indicate more severe depressive symptoms. The internal consistency of the scale is.85 (coefficient Alpha).	Prenatal depression scores above 22.
State-Trait Anxiety Inventory (STAI) (Spielberger, 1989)	A measure of trait and state anxiety composed of 20 items for each construct. Internal consistency coefficients for the scales ranged from.86 to.95.	Pregnancy anxiety score above 1.95.
Abuse Assessment Screen (Newberger et al., 1992)	Presence of domestic violence or sexual abuse during pregnancy.	One point for the presence.
Marital Strain Scale (Pearlin and Schooler, 1978)	The Marital Strain Scale of Pearlin and Schooler is used to assess chronic stress with the romantic partner.	Marital strain score less than 2.9.
Health during pregnancy	Presence of chronic diseases during pregnancy: diabetes, hypertension, asthma, current or resolved), current severe vomiting, vaginal spotting or bleeding during the past 4–6 weeks, current anemia/constipation/blood in stool, or current vaginal/cervical/urinary tract infection/diarrhea.	One point for the occurrence of any pathology.
Smoking	Smoking anytime during pregnancy.	One point for the presence.
Gestational age	Gestational age in weeks.	One point if gestational age $\leq$ 37 weeks.
Birth size	Birth size percentile bellow 10th percentile or above 90th percentile	One point for the presence.
Income	Household total gross income.	One point if less than $30,000 a year.

diabetes, hypertension, vaginal spotting or bleeding, smoking during pregnancy, low birth size percentile, gestational age, and socioeconomic characteristics. Further descriptions of all instruments included in the cumulative prenatal adversity environment are presented in **Table 2**. For each met criterion - such as size percentile below 10th percentile or above 90th percentile or smoking during pregnancy - one point was given and all points were summed to obtain the adversity score. For psychometric scales, we considered 85th percentile as a cut-off value for positive screening stated by the instrument.

Cognitive Development Measure

The Bayley Mental Scale of Infant Development

The Bayley Mental Scales (BSID-II) development index (MDI) is a composite of children's language and cognitive abilities. It assesses age-appropriate levels of memory, problem-solving, habituation, incipient number concepts, generalization, classification, vocalizations, and language skills (Bayley, 1993). Psychometric properties of the Bayley scale indicated good to excellent evidence for the validity and reliability of the scale (Silva et al., 2020). Children's development assessment was performed by trained and experienced professionals.

Brain-Derived Neurotrophic Factor Gene Network Score

Genotyping

At first, genetic variation in children was described using genome-wide platforms PsychChip and PsychArray (Illumina) using 200 ng of genomic DNA collected from buccal epithelial cells. SNPs with low call rate (bellow 95%), low *p*-values on Hardy-Weinberg Equilibrium exact test ($p < 1e\text{-}40$), and minor allele frequency smaller than 5% were removed. Quality control (QC) procedure was carried out using PLINK 1.951 (Purcell et al., 2007). Samples of individuals with a call rate less than 90% were also excluded. Imputation was performed using the Sanger Imputation Service and the Haplotype Reference Consortium (HRC) as the reference panel (release 1.1) by McCarthy et al. (2016) resulting in 20,790,893 SNPs with an information score higher than 0.80 and posterior genotype probabilities over 0.90.

Brain-Derived Neurotrophic Factor Expression Polygenic Score

The BDNF ePRS was calculated considering genes co-expressed with the BDNF gene in the PFC following the protocol described at Silveira et al. (2017) and Hari Dass et al. (2019). Three genetic databases were involved in thesis process: the *Genenetwork*[1], *Brainspan*[2], and *GTEx* (Genotype-Tissue Expression The GTEx Consortium, 2013[3]).

First, using the *Genenetwork*, the genes co-expressed with the BDNF gene in the PFC in mice were selected considering an absolute co-expression correlation equal to or higher than 0.5. Based on the Mouse Genome Informatics (MGI) database we identified human homologous genes. Then, we considered the *Brainspan* database to select human homologous transcripts that are enriched during the prenatal period to five years of age in the human PFC. At this point, we selected only transcripts that were differentially expressed in the PFC at $\geq$ 1.5-fold in comparison with adult samples, this list had 51 genes. This list was used to select individual SNPs within start/end $\pm$ 500 bp position of the genes according to NCBI in humans (the National Center for Biotechnology Information, United States National

[1]http://genenetwork.org

[2]http://www.brainspan.org/rnaseq/search/index.html

[3]https://www.gtexportal.org/home/

TABLE 3 | List of genes co-expressed with the BDNF gene and included in the PFC BDNF ePRS.

Gene Symbol	Ensembl	Description	PFC Co-expression Correlation with the BDNF gene in mice
PGD	ENSG00000142657	Phosphogluconate dehydrogenase	−0.81
CBX5	ENSG00000094916	Chromobox 5	0.7
SET	ENSG00000119335	SET nuclear proto-oncogene	0.65
NUP62	ENSG00000213024	Nucleoporin 62	0.63
PFDN2	ENSG00000143256	Prefoldin subunit 2	0.62
SMARCD1	ENSG00000066117	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 1	−0.62
CCT4	ENSG00000115484	Chaperonin containing TCP1 subunit 4	0.61
CCT2	ENSG00000166226	Chaperonin containing TCP1 subunit 2	0.60
GTF2F2	ENSG00000188342	General transcription factor IIF subunit 2	0.59
EIF3E	ENSG00000104408	Eukaryotic translation initiation factor 3 subunit E	0.59
SLC39A6	ENSG00000141424	Solute carrier family 39 member 6	−0.59
SEZ6	ENSG00000063015	Seizure related 6 homolog	−0.58
BTG3	ENSG00000154640	BTG anti-proliferation factor 3	0.57
MYCN	ENSG00000134323	MYCN proto-oncogene, bHLH transcription factor	0.57
ODC1	ENSG00000115758	Ornithine decarboxylase 1	0.57
ANTXR2	ENSG00000163297	ANTXR cell adhesion molecule 2	0.56
BCL10	ENSG00000142867	BCL10 immune signaling adaptor	0.56
CCT3	ENSG00000163468	Chaperonin containing TCP1 subunit 3	0.56
MYL12A	ENSG00000101608	Myosin light chain 12A	0.56
SERBP1	ENSG00000142864	SERPINE1 mRNA binding protein 1	0.56
NR4A2	ENSG00000153234	Nuclear receptor subfamily 4 group A member 2	0.55
IGSF9	ENSG00000085552	Immunoglobulin superfamily member 9	0.55
PTPRS	ENSG00000105426	Protein tyrosine phosphatase receptor type S	−0.54
PHF5A	ENSG00000100410	PHD finger protein 5A	0.54
RSL1D1	ENSG00000171490	Ribosomal L1 domain containing 1	0.54
ARF4	ENSG00000168374	ADP ribosylation factor 4	0.54
NFIL3	ENSG00000165030	Nuclear factor, interleukin 3 regulated	0.54
SEC61A1	ENSG00000058262	SEC61 translocon subunit alpha 1	0.53
PSMA2	ENSG00000106588	Proteasome 20S subunit alpha 2	0.53
DNAJB5	ENSG00000137094	DnaJ heat shock protein family (Hsp40) member B5	0.53
GDI2	ENSG00000057608	GDP dissociation inhibitor 2	0.53
NFIB	ENSG00000147862	Nuclear factor I B	−0.53
LMO3	ENSG00000048540	LIM domain only 3	−0.53
RPL11	ENSG00000142676	Ribosomal protein L11	−0.52
NA	ENSG00000155130	Myristoylated alanine rich protein kinase C substrate	0.52
DACT1	ENSG00000165617	Disheveled binding antagonist of beta catenin 1	0.52
KDM6B	ENSG00000132510	Lysine demethylase 6B	0.52
NPM1	ENSG00000181163	Nucleophosmin 1	0.51
CDK8	ENSG00000132964	Cyclin dependent kinase 8	−0.51
OBSCN	ENSG00000154358	Obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF	−0.51
ING1	ENSG00000153487	Inhibitor of growth family member 1	0.50
RBM7	ENSG00000076053	RNA binding motif protein 7	0.50
MTHFD2	ENSG00000065911	Methylenetetrahydrofolate dehydrogenase (NADP + dependent) 2, methenyltetrahydrofolate cyclohydrolase	0.50
BAZ1A	ENSG00000198604	Bromodomain adjacent to zinc finger domain 1A	0.50
SEC11A	ENSG00000140612	SEC11 homolog A, signal peptidase complex subunit	0.50
MMP24	ENSG00000125966	Matrix metallopeptidase 24	−0.50

Library of Medicine[4]). From the gathered SNPs we retained only common SNPs between MAVAN genodata and GTEx and applied a linkage disequilibrium clumping procedure ($r^2 > 0.2$), to keep independent SNPs with the lowest association p-values in the across 500 kb region. The final list consisted of 46 genes, with a 473 SNPs included in the BDNF ePRS.

[4]https://www.ncbi.nlm.nih.gov/variation/view/

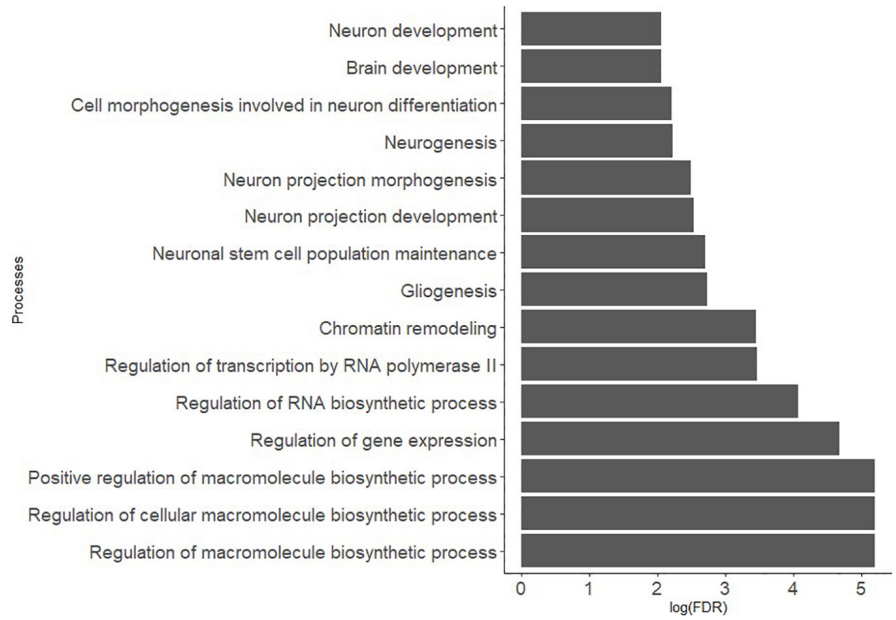

FIGURE 1 | Gene ontology processes related to the genes included in the co-expression PFC BDNF ePRS.

Finally, to calculate the BDNF ePRS score we used the GTEx as a reference to weight the selected 473 SNPs. We multiplied the number of effect alleles for each SNP by the estimated coefficient of the association between each SNP and the genes' expression in the PFC and by the sign of correlation between the gene expression of the particular gene and the BDNF. We summed all weighted SNPs to obtain the PFC BDNF ePRS score. High ePRS scores indicate higher predicted expression levels of genes that composed the BDNF network. The calculation of the BDNF ePRS score was done using PRSoS software tool (Chen et al., 2018).

In order to control for population stratification a principal component analysis was performed using SMARTPCA on the pruned dataset. For the pruned dataset we kept common variants (MAF $>$ 0.05), not in linkage disequilibrium ($r^2 < 0.20$, with a sliding window of 50 kb and an increment of 5 SNPs). Pruning was performed using PLINK 1.9. Based on a screen plot inspection the first three principal components that were the most informative of population structure were retained (Price et al., 2006). For validation of how the gene network scores change across brain regions, developmental stage, and gene of interest see Hari Dass et al. (2019).

Data Analysis

Brain-Derived Neurotrophic Factor Expression Polygenic Score Enrichment Analysis

Biological interpretation of genes that comprised our genetic score was performed using enrichment analysis using MetaCore^{TM} (Clarivate Analytics). The enrichment identifies statistically significant pathway maps and gene ontology processes associated with this list of genes after false discovery rate (FDR) correction, to summarize the most enriched and pertinent biology associated with the set of genes under investigation (Huang et al., 2009). We also performed enrichment analysis to identify genes differentially expressed at different developmental phases, *via* functional mapping of genetic and expression using the FUMA tool (Watanabe et al., 2017).

Cognitive Development Trajectories

With the aim of exploring cognitive development longitudinally, we first run item analysis across different age-based forms of the Bayley Mental Scale using 1-parameter (Rasch) Item Response Theory (IRT). IRT modeling assumes that the probability of a correct response to an item is based only on the ability of the subject and the difficulty of items (Rasch, 1960; de Ayala, 2009), and thereby yields both sample and test independent estimates of item parameters and individual abilities on the latent trait being measured (DeMars, 2010). To scale infant performance for growth interpretations, concurrent vertical scaling was performed taking advantage of an overlapping common item structure (Kolen and Brennan, 2014). This analytical approach provides information on the developmental ordering of items, and the measurement precision associated with the reliability of items and the scores of participants. The calculated separation index shows the scale scores' capacity to discriminate among children with high, medium, and low ability. The higher the value, the better the separation that exists between the items and between persons and the more precise the representation of the measured ability. Reliability values above 0.80 are considered adequate and separation index above 3 suggests that the scores are sensitive enough to discriminate participants (Linacre, 2010). At this stage cognitive development was estimated using Winsteps Version 3.7 (Linacre, 2010); psychometric properties of the Bayley Mental scaled items and estimates of items' fit can be found in the **supplementary materials**.

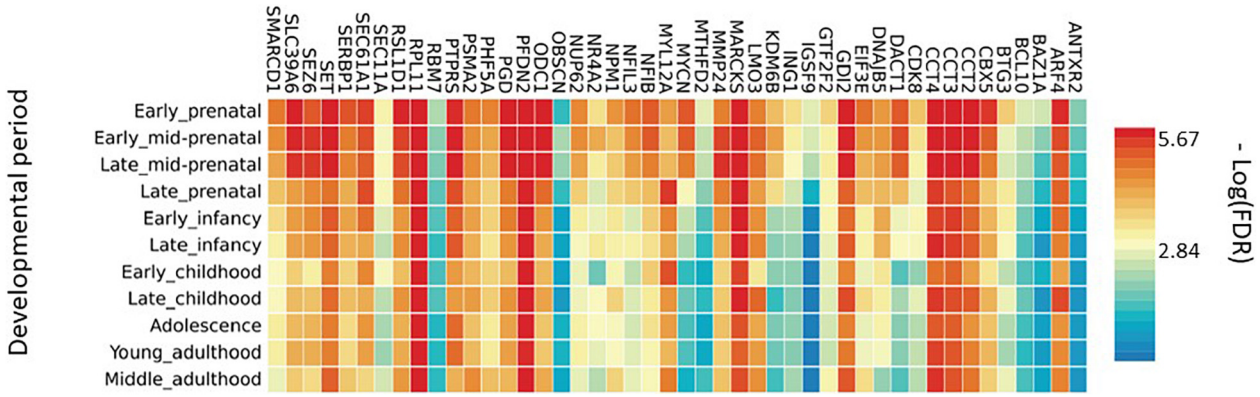

FIGURE 2 | Average gene expression in brain areas at different developmental stages.

Modeling of the cognitive development curve was performed using Linear Mixed Effects Model (LME) (Gałecki and Burzykowski, 2013; Fox and Weisberg, 2019). Models were fitted including the fixed effect of prenatal adversity score, BDNF ePRS, three population stratification principal components, children's sex, and age at data collection time point, and a quadratic term to model the observed non-linear pattern between age and the outcome. We also considered an interaction term between prenatal adversity, BDNF ePRS, and age. For random effects, participants' age and the quadratic age term were specified as nested effects with an autoregressive error correlation structure (Fox and Weisberg, 2019), to model individual cognitive development. The pseudo R^2 for generalized mixed-effect models (Nakagawa and Schielzeth, 2013) was used to compute Cohen's f^2 measure of local effect size, in which values bellow 0.02 indicate small effect sizes, medium values from 0.02 to 0.15, and values greater than 0.15 are considered large effect (Selya et al., 2012). Packages *lme4* (Pinheiro et al., 2018) and *reghelper* (Hughes, 2020) from R software (R Core Team, 2019) were used to perform the statistical analysis.

Structural Magnetic Resonance Imaging

Acquisition and Data Preparation

High-resolution T1-weighted images for the whole brain of 47 children from MAVAN cohort were acquired using a 3T Trio Siemens scanner in Montreal and GE MR750 Discovery 3T Magnetic Resonance Imaging (MRI) scanner in Hamilton. We used the following parameters: Montreal) 1 mm isotropic 3D MPRAGE, sagittal acquisition, 256 × 256 mm grid, TR = 2300 ms, TE = 4 ms, FA = 9degrees; Hamilton) a 3D inversion recovery-prepped, T1-weighted anatomical data set, fSPGR, axial acquisition, TE/TR/flip angle = 3.22/10.308/9, 512 × 512 matrix with 1mm slice thickness and 24cm FOV. Computational Anatomy Toolbox (CAT12) from the Statistical Parametric Mapping software (SPM12) was used to process the T1-weighted images. In the preprocessing step, the images were normalized, registered to Montreal Neurological Institute (MNI) space, and segmented into gray matter (GM) and white matter (WM) by voxel-based morphometry. After a high-dimensional Diffeomorphic Anatomical Registration Through Exponentiated Lie Algebra (DARTEL) normalization, that takes into account the sample specific spatial intensity distribution of structural MRI, a smoothing process was applied using 8mm full width half maximum kernel.

Parallel Independent Component Analysis

A multivariate Parallel Independent Component Analysis (p-ICA) was performed to identify the relationship between two different data modalities in a data-driven manner (Khadka et al., 2016). In this case, the components of BDNF ePRS (genotype * GTEx gene expression slope for each SNP) and whole-brain voxel-based gray matter density were used. This analysis estimates the maximally independent components within each data modality separately while also maximizing the association between modalities using entropy terms based on information theory (Liu and Calhoun, 2014; Pearlson et al., 2015). This process results in each identified independent component resultant from the p-ICA, being an additive subcomponent of the overall multi variant signal that also considers the relationship with a second data modality The prenatal adversity score was used to define the groups for comparison (23 children high environmental score, 24 children low environmental score), aggregated with the most significant principal components from population stratification for adjustment (ethnicity). The Fusion ICA Toolbox[5] within MATLAB® R2019 was used to run the analysis. The number of independent components estimated using minimum description length criteria (Calhoun et al., 2010; Pearlson et al., 2015) was 15 for genetic data and 8 for MRI data. The different resulting ICs are interpretable as brain Talairach coordinates are extracted from the MRI components, indicating brain regions that contribute to the overall independent component. As for the genetic modality, the biological relevance of the functionally related SNPs statistically correlated with brain phenotypes is inferred by subsequent enrichment analysis, using annotation software such as the Metacore, thus providing information for interpretation of the genetic independent components. To identify significant brain

[5]http://mialab.mrn.org/software/fit/

TABLE 4 | Descriptive statistics of scaled cognitive developmental ability estimates of the Bayley Mental items.

Timepoint	N	Mean	SD
6 months	157	−4.82	13.26
12 months	157	43.61	12.41
18 months	157	100.52	17.72
36 months	157	217.25	16.42

regions and SNPs that contributed the most to the ICs, IC weights were converted to z-scores and a threshold at $|z| > 2.5$ was used. Loading coefficients, which describe the presence of the identified component across subjects (Liu et al., 2012), were extracted for each component, modality, and subject. The mean subject-specific loading coefficients of these components between children from high and low prenatal adversity groups were compared using Student's t-test.

TABLE 5 | Results of the linear mixed-effect regression analysis of cognitive developmental trajectories.

	β	SE	t^2	*P*
Intercept	−12.65	1.66	0.18	< 0.001
BDNF ePRS	−3.19	1.35	0.01	0.02
Prenatal Adversity	0.31	0.75	0.03	0.68
BDNF ePRS x Prenatal Adversity	1.73	0.79	0.01	0.03
Age (months)	10.60	0.23	3.20	< 0.001
BDNF ePRS x Age	0.17	0.06	0.01	0.01
Prenatal Adversity x Age	−0.14	0.04	0.02	< 0.001
BDNF ePRS x Prenatal Adversity x Age	−0.12	0.04	0.02	< 0.001
Age quadratic term	−0.07	0.01	0.26	< 0.001
Sex female	1.87	1.67	0.00	0.27
PC1	−34.50	20.64	0.01	0.10
PC2	21.31	15.79	0.01	0.18
PC3	−8.11	16.30	0.00	0.62

RESULTS

Establishment of the Early Life Brain-Derived Neurotrophic Factor Gene Network

The biologically-informed method for selecting SNPs is designed to capture the genes intricately acting in conjunction with the BDNF gene in the prenatal and early life period, hence describing the gene network of interest acting during a specific sensitive period of development. The final list consisted of 46 genes and can be seen in **Table 3**.

Metacore® enrichment analysis of the 46 genes that contributed to the BDNF ePRS shows false discovery rate (FDR) for pathway maps (**Figure 1**). Gene ontology processes were enriched for several epigenetic processes, neuron differentiation, and cellular transport. The main biological processes involved in the BDNF ePRS network included biosynthesis of complex macromolecules, regulation of gene expression and RNA transcription, maintenance of neuronal stem cells, neurogenesis, and neuron development.

To enlighten which genes of the BDNF ePRS are differentially expressed at different developmental phases, we performed a functional mapping of genetic and expression using the FUMA tool (Watanabe et al., 2017). In **Figure 2**, it is possible to observe that some genes comprising our genetic score have specific expression patterns across distinct developmental periods, suggesting that the function of this gene network varies during development. It is important to notice that our score is enriched for early life developmental periods (transcripts differentially expressed in the PFC in comparison with adult samples), so it is expected that these genes would be highly expressed in early life.

To further understand these different expressions across development, we performed enrichment analysis in the subset of genes that co-variated with age (ARF4, CBX5, CCT2, DACT1, KDM6B, MYCN, MYL12A, NFIB, ODC1, PGD, RSL1D1, SERBP1, SET, SEZ6, SLC39A6, SMARCD1). These genes are significantly enriched for the gene ontology process of regulation of gene expression, DNA transcription, biosynthesis of RNA, and macromolecules. The subset list of genes was also related to chromatin remodeling, axogenesis, and nervous system development.

Cognitive Development Trajectories

Repeated measures analysis of variance yielded significant mean differences of cognitive development at each time point, $F(3,468) = 962.8$, $P < 0.001$, with significant Bonferroni adjusted p-values for pairwise comparisons between all age groups. Descriptive data on cross-sectional scaled cognitive development at 6, 12, 18, and 36 months are presented in **Table 4**.

To best characterize the cognitive developmental trajectories from 6 to 36 months we visually inspected the scaled cognitive scores, and data suggested that cognitive skills followed a curvilinear trajectory, which we modeled by adding age quadratic term that reached statistical significance. Our final LME model is presented in **Table 5**. The model considered growth velocity (age linear term), and acceleration (age quadratic term) of cognitive development, and an interaction effect between BDNF ePRS, prenatal adversity, and age. Neither of the covariates (population stratification components and sex) significantly predicted the outcome.

The BDNF ePRS score, prenatal adversity and age presented a significant interaction on cognitive development trajectory ($\beta = -0.12$, $P < 0.001$). Cognitive development differences for children with higher BDNF ePRS scores exposed to low and to high prenatal adversity were larger (**Figure 3**, red line [low adversity] vs purple line [high adversity]) in comparison to children with low BDNF ePRS scores (**Figure 3**, green line [low adversity] vs blue line [high adversity]). The model shows that, on average, infants with high BDNF genetic scores were more susceptible to prenatal adversity exposure (higher BDNF ePRS and

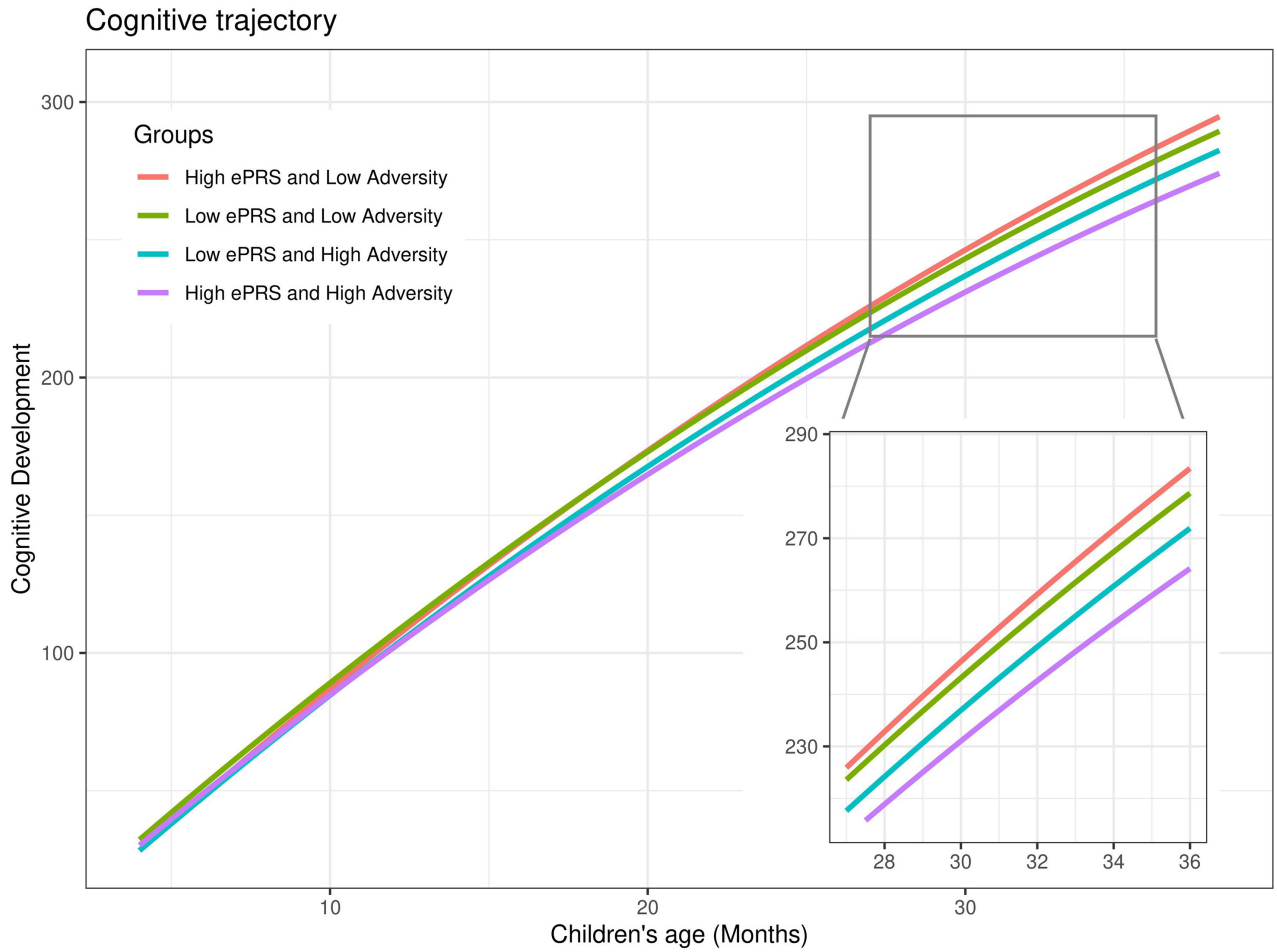

FIGURE 3 | Cognitive developmental growth as function of age, BDNF gene network and cumulative prenatal adversity. Predicted estimates of cognitive development were plotted considering high (+ 1SD) and low (–1SD) BDNF ePRS and high (+ 1SD) and low (–1SD) prenatal adversity for sake of the interaction visualization. Prenatal adversity effects on cognitive development trajectories are larger for children with high BDNF ePRS scores (red vs purple lines) in comparison with children with low BDNF ePRS scores (green and blue lines).

higher prenatal adversity was associated with slower cognitive development trajectory).

Brain-Derived Neurotrophic Factor Expression Polygenic Score and Gray Matter Associations

Magnetic Resonance Imaging scans from 47 participants at age nine, indicated significant pairs of ICs from two data modalities, the whole-brain voxel-based gray matter density and SNPs from the BDNF ePRS. This means that the pICA identified relationships between the two data modalities, allowing the characterization of the associations between specific portions of our gene network and specific brain regions, suggesting an anatomo-functional basis of the phenotypic differences in neurodevelopmental trajectories. These associations indicated that the genetic IC 10 (G10) was significantly correlated to MRI IC (B6), $r = -0.65$, $p = 5.76\text{e-}07$; genetic component 13 (G13) and MRI component (B8), $r = 0.63$, $p = 1.45\text{e-}06$; and genetic component 11 (G11) and MRI component 5 (B5), $r = -0.42$, $p = 2.98\text{e-}03$.

Comparison of the mean loading coefficients of these three ICs between children from high and low prenatal adversity groups indicated statistically significant differences for G10 ($t = 2.36$, $p = 0.02$), G11 ($t = -2.05$, $p = 0.04$), B8 ($t = -3.34$, $p = 0.001$) and B5 ($t = 2.09$, $p = 0.04$), see **Supplementary Table 5**. This means that participants from the two prenatal adversity groups contributed differently to the overall IC data pattern.

The G11-B5 IC pair showed significant differences for both the genetic and brain-phenotype components concerning high and low prenatal adversity and was selected for further analysis. This pair is of primary interest to our study aims and suggests that the relationship between these components is moderated by variations in the quality of the perinatal environment (**Figure 4**). The G10-B6 and G13-B8 pairs were less informative regarding our main objective, as for the G10-B6 pair only the genetic modality had a significant difference between our groups of interest, and for the G13-B8 pair, only the brain-phenotype

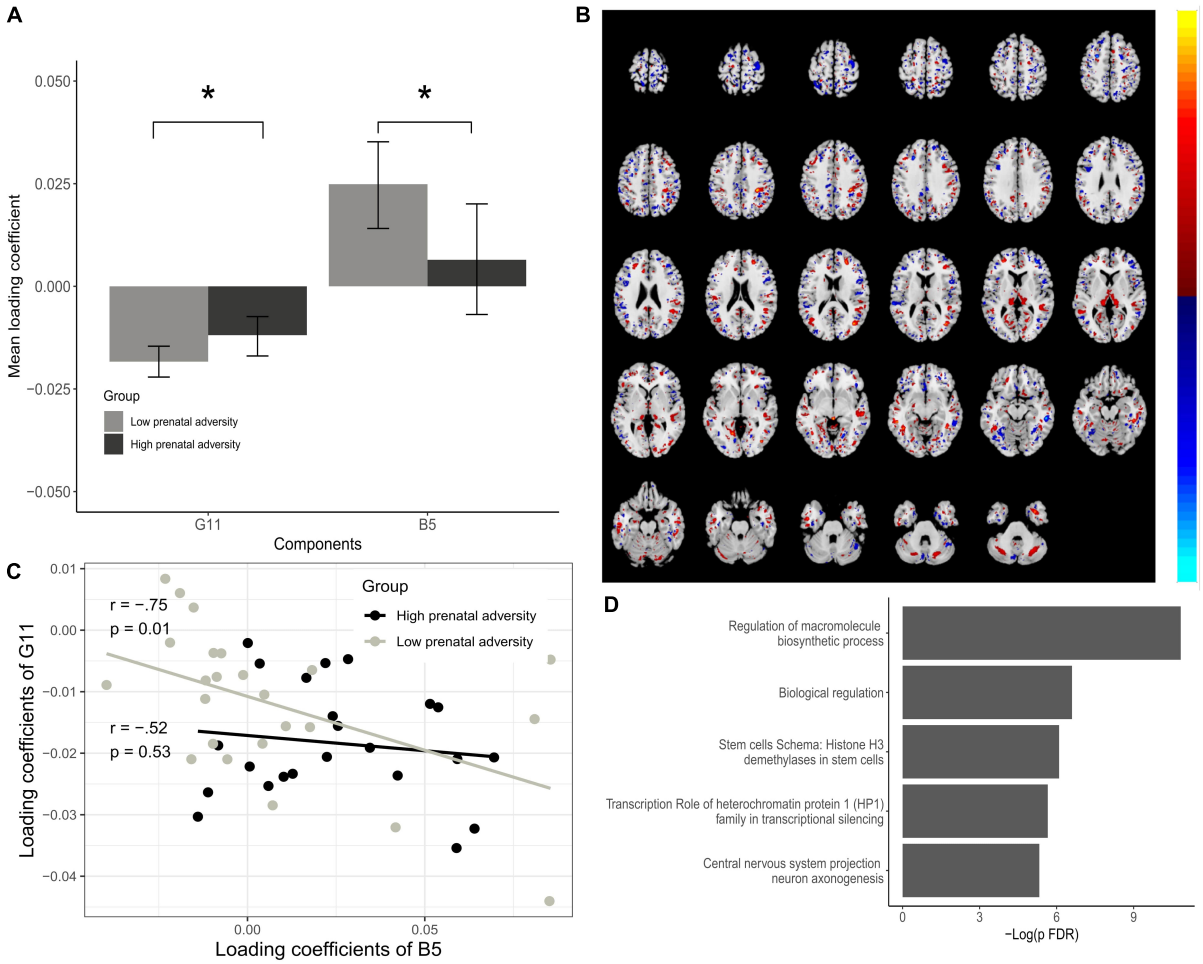

FIGURE 4 | Associations between Gene IC G11 and Brain IC B5. **(A)** Bar plots of estimated loading coefficients for statistically significant brain phenotype (B-5) and genetic component (G11) pair. **(B)** Brain areas comprising the B5 component according to group differences. **(C)** Scatter plot of loading coefficients of B5 and G11 and association between them for low and high prenatal adversity groups. Lines and dots colors represent the High and Low prenatal adversity score groups. **(D)** Enrichment analysis of G11 dominant SNPs. Loading coefficients represent the weight of the overall components for each subject. Significant differences in components are seen contrasting high and low adversity groups, indicating that individuals from high and low adversity groups contributed differently to the overall pattern of the component. *$P < 0.05$.

component was significant. Brain regions and SNPs comprising these components are described in **Supplementary Tables 3**, **4**.

The interpretation of the significant ICs pair was done by extracting brain Talairach coordinates from MRI IC and by enrichment analysis of the genetic IC, proving interpretable information from the observed patterns. All the significant brain regions for the B5 component as well as for the other brain-phenotype components (B6 and B8) are listed in Supplementary Materials. Many regional variations contribute to the B5 component, located mainly in distinct portions of the occipital, frontal, and parietal cortex. The most prominent regions according to Brodmann areas were 19 and 18 (occipital cortex), 7 (parietal cortex), 6 (frontal cortex) that contributed bilaterally and both negatively and positively to the overall pattern. Brodmann area 10 (anterior PFC) was also associated with the genetic component, although with a less prominent contribution to the overall pattern of the component (**Figure 4B**). In the G11 component, from the 473 SNPs used, 16 significantly contributed to the component (Z-Threshold $> \pm 2.5$). Enrichment analysis showed significant pathway maps related to these SNPs such as the transcription role of heterochromatin protein 1 (HP1) family in transcriptional silencing (FDR = 0.001) and start of DNA replication in early S phase on cell cycle (FDR = 0.018). As for process networks, cell cycle S phase and mitosis (FDR = 0.016) were significant, and gene ontology processes were related to central nervous system development, more specifically commissural neuron axon guidance (FDR = 0.001) and regulation of mRNA processing (FDR = 0.025), response to dsRNA (FDR = 0.011) and negative regulation of transcription by RNA polymerase II (FDR = 0.017). This suggests that variations in gray matter density from the identified regions (from B5) and identified SNPs (from G11) vary together across the sample

subjects and that subjects from high and low prenatal adversity groups contribute differently to the overall data pattern of B5 and G11 (**Figures 4A,C**).

DISCUSSION

This study aimed at examining the hypothesis that the effects of prenatal exposure to adversity on cognitive trajectories are moderated by the prefrontal BDNF gene network. Differential response to prenatal exposure was captured using a novel bioinformatics approach that provides a biologically-informed genetic score, based on genes co-expressed with the BDNF in the PFC. Significant associations between SNPs weighted by gene expression and gray matter density at 8 to 10 years of age were located mainly in distinct portions of the occipital, frontal, and parietal cortex.

Longitudinally, high BNDF ePRS levels at the PFC were associated with higher environmental susceptibility in predicting the cognitive growth trajectory. Our data support the differential susceptibility model that postulates that individuals that are more likely to be affected by adverse environmental conditions are also most likely to benefit from positive conditions (Belsky, 2013; Belsky et al., 2018). The highest differences were observed in later development (36 months). This result might be related to the delayed messenger RNA expression in the PFC (Somel et al., 2009) and corroborates the enrichment analysis done with FUMA that shows different patterns of gene expression especially at beginning of infancy (**Figure 4**). Previous research found constancy on BDNF mRNA levels through development in the hippocampus, and variability at the temporal cortex with the highest expression in neonates that decreased with age (Webster et al., 2006).

The neurobiological processes enriched in the BDNF ePRS network were mostly associated with biosynthesis of complex macromolecules, regulation of gene expression and RNA transcription, maintenance of neuronal stem cells, neurogenesis, and neuron development. This is in line with previous animal research that proposes that BDNF system is critically involved in neuron development (Jones et al., 1994), regulation of genes that are associated with synaptic function (Mariga et al., 2015), dendritic growth of cortical neurons (Martin and Finsterwald, 2011), and formation of the neural networks being secreted locally by activity-dependent manner (Hayashi et al., 2007).

Going beyond the analysis of polymorphisms in G × E studies, we integrated information about the gene network of the BDNF with its function at the PFC in a specific developmental period, taking advantage of a cumulative measure of prenatal adversity that reflects a more global level of environmental influence (Silveira et al., 2017; Camerota and Willoughby, 2019). Several mechanisms might be involved in the relation of prenatal adversity and cognitive development, and the observed moderation by the BDNF ePRS. For example, activity-dependent transcription of BDNF is controlled by at least 9 distinct promoters, partially mediated by dynamic changes in DNA methylation (Martinowich et al., 2003; Sakata et al., 2009). Boersma et al. (2013) have found evidence of reduced BDNF expression in response to increased methylation of BDNF at the exon IV in both the amygdala and the hippocampus of prenatally stressed rat's offspring. In humans, prenatal depressive symptoms were also associated with the BDNF promoter IV region along with NR3C1 1F (Braithwaite et al., 2015), suggesting that this epigenetic marker is developmentally sensitive to the quality of the early environmental exposure (Romens et al., 2015).

Another mechanism that may be involved, is the variability of gene expression in different brain regions at different developmental stages. In order to verify if the genes that composed the PFC BDNF ePRS are co-expressed in infancy and if the list of co-expressed genes is maintained in adulthood, we used databases that included gene expression levels in the human cortex. The heatmap obtained (**Figure 2**) demonstrates that a high proportion of these genes have different expression levels from early prenatal to late infancy, but others maintained a similar pattern. The pattern of the specific genes that varied from early prenatal to late infancy points to a special role of the network during development, which may be implicated in the relation of early life adversity effect on cognitive development (Gold et al., 2003; Braithwaite et al., 2015).

To further explore the interaction between cumulative prenatal adversity exposure with our genetic scores, we analyzed the association of the BDNF ePRS weighted SNPs in relation and brain matter density considering groups of high x low adversity. The strongest associations were observed at Brodmann areas 19, 6, 18, and 7 that contributed bilaterally and both negatively and positively to the overall pattern. The areas are related to visual association processes, including visual-motor integration, feature extracting, interpretation of images, attentional and multimodal integrating functions, as well as planning of complex and coordinated movements and convergence between vision and proprioception (Gentile et al., 2011; Bear et al., 2016). Significant associations were also found at the anterior PFC (Brodmann area 10). This area is involved in higher-order cognitive functions, for instance, the processing of internal states, strategic processes in memory recall, reallocation of attention, and more broadly the integration of information from across the supramodal cortex (Ramnani and Owen, 2004; Baird et al., 2013), all of which may be associated with the differential responsiveness to environmental adversity, as reflected in our main interaction finding.

Previous research indicates that prenatal exposure to tobacco correlates with a decrease in cortical thickness in the orbitofrontal cortex, in addition to the reduction in BDNF mRNA and protein levels (Lotfipour et al., 2009; Yochum et al., 2014). Experimental and prospective studies have shown that high pregnancy anxiety is negatively associated with gray matter volume, spine density, and dendritic complexity in the PFC (Murmu et al., 2006; Buss et al., 2010) supporting the idea that prenatal adversity has implications at the neurobiological and structural level.

The integration of genotype and gray matter data using p-ICA analysis suggests that environmental conditions have an especial impact on important neurodevelopmental processes. The G11 component is implicated in neural growth, DNA replication, regulation of mRNA processing, and commissural neuron axon guidance. The aforementioned processes are highly susceptible to environmental influences *via* epigenetic factors including

DNA methylation and histone acetylation changes (Martinowich et al., 2003; Boulle et al., 2012; Braithwaite et al., 2015). The central role that the BDNF plays in neural development, learning, and memory processes suggests that prenatal exposure to unfavorable intrauterine conditions may compromise proper cognitive function *via* dysfunction of the BDNF system (Jones et al., 1994; Gomez-Pinilla and Vaynman, 2005; Boersma et al., 2013). The disruption of the BDNF network could be even more critical to the more susceptible individuals identified in our study since BDNF function have been repeatedly related to learning and memory, as well as the somatosensory and visual cortices (Gold et al., 2003; Lotfipour et al., 2009; Chiang et al., 2011; Xing et al., 2012). Thus, the observed environmental groups' differences in common components of gray matter density and the weighted SNPs appear to play a role in a complex phenotype such as cognitive development.

Although long-lasting effects of prenatal adversity exposure were observed in cognitive behavior and gray matter density, we acknowledge that a continued influence of prenatal maternal adversity during the postnatal period is mediated through the quality of mother-infant interactions and the environmental conditions (Monk et al., 2012). The quality of interactions between caregivers and infants during the postnatal period can have a profound impact on several developmental domains predicting neuronal excitability and synaptic plasticity *via* epigenetic pathways (Meaney, 2010; Nguyen et al., 2015; Ohta et al., 2017). It is important to highlight that prenatal negative exposure is not determinant of a negative outcome, but rather offers possible optimistic opportunities for intervention during postnatal development (Bos et al., 2009; Silva et al., 2020).

With this work we expect to contribute with the understanding of how prenatal adversity and the BDNF gene network shape neural and cognitive development, aiming at ultimately inform and improve both prevention and intervention endeavors, yet a few limitations should be addressed. This study would benefit from replication in a different longitudinal cohort specific to the age bands that comprised our sample since during this period children go through several important sensitive periods of development. The smaller sample size of our neuroimaging study is also an aspect that suggests a need for replication using a falsification approach to avoid Type-I errors. Also, PFC subregions have been reported to develop following temporally different trajectories (Shapiro et al., 2017). Therefore, depending on the time when the stressor is applied, distinct effects could be expected in these different subregions, leading to later effects on specific aspects of cognitive behavior. Distinct PFC regions have also been shown to interact differently with the HPA axis: in rodents, GR gene knockdown in the IL cortex potentiated CORT response to a novel stressor in animals previously subjected to chronic stress, while GR knockdown in the PL cortex did not result in the same effect (McKlveen et al., 2013). In addition, functions such as attentional flexibility, reversal learning, and working memory, for example, are dependent on distinct PFC regions (Birrell and Brown, 2000; Manes et al., 2002; McAlonan and Brown, 2003; Gisquet-Verrier and Delatour, 2006). Although exposure to post-natal stress can have opposing effects on dendrite structure and spine density in distinct PFC regions, such as mPFC and OFC (Liston et al., 2006), specific effects of prenatal stress on neuronal structure according to different PFC regions are less studied. Unfortunately, the database (GTEx) used to calculate our polygenic score did not have expression data available from distinct PFC regions. We believe that future studies approaching this point considering specific PFC regions are warranted.

The broader literature on G x E contains few reports of a network approach specific to a determined brain region, use of psychometric modeling to obtain cognitive development trajectories, and the integration of genotype data with neuroimage. We demonstrated that the PFC BDNF gene network moderates the association between exposure to cumulative prenatal adversity and cognitive growth. Our results provide support for the developmental origins of health and disease (DOHaD), along with prenatal fetal programing of biological mechanisms, and differential susceptibility hypotheses (Silveira et al., 2007; Belsky, 2013; Barth et al., 2019). The focus on genes co-expressed with the BDNF allowed us to identify different patterns of enrichment throughout developmental stages that are in line with the multiple sensitive periods of brain development (Knudsen, 2004). It also made it possible to inspect specific pathways more comprehensively than the candidate-gene approach (Silveira et al., 2017). Thus, we expect to contribute to the understanding of neurobiological processes of cognitive development, and how prenatal adversity exerts a long-term influence on this complex phenotype.

DATA AVAILABILITY STATEMENT

The data are not publicly available due to information that could compromise the privacy of research participants. Requests to access the datasets should be directed to PS.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Douglas Hospital Research Centre, Montreal, and St. Joseph Healthcare, Hamilton (protocol number IUSMD-03-45/IUSMD-06-09). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

EM was involved in data analysis, preparation, and review of the manuscript. BB was involved in parallel independent component analysis and preparation of the manuscript. DB was involved in the review of the cognitive development trajectories measure and manuscript. RL, DA, and CD were involved in enrichment analysis and review of the manuscript. IP was involved in preparation, data analysis interpretation, and review

of the manuscript. RS and GH were involved in MRI acquisition and processing. MM and PS were involved in the design of study, preparation, and review of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.744743/full#supplementary-material

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Lack of Vesicular Zinc Does Not Affect the Behavioral Phenotype of Polyinosinic:Polycytidylic Acid-Induced Maternal Immune Activation Mice

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Zinc is important in neural and synaptic development and neuronal transmission. Within the brain, zinc transporter 3 (ZnT3) is essential for zinc uptake into vesicles. Loss of vesicular zinc has been shown to produce neurodevelopmental disorder (NDD)-like behavior, such as decreased social interaction and increased anxiety- and repetitive-like behavior. Maternal immune activation (MIA) has been identified as an environmental factor for NDDs, such as autism spectrum disorders (ASDs) and schizophrenia (SZ), in offspring, which occurs during pregnancy when the mother's immune system reacts to the exposure to viruses or infectious diseases. In this study, we investigated the interaction effect of a genetic factor [ZnT3 knockout (KO) mice] and an environmental factor (MIA). We induced MIA in pregnant female (dams) mice during mid-gestation, using polyinosinic:polycytidylic acid (polyI:C), which mimics a viral infection. Male and female ZnT3 KO and wild-type (WT) offspring were tested in five behavioral paradigms: Ultrasonic Vocalizations (USVs) at postnatal day 9 (P9), Open Field Test, Marble Burying Test, three-Chamber Social Test, and Pre-pulse Inhibition (PPI) in adulthood (P60–75). Our results indicate that loss of vesicular zinc does not result in enhanced ASD- and SZ-like phenotype compared to WT, nor does it show a more pronounced phenotype in male ZnT3 KO compared to female ZnT3 KO. Finally, MIA offspring demonstrated an ASD- and SZ-like phenotype only in specific behavioral tests: increased calls emitted in USVs and fewer marbles buried. Our results suggest that there is no interaction between the loss of vesicular zinc and MIA induction in the susceptibility to developing an ASD- and SZ-like phenotype.

Keywords: vesicular zinc, maternal immune activation, autism spectrum disorders, schizophrenia, polyI:C, behavior, ZnT3, *SLC30A3*

INTRODUCTION

Brain development is a complex process that is influenced by genetic and environmental factors. The development of the central nervous system, which begins in the early embryonic stages, includes various critical periods of vulnerability, at which point alterations to the fetal environment can result in structural and functional abnormalities in offspring organs, including the brain (Rice and Barone, 2000; Schlotz and Phillips, 2009). This could lead to increased susceptibility to diseases and psychiatric disorders later in life as physiological changes may occur in the process (Rice and Barone, 2000; Schlotz and Phillips, 2009). For instance, during pregnancy, maternal immune activation (MIA) can lead to changes in the fetal environment, contributing to the disruption of brain development in exposed offspring (Meyer, 2014). Zinc deficiency is another risk factor that has been identified as a contributor to neurodevelopmental disorders (NDDs), such as autism spectrum disorders (ASDs) and schizophrenia (SZ) (Yasuda et al., 2011; Grabrucker, 2013; Nuttall, 2017; Ha et al., 2018; Joe et al., 2018). Zinc is an essential component of the structure and functioning of the brain. It plays a role in the development of neurons and synaptic connections, as well as in neural transmission (Sandstead et al., 2000). Within the brain, zinc transporter 3 (ZnT3) is important for the uptake of zinc into vesicles and it is expressed in zinc-enriched areas such as the cerebral cortex, amygdala, and hippocampus [as depicted by Cole et al. (1999) using modifications of Timm's silver-sulfide stain].

Zinc concentration in the brain increases with age and remains constant in adulthood (Takeda, 2001). Dysregulation of zinc homeostasis has the potential to modify the functioning of neurotransmitter receptors and second-messenger systems, potentially causing brain dysfunctions and neurological diseases (Takeda, 2000; Nakashima and Dyck, 2008). Loss of vesicular zinc has been shown to produce NDD-like behavior, including decreased social interaction and preference for social novelty; decreased time spent in the center of the open field test; increased repetitive behavior in the marble burying test; and age-related cognitive decline, as shown by deficits in the novel object preference test, the Morris water task, and T-maze test (Cole et al., 1999; Yoo et al., 2016; McAllister and Dyck, 2017). Dietary zinc deficiency has also been associated with different conditions including neural development disorders, impaired immunity, and degenerative diseases (Yasuda et al., 2011; McAllister and Dyck, 2017).

Autism spectrum disorders and SZ are associated to prenatal risk factors, such as MIA (Canetta and Brown, 2012; Canetta et al., 2016; Scola and Duong, 2017). ASDs are defined as NDDs characterized by key behavioral features: social impairments, difficulties communicating, stereotyped behaviors, and abnormal responses to sensory stimulation (Patterson, 2006; Theoharides and Zhang, 2011; Yoo et al., 2016). According to the Public Health Agency of Canada, in 2018, 1 in 66 children and youth have been diagnosed with ASDs, and it is predominantly diagnosed in males (Public Health Agency of Canada, 2018). In humans, ASDs are usually noticed in the first or second year of life, indicating that prenatal and/or early postnatal development may be critical (Koh et al., 2014).

As previously mentioned, genetic risk is thought to be the leading cause of ASDs. More than 500 genes may be involved (Parellada et al., 2014; Ronemus et al., 2014). One of the well-known genes associated with ASDs is *SHANK3*. Mutations of this gene have been found in people with ASDs, making it a candidate gene to study for these disorders (Fourie et al., 2018). *SHANK3* is involved in synapse formation and synaptic transmission, providing support to organize other proteins at the synapse (Koh et al., 2014; Arons et al., 2016; Tao-Cheng et al., 2016). Interestingly, *SHANK3* activation and function requires zinc, and an endogenous source of free zinc for *SHANK3* modulation is the release of zinc from synaptic vesicles (Koh et al., 2014; Arons et al., 2016; Ha et al., 2018).

Schizophrenia is a psychotic disorder that affects approximately 1% of the population worldwide (Meyer and Feldon, 2012). It is defined by positive symptoms (e.g., hallucinations and/or delusions), negative symptoms (e.g., anhedonia), and cognitive deficits associated with the positive and negative dichotomy (e.g., attention and memory deficits) (Missault et al., 2014). SZ is usually noticed during adolescence or early adulthood (Meyer and Feldon, 2009). The disruptions in behavioral, mental, and emotional functions are believed to be a product of genetic and environmental factors in brain development early in life and peri-adolescence (Meyer and Feldon, 2009).

A gene that has been associated with SZ is *SLC30A3* (ZnT3) expressed in the glutamate synapse in the hippocampus and cerebral cortex; it has been shown to be downregulated in three SZ patient cohorts (Maycox et al., 2009; Perez-Becerril et al., 2014, 2016). The findings suggested that ZnT3-gene is a SZ susceptibility gene that could have sex-dependent effects, impacting females more than males. Another gene that has been associated with SZ is *SHANK3*, the same gene previously mentioned to be associated with ASDs (Arons et al., 2016; Zhou et al., 2016).

Zinc deficiency during pregnancy has been shown to increase the risk of impairments in offspring (Vela et al., 2015). There is clinical evidence that zinc deficiency may impact brain development, as low levels of serum zinc are commonly observed in patients with ASDs and SZ (Grabrucker, 2013; Joe et al., 2018).

Zinc deficiency, from diet in humans and ZnT3 KO in mice, has been shown to trigger a suppressed immune response to pathogens making animals with zinc deficiency more susceptible to infections (Fukada and Kambe, 2018). A study by Yoo et al. (2016) used ZnT3 KO mice to study the role of vesicular zinc in ASDs. Their results suggest that ZnT3 KO leads to a sex-dependent autistic-like phenotype (Yoo et al., 2016).

Maternal immune activation has been modeled in rodents by exposure to a pathogen, which triggers an immune response in the mother. Epidemiological studies looking at the effects of MIA have shown a recurrent link with NDDs in adult offspring (Fortier et al., 2007; Atladóttir et al., 2010; Bitanihirwe et al., 2010; Vuillermot et al., 2012; Zhang et al., 2012). A well-known model of viral-like immune activation is achieved by injection of polyinosinic:polycytidylic acid (polyI:C), which mimics a viral

infection as a synthetic viral-like double stranded RNA (dsRNA) (Fortier et al., 2004). Injections of polyI:C in rodents have been shown to induce sickness behavior, such as reduced appetite, decreased body weight, and increased body temperature (Fortier et al., 2004; Ratnayake et al., 2014).

Polyinosinic:polycytidylic acid has been associated with Toll-like receptor-3 (TLR3), a receptor that is specific to dsRNA viral infection, as it activates cytokines (including interleukin-1β, interleukin-6, and tumor necrosis factor-α), causing an inflammatory response (Fortier et al., 2004; Cunningham et al., 2007). It is not surprising that the use of polyI:C in rodents increases the level of pro-inflammatory factors, such as cytokines, due to its interaction with TLR3 (Fortier et al., 2004; Cunningham et al., 2007; Ratnayake et al., 2014). Cytokines are small proteins that are involved in cell-to-cell communication produced by immune cells in response to inflammation and are involved in the regulation of neurodevelopment processes (Scola and Duong, 2017).

Maternal immune activation models exhibit disturbances in a variety of brain regions, including the hippocampus, prefrontal cortex, insula, cingulate cortex, mid-temporal lobe, and parietal lobe (Spann et al., 2018). These brain regions are involved in deciphering emotions, behavioral reactivity, attention, and learning and memory (Scola and Duong, 2017; Spann et al., 2018). Non-human animal studies have identified behavioral abnormalities in MIA exposed offspring using various behavioral tasks relevant to NDD-like symptoms: anxiety (open-field test, elevated plus maze), communication [ultrasonic vocalizations (USVs), olfactory sensitivity], social interaction (three-chamber social test, social recognition), repetitive behavior (marble burying test, self-grooming), and sensory stimuli sensitivity [pre-pulse inhibition (PPI), latent inhibition] (Meyer et al., 2005; Crawley, 2007; Smith et al., 2007; Malkova et al., 2012; Ratnayake et al., 2014; Kim et al., 2017; Mueller et al., 2021).

To our knowledge, it is unknown whether or not there is an interaction between an environmental factor (MIA) and the genetic factor of ZnT3 deletion. To elucidate the behavioral consequences following MIA exposure, offspring underwent a battery of behavioral assays to assess core symptoms associated with ASD- and SZ-like symptoms, as well as comorbid features, often observed in human patients: USVs, open field, marble burying, three-chamber social test and PPI. Based on previous studies, we hypothesized that offspring of a polyI:C exposed mothers would demonstrate an NDD-like phenotype compared to the control offspring. Additionally, we expected that this phenotype would be more severe in ZnT3 KO mice than in wild-type (WT) mice. More specifically, we expected to observe either an increased or decreased number and length of USVs in pups. In adult mice, it was anticipated that they would have decreased time spent in the center of the open field test—indicating increased anxiety-like behavior—as well as less distance traveled in the open field, increased stereotyped behavior in the marble burying test, decreased social interaction in the three-chamber social test, and low inhibition in the PPI test. Furthermore, we hypothesized that this phenotype would be more pronounced in male ZnT3 KO mice compared to female ZnT3 KO mice. Lastly, we hypothesized that there would be an interaction effect between genotype and treatment in which case ZnT3 KO offspring of polyI:C-injected mothers would show the more severe NDD-like phenotype.

MATERIALS AND METHODS

Animals

All procedures were approved by the Life and Environmental Sciences Animal Care Committee at the University of Calgary and conformed to the guidelines established by the Canadian Council on Animal Care. Male and female C57BL/6 × 129Sv mice heterozygous for the ZnT3-coding gene (*slc30A3*) were paired and housed in standard cages (28 cm × 17.5 cm × 12 cm, bedding, nesting material and a house as an enrichment object). Offspring, both male and female, were housed with the mother until postnatal day 21 (P21), after which they were weaned and housed in standard cages with 2–5 littermates of the same sex. They were kept on a 12 h light/dark cycle with lights on at 7 a.m. at an ambient room temperature of 22°C, and food and water available *ad libitum*.

Experimental Design

We generated seven cohorts of offspring for this experiment. In total, 71 heterozygous (ZnT3+/−) females were paired with 57 heterozygous males, but only 45 were impregnated and gave birth. From the 45 pregnant females, 7 dams did not deliver live pups, and 1 dam had complications during delivery, resulting in a total of 37 viable litters.

To determine the beginning of gestation, the appearance of a seminal plug was considered embryonic day 0.5 (E0.5). At E12.5, dams were injected with polyI:C (20 mg/kg; Sigma-Aldrich, St. Louis, Mo, United States), which was dissolved in 0.9% saline and administered *via* intraperitoneal (i.p.) injection. Control females were given an equivalent volume of 0.9% saline (0.001 mL/g), also *via* i.p. injection. That same day (E12.5) male breeders were removed from the cage and the dams were single housed until birth. The body weights of dams were recorded on a daily basis until E16.5 to confirm that the polyI:C induced an acute response, identified by a decrease in weight, 24-h post-injection (Fortier et al., 2004).

Offspring were genotyped after being weaned in order to determine which mice to use for behavioral testing. In this case, only WT and ZnT3 KO male (WT-Saline: $n = 12$; KO-Saline: $n = 7$; WT-PolyI:C: $n = 13$; KO-PolyI:C: $n = 7$) and female (WT-Saline: $n = 9$; KO-Saline: $n = 9$; WT-PolyI:C: $n = 5$; KO-PolyI:C: $n = 8$) offspring were selected for testing. Ear tissue sample was taken from mice to extract DNA using proteinase K. Polymerase chain reaction (PCR) was used to amplify DNA, using primers oIMR3663 (mutant), oIMR3693 (WT), oIMR3694 (common). To determine the alleles contained in the sample, we ran gel electrophoresis accompanied by positive and negative controls. For a diagram of the experimental timeline, see **Figure 1**.

Behavioral Assessments

For the following tests, experimental mice were habituated to the test room for 30-min prior to each testing day. All tests were

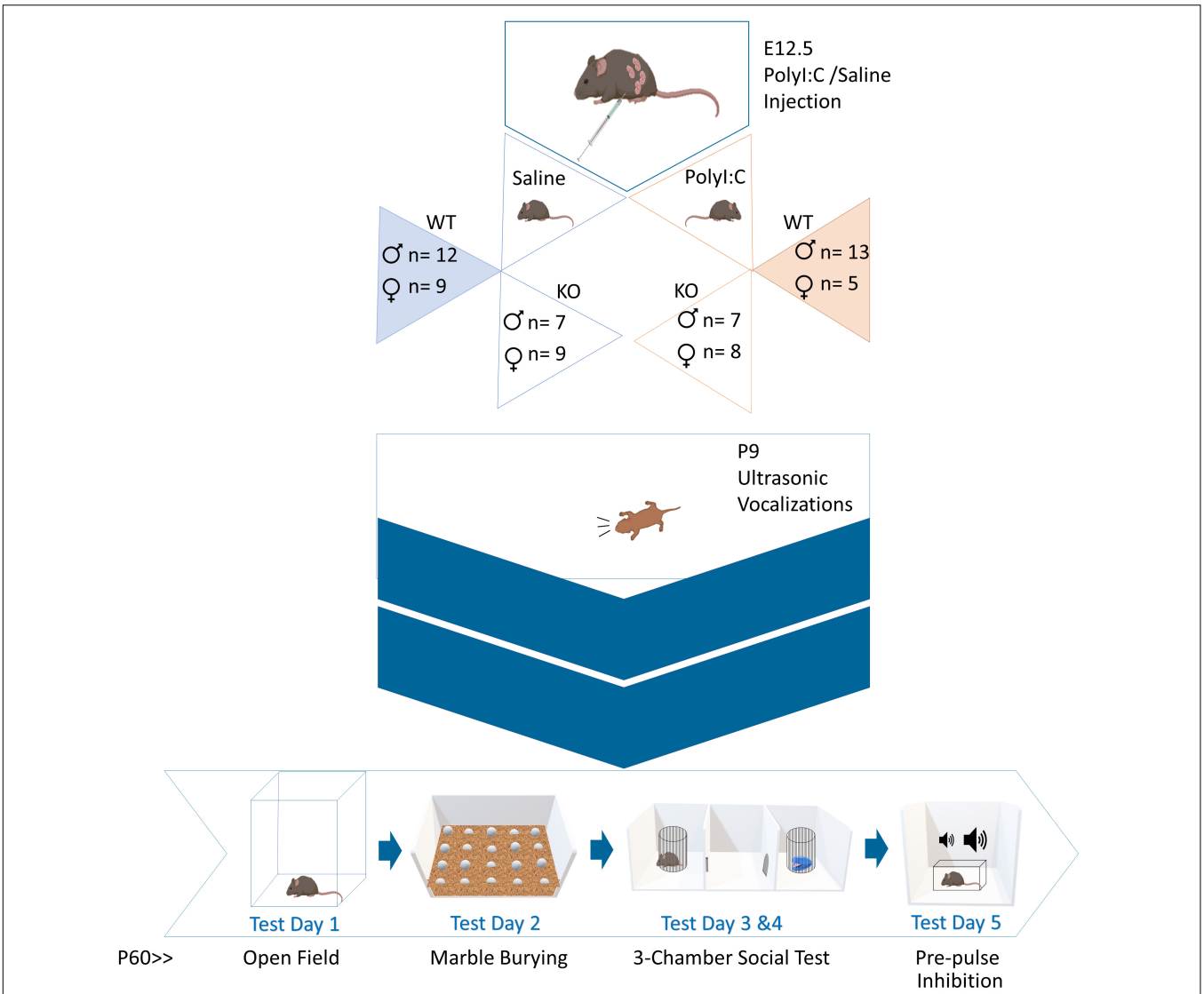

FIGURE 1 | Experimental timeline. Pregnant dams were administered polyinosinic:polycytidylic acid (polyI:C) at embryonic day 12.5. Female and male, wild-type (WT) and zinc transporter 3 knockout (ZnT3 KO) offspring underwent five behavioral tests. The first behavioral test was completed on P9. The other tests were done in adulthood, between P60-P75, across five consecutive days.

conducted during the light phase of the light/dark cycle between 10 a.m. and 5 p.m.

Ultrasonic Vocalizations

At P9, the dams were removed from the nest and placed in a clean holding cage while their pups were left in their home cage. The home cage, containing the pups, was moved to the testing room. Following habituation, pups were removed from the home cage, one at a time, isolated from their siblings and placed in a Plexiglas recording chamber (28 cm × 17.5 cm × 12 cm). The test was performed in a room with the lights turned off. USVs were recorded for 4-min using an UltraSoundGate 116Hm microphone (Avisoft Bioacoustics, Berlin, Germany) and collected using Avisoft Recorder USGH (Kim et al., 2017). After the USV recording, pups were placed in a separate holding cage to avoid re-testing the same pup.

To avoid any stress in the mother and offspring due to foreign olfactory cues, gloves were changed after handling each litter. Additionally, gloved hands were rubbed in the cage bedding of the home cage for a few seconds, prior to handling the pups to ensure the transfer of the litter smell to the gloves. The recording cage was cleaned with 70% ethanol after each litter.

The recorded USV calls were scored using DeepSqueak 2.6.1 software (Coffey et al., 2019). DeepSqueak is a free software that runs through MATLAB (R2018b, Natick, MA, United States). The software was used to determine the number of calls made by the pups and the length of their calls.

Open Field Test

To assess anxiety-like behavior, mice were placed individually near the center of an arena (40 cm × 40 cm × 40 cm) containing approximately 1.3 cm bedding. We have found that the addition of bedding, which is not typical, reduces stress in the mice

and has allowed us to probe for any stereotypies. Movement was recorded for a period of 10-min (Kim et al., 2017). We used an overhead camera (Basler acA1300-60mg GigE, Basler AG, Berlin, Germany) fixed to the ceiling above the arenas, or a Sony Handycam HDR-SR8 camera for the 2nd cohort. The total distance traveled, and the total time spent in the center of the field (20 cm × 20 cm) were measured using EthoVision XT 14 software (Noldus Information Technology Inc., Leesburg, VA, United States).

The arena was cleaned with 70% ethanol after each mouse. Mice were tested by sex group, and the bedding was changed between male and female mice.

Marble Burying Test

To measure repetitive behavior, mice were placed in a cage (28 cm × 17.5 cm × 12 cm) containing 4 cm of bedding. We used 20 identical black-painted glass marbles placed in an equidistant 4 × 5 array on top of the bedding. Mice were left in the cage for a duration of 10-min, after which they were placed back into their home cage (Kim et al., 2017). The number of marbles buried in 10-min was counted and converted to a three-level scale: if the marble was not buried, it was given a score of 0; if it was 50% buried or less it was given a score of 0.5; and if it was buried more than 50% buried a score of 1 was given. The sum of these scores was divided by 20 to obtain the ratio of marbles buried.

The cage with bedding and the marbles were cleaned with 70% ethanol after each mouse. Mice were tested by sex group, and separate cages were used for male and female mice.

Three-Chamber Social Test

Social interaction was assessed using the three-chamber test across 2-days. On the first day, mice were placed individually in the three-chamber arena (40 cm × 60 cm × 22 cm partitioned equally lengthwise) and left to freely explore all chambers for 10-min. The right and left chambers held an empty wire-mesh cylinder (8.5 cm diameter × 9.5 cm) (Kim et al., 2017). Therefore, this exploration allowed for the evaluation of a mouse's preferred cylinder and chamber. The next day, the doors to the right and left chambers were initially blocked, and the mouse was placed in the middle chamber for 5-min (habituation). After this period, the doors were opened to allow free exploration in all chambers for 10-min. The previously determined preferred side had a novel object (a mini stapler) in the mesh cage, and the other side had a novel conspecific, a heterozygous mouse of the same sex and age as the experimental mouse, in the mesh cage. In the cases where a mouse did not have a preference, cylinders were placed randomly.

An overhead camera (Basler acA1300-60mg GigE, Basler AG, Germany) was used to record the total time spent exploring each mesh cage containing the novel conspecific/novel object. EthoVision XT 14 software (Noldus Information Technology Inc., Leesburg, VA, United States) was used to automatically assess the total time spent exploring each mesh cage. The social index was calculated using the time spent exploring mesh cages: $\frac{novel\ conspecific}{novel\ conspecific + novel\bar{o}bject}$.

The arena was cleaned with 70% ethanol after each mouse. Mice were tested by sex group, and the bedding was changed between male and female mice.

Pre-pulse Inhibition

Sensory-motor gating was assessed using PPI of the acoustic startle response. This test involves the presentation of a lower intensity sound prior to the acoustic startle stimulus and the measurement of the startle response. The mice were individually placed in an isolation chamber (5 cm × 10 cm × 5 cm), which includes a force sensor to detect the movement of the mouse. The startle response was measured and analyzed using a SM100SP Startle Monitor system (Hamilton-Kinder LLC, San Diego, CA, United States).

The session began with a 5-min acclimatization period with background white noise (65 dB) and proceeded with four 115 dB, 40 ms sound bursts—which were not included in the analysis as they measure the baseline of the acoustic startle response. The session included six of each of the following trial types for a total of 18 trials: 20 ms pre-pulse stimuli at 70 dB (PPI 5 dB higher than background), 75 dB (PPI 10 dB higher than background), and 85 dB (PPI 20 dB higher than background) (Thackray et al., 2017). Each session lasted approximately 18-min. Percent PPI was calculated using the formula: $\frac{pulse - prepulse}{pulse}$ 100.

The chamber was cleaned with 70% ethanol after each mouse. Mice were tested by sex group.

Statistical Analysis

Statistical analyses were conducted using IBM SPSS Statistics (version 25). A 2-way Analysis of Variance (ANOVA) with [Genotype (WT vs. ZnT3 KO) × Treatment (Saline vs. PolyI:C)] as factors was performed to analyze each behavioral test, unless otherwise specified. A separate ANOVA was run for each sex (male and female). A critical alpha of $p < 0.05$ was used to assess statistical significance, and significant interactions were followed up with (Bonferroni-corrected) *post-hoc* tests. GraphPad Prism 8 software was used to create all the graphs presented in the section "Results."

RESULTS

Weight of Mothers and Litter Size

A repeated-measures ANOVA [Treatment (Saline vs. PolyI:C) × Days (day 1.5–day 16.5)] was performed to assess the weight of the dams during pregnancy. Sphericity was violated according to Mauchly's Test of Sphericity, $\chi^2(119) = 1040.171$, $p < 0.001$, and therefore, a Huynh-Feldt correction was used ($\varepsilon = 0.083$). All pregnant females (dams) increased in body weight as pregnancy progressed [$F_{(1.228,34.373)} = 146.500$, $p < 0.001$], with no difference in weight progression between saline- and polyI:C-injected dams [$F_{(1.228,34.373)} = 0.848$, $p = 0.386$]. We previously hypothesized that dams injected with polyI:C would demonstrate a loss of weight 24-h post-injection (day 13.5), and saline-injected dams would continue gaining weight consistently. *A priori* comparison revealed that the weight significantly decreased in polyI:C-injected dams by an average of 2.956 g [$F_{(1, 28)} = 5.877$, $p = 0.022$] at day 13.5 (**Figure 2**). Furthermore, 72% of the dams from the saline group gave birth to viable litters, but only 49% of polyI:C-injected dams gave birth to viable litters.

However, litter size did not differ between the saline-injected (7.40 ± 2.542) and polyI:C-injected (7.29 ± 2.544) groups [$t(35) = 0.126$, $p = 0.900$].

Ultrasonic Vocalizations

We determined the latency to emit the first call, counted the number of USV calls emitted by pups, and measured the average length of calls. Means ± SD values can be found in **Supplementary Table 1**.

Latency

In males, there was a significant difference between genotypes [main effect of genotype: $F_{(1,\ 34)} = 4.695$, $p = 0.037$; genotype × treatment interaction: $F_{(1,\ 34)} = 0.008$, $p = 0.931$] in which ZnT3 KO male offspring emitted their first call sooner than their WT counterparts. There was no significant difference between treatments [main effect of treatment: $F_{(1,\ 34)} = 0.061$, $p = 0.807$] (**Figure 3A**).

In females, there was no significant difference between genotypes or treatments on the latency to emit the first call [main effect of genotype: $F_{(1,\ 27)} = 0.016$, $p = 0.899$; main effect of treatment: $F_{(1,\ 27)} = 0.717$, $p = 0.404$; genotype × treatment interaction: $F_{(1,\ 27)} = 2.253$, $p = 0.145$] (**Figure 3B**).

Number of Calls

In males, there was a significant difference between the two genotypes in the number of calls emitted [main effect of genotype: $F_{(1,\ 34)} = 5.165$, $p = 0.029$; genotype × treatment interaction: $F_{(1,\ 34)} = 0.498$, $p = 0.485$], with ZnT3 KO male offspring emitting a greater number of calls than WT offspring. There was also a significant difference between the two treatments observed [$F_{(1,\ 34)} = 4.734$, $p = 0.037$], in which MIA-offspring emitted more calls than male offspring of saline-injected mothers (**Figure 3C**).

In females, there was no significant difference between genotypes or treatments for the number of calls emitted [main effect of genotype: $F_{(1,\ 27)} = 0.491$, $p = 0.490$; main effect of treatment: $F_{(1,\ 27)} = 0.029$, $p = 0.865$; genotype × treatment interaction: $F_{(1,\ 27)} = 0.216$, $p = 0.646$] (**Figure 3D**).

Calls Length

In males, there was no significant difference between genotypes or treatments for the number of calls emitted [main effect of genotype: $F_{(1,\ 34)} = 0.569$, $p = 0.456$; main effect of treatment: $F_{(1,\ 34)} = 0.326$, $p = 0.572$; genotype × treatment interaction: $F_{(1,\ 34)} = 0.193$, $p = 0.663$] (**Figure 3E**).

In females, there was no significant difference between genotypes or treatments for the number of calls emitted [main effect of genotype: $F_{(1,\ 27)} = 1.499$, $p = 0.231$; main effect of treatment: $F_{(1,\ 27)} = 0.488$, $p = 0.491$; genotype × treatment interaction: $F_{(1,\ 27)} = 0.325$, $p = 0.573$] (**Figure 3F**).

Open Field

Distance Traveled

In males, there was no significant difference between genotypes or treatments on the distance traveled [main effect of genotype: $F_{(1,\ 34)} = 0.434$, $p = 0.514$; main effect of treatment: $F_{(1,\ 34)} = 3.617$, $p = 0.066$; genotype × treatment interaction: $F_{(1,\ 34)} = 0.306$, $p = 0.584$] (**Figure 4A**).

In females, there was a statistically significant interaction between treatments and genotypes on the distance traveled [$F_{(1,\ 27)} = 4.569$, $p = 0.042$]. Follow-up t-tests did not identify significant effects of polyI:C treatment in either genotype [WT: $t(4.764) = -1.961$, $p = 0.110$; KO: $t(15) = 0.007$, $p = 0.994$]. No significant difference was observed between genotypes [$F_{(1,\ 27)} = 1.530$, $p = 0.227$]. There was a significant main effect of treatment [$F_{(1,\ 27)} = 4.533$, $p = 0.043$] in which MIA-offspring traveled longer distances (**Figure 4B**). Means ± SD can be found in **Supplementary Table 1**.

Time Spent in the Center

In males, there was no significant difference between genotypes or treatments on the time spent in the center of the open field [main effect of genotype: $F_{(1,\ 34)} = 0.963$, $p = 0.333$; main effect of treatment: $F_{(1,\ 34)} = 0.219$, $p = 0.643$; genotype × treatment interaction: $F_{(1,\ 34)} = 1.099$, $p = 0.302$] (**Figure 4C**).

In females, there was no significant difference between genotypes or treatments on the time spent in the center of the open field [main effect of genotype: $F_{(1,\ 27)} = 0.730$, $p = 0.400$; main effect of treatment: $F_{(1,\ 27)} = 0.495$, $p = 0.488$; genotype × treatment interaction: $F_{(1,\ 27)} = 0.341$, $p = 0.564$] (**Figure 4D**).

Marble Burying

In males, there was no significant difference between genotypes or treatments on the proportion of marbles buried [main effect of genotype: $F_{(1,\ 34)} = 1.524$, $p = 0.225$; main effect of treatment: $F_{(1,\ 34)} = 3.007$, $p = 0.092$; genotype × treatment interaction: $F_{(1,\ 34)} = 0.643$, $p = 0.428$] (**Figure 5A**).

In females, there was no significant difference between the two genotypes on the proportion of marbles buried [main effect of genotype: $F_{(1,\ 27)} = 3.148$, $p = 0.087$; genotype × treatment interaction: $F_{(1,\ 27)} = 0.967$, $p = 0.334$]. There was a significant difference observed between the two treatments [$F_{(1,\ 27)} = 21.077$, $p < 0.001$], indicating that female MIA-offspring buried fewer marbles than offspring from saline-injected mothers (**Figure 5B**). Means ± SD can be found in **Supplementary Table 1**.

Three-Chamber Social Test

Social behavior was measured by determining the time offspring spent in the chambers containing the mesh cages. The social index was calculated using the time spent exploring mesh cages: $\frac{novel\ conspecific}{novel\ conspecificnovel\ object}$. Means ± SD can be found in **Supplementary Table 1**.

In males, there was no significant difference between genotypes or treatments on the social index [main effect of genotype: $F_{(1,\ 34)} = 0.140$, $p = 0.710$; main effect of treatment: $F_{(1,\ 34)} = 0.482$, $p = 0.492$; genotype × treatment interaction: $F_{(1,\ 34)} = 0.537$, $p = 0.468$] (**Figure 6A**).

Likewise, in females, there was no significant difference between genotypes or treatments on the social index [main effect of genotype: $F_{(1,\ 27)} = 0.055$, $p = 0.817$; main effect of treatment: $F_{(1,\ 27)} = 0.007$, $p = 0.936$; genotype × treatment interaction: $F_{(1,\ 27)} = 0.307$, $p = 0.584$] (**Figure 6B**).

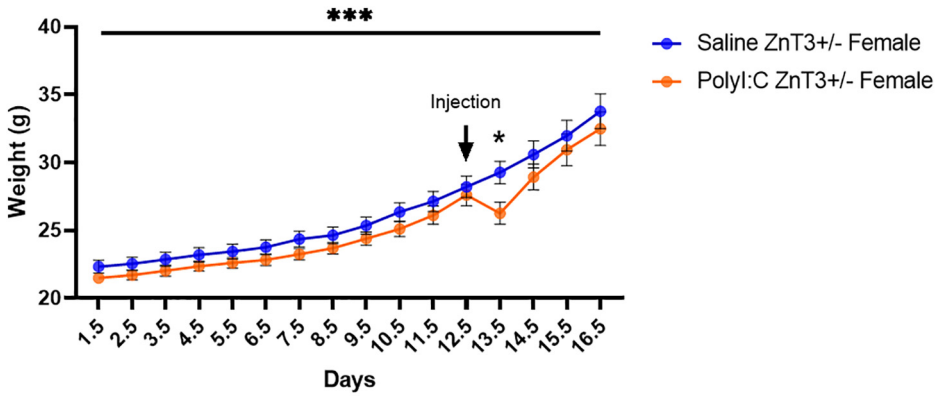

FIGURE 2 | Body weight of ZnT3+/– dams, recorded from gestational day 1–16. A significant body weight decrease was observed 24-h after polyinosinic:polycytidylic acid (polyI:C) administration (Day 13). Error bars depict SEM, $^{*}p < 0.05$, $^{***}p < 0.001$.

Pre-pulse Inhibition

Means ± SD can be found in **Supplementary Table 1**.

In males, the interaction between genotype and treatment at PPI 5 was close to significance, but this interaction was not significant at PPI 10 or 20 [PPI 5: $F_{(1,\ 34)} = 3.487$, $p = 0.070$; PPI 10: $F_{(1,\ 34)} = 0.027$, $p = 0.871$; PPI 20: $F_{(1,\ 34)} = 2.655$, $p = 0.112$]. A follow-up independent *t*-test did not identify a statistically significant difference between WT and ZnT3 KO offspring of saline-injected mothers at PPI 5 [$t(16) = 0.398$, $p = 0.696$] or ZnT3 KO MIA-offspring were less inhibited by the pre-pulse at 70 dB (PPI 5) compared to the WT MIA-offspring [$t(18) = 2.904$, $p = 0.009$, Bonferroni-corrected $\alpha = 0.008$]. There was no significant main effect of treatment [PPI 5: $F_{(1,\ 34)} = 0.058$, $p = 0.811$; PPI 10: $F_{(1,\ 34)} = 0.192$, $p = 0.664$; PPI 20: $F_{(1,\ 34)} = 0.339$, $p = 0.564$] or genotype, with the exception of PPI 5, in which case ZnT3 KO male offspring were less inhibited by the pre-pulse at 70 dB (PPI 5) compared to WT offspring, [PPI 5: $F_{(1,\ 34)} = 5.741$, $p = 0.022$; PPI 10: $F_{(1,\ 34)} = 0.054$, $p = 0.817$; PPI 20: $F_{(1,\ 34)} = 1.227$, $p = 0.276$] (**Figure 7A**).

In females, there was no significant interaction between treatments and genotype on PPI 5, 10, or 20 [PPI 5: $F_{(1,\ 27)} = 0.097$, $p = 0.758$; PPI 10: $F_{(1,\ 27)} = 0.286$, $p = 0.597$; PPI 20: $F_{(1,\ 27)} = 0.190$, $p = 0.666$]. There was no significant main effect of treatment, except at PPI 20 where MIA-offspring had greater inhibition than offspring of saline-injected mothers [PPI 5: $F_{(1,\ 27)} = 2.089$, $p = 0.160$; PPI 10: $F_{(1,\ 27)} = 1.867$, $p = 0.183$; PPI 20: $F_{(1,\ 27)} = 5.891$, $p = 0.022$]. There was no genotype effect at any PPI intensity [PPI 5: $F_{(1,\ 27)} = 0.360$, $p = 0.554$; PPI 10: $F_{(1,\ 27)} = 0.215$, $p = 0.646$; PPI 20: $F_{(1,\ 27)} = 0.750$, $p = 0.394$] (**Figure 7B**).

DISCUSSION

The aim of this study was to determine the effects of prenatal immune challenge, MIA, in mice lacking vesicular zinc. We hypothesized that offspring of polyI:C-exposed dams would demonstrate an ASD- and SZ-like phenotype. Furthermore, we hypothesized that ZnT3 KO mice would show an enhanced ASD- and SZ-like phenotype compared to WT mice. Finally, we hypothesized that this phenotype would be more pronounced in male ZnT3 KO offspring compared to female ZnT3 KO offspring.

The results, summarized in **Table 1**, show that polyI:C-induced MIA at E12.5 leads to alterations in behavior of males that fall within one key diagnostic criterion for ASDs and SZ: communication. Anxiety-like, social, and repetitive-like behaviors remained unchanged. In females, MIA led to behavior alterations opposite to that of what was expected, changing one key diagnostic criteria for ASDs and SZ: repetitive-like behavior. Other measures of behavior such as communication, anxiety-like behavior, and social behavior, remained unchanged. As for sensorimotor gating measured in the PPI test, a deficit was observed in female (at 85 dB) ZnT3 KO offspring of polyI:C-injected mothers and greater inhibition was observed in female offspring of polyI:C-injected mothers (at 85 dB).

Offspring of a Polyinosinic:Polycytidylic Acid Exposed Dams Demonstrated an Autism Spectrum Disorder- and Schizophrenia-Like Phenotype Only in Certain Behavior Tests

Maternal immune activation induction by polyI:C has been shown to produce an ASD- and SZ-like phenotype in offspring (Giovanoli et al., 2014; Grabrucker, 2014; Spann et al., 2018). Previous studies reported changes in USVs (increased or decreased calls), decreased distance traveled and decreased time spent in the center of the open field, increased marble burying, decreased social interaction, and deficits in PPI (Malkova et al., 2012; Schwartzer et al., 2013; Careaga et al., 2017; Kim et al., 2017). It is interesting that we observed different results in some of the tests. These difference could be due to the timing, dosage, and number of polyI:C injections (Kentner et al., 2018). Timing and dosage of polyI:C injection have been studied before, and the most common gestational points are E9, E12.5, and E17.

Changes in offspring behavior tests are observed when polyI:C is administered at any of the three gestational points; however, results vary depending on the tests performed. A study compared

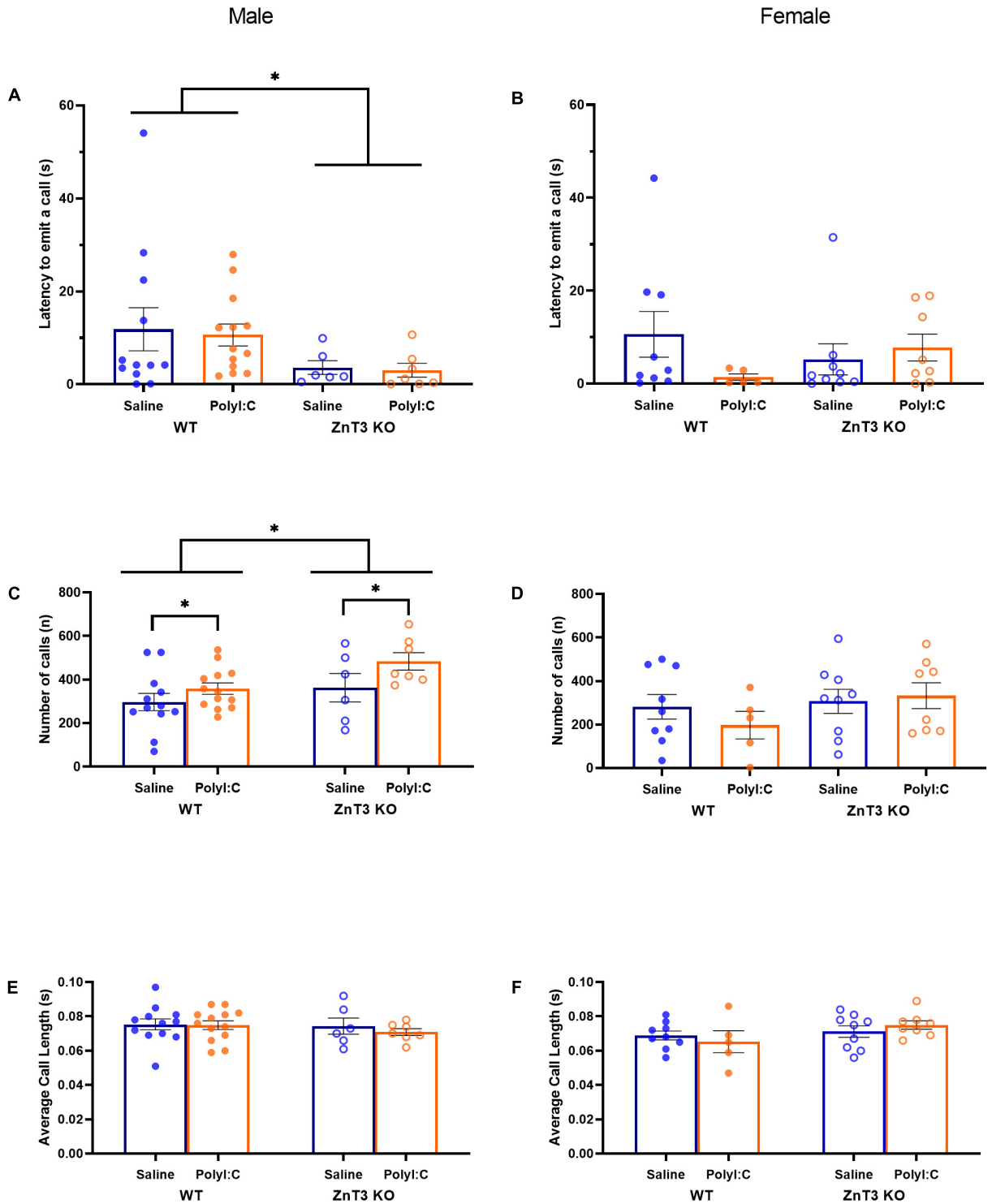

FIGURE 3 | Ultrasonic vocalizations (USVs) by postnatal day 9 pups. Latency to emit a call **(A,B)**, number of calls **(C,D)** and average call length **(E,F)** were measured. **(A)** Male zinc transporter 3 knockout (ZnT3 KO) offspring took less time to emit their first call compared to wild-type (WT) offspring. No effect of polyinosinic:polycytidylic acid (polyI:C) treatment was observed. **(B)** In female offspring, no significant difference was observed. **(C)** Male maternal immune activation (MIA)-offspring emitted more calls than saline-injected offspring and male ZnT3 KO offspring emitted more calls than WT offspring. **(D)** No significant difference was observed in female offspring. **(E)** Male offspring did not demonstrate a statistically significant difference between treatments or genotypes. **(F)** Female offspring did not demonstrate statistically significant differences between treatments or genotypes. Error bars depict SEM, $*p < 0.05$.

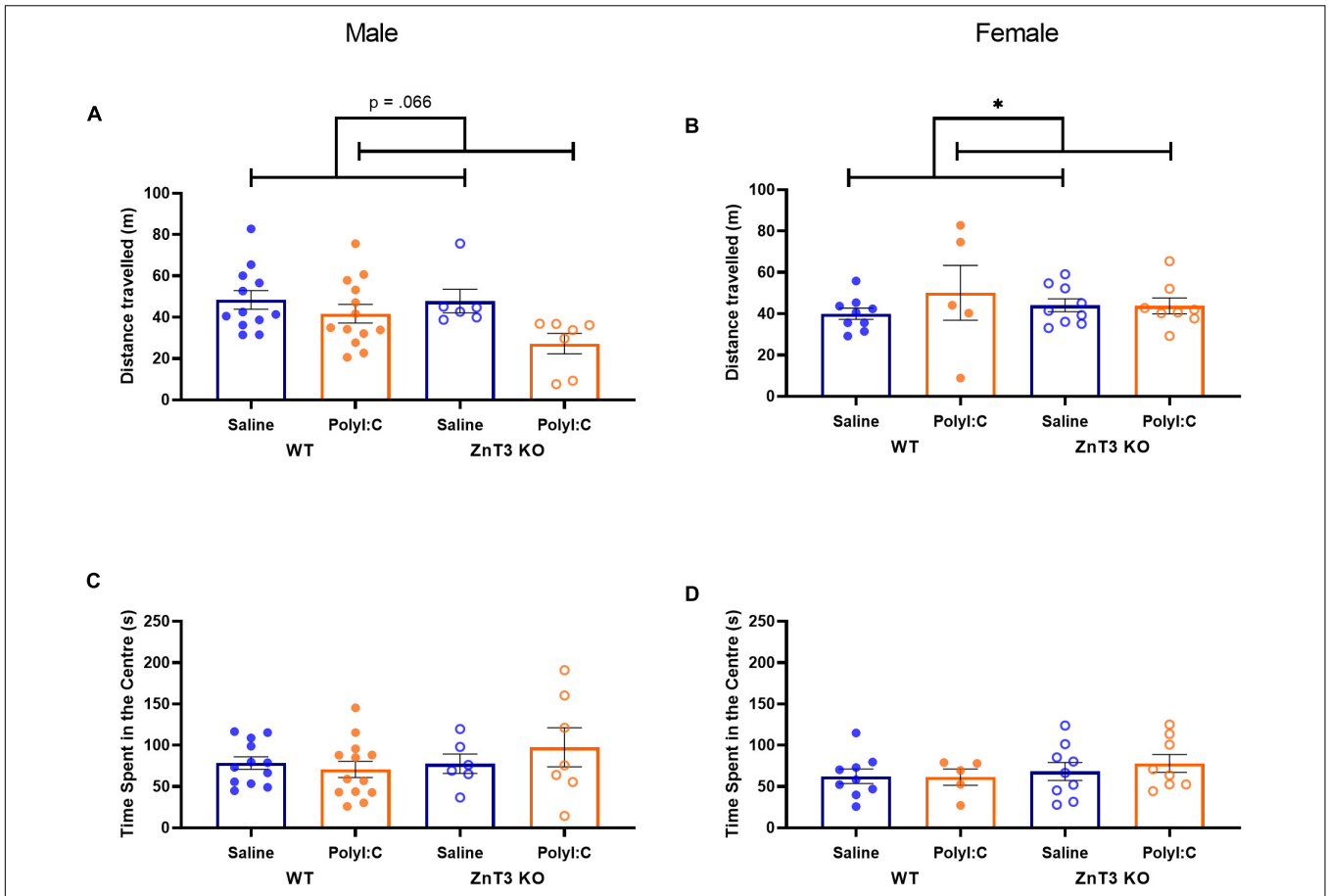

FIGURE 4 | Distance traveled **(A,B)** and time spent in the center **(C,D)** in the open field test. **(A)** Male maternal immune activation (MIA)-offspring tended to travel shorter distances than male offspring of saline-injected mothers, though the difference was not significant. **(B)** Female MIA-offspring traveled greater distances than female offspring from saline-injected mothers. **(C,D)** No significant differences were observed in the time spent in the center between treatment and genotype, in either male or female offspring. Error bars depict SEM, $*p < 0.05$.

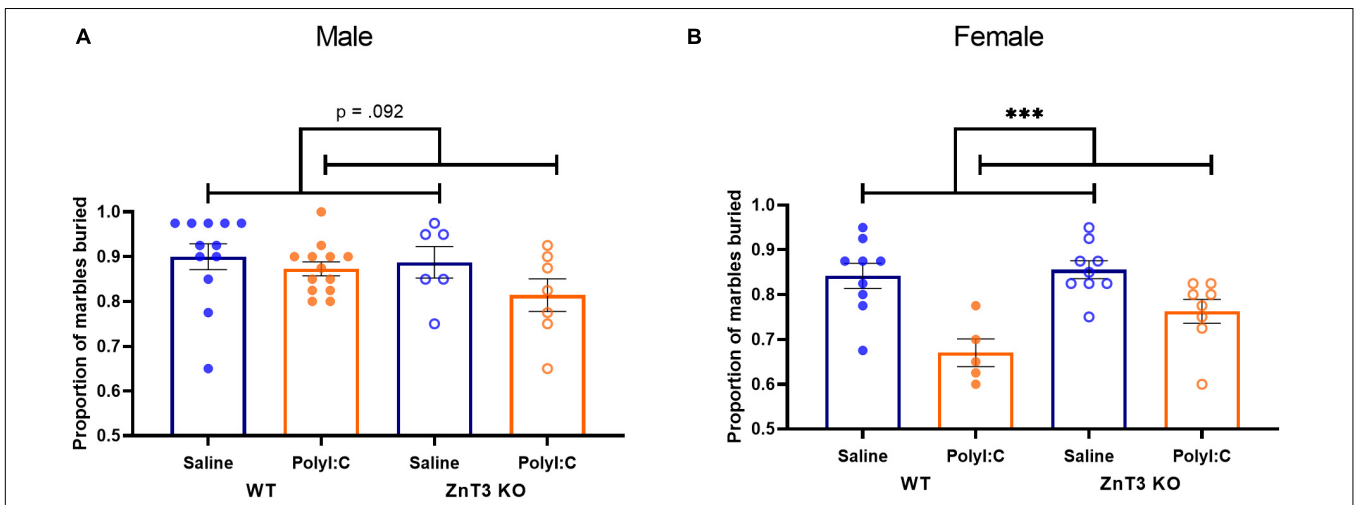

FIGURE 5 | Marble burying test. **(A)** Male maternal immune activation (MIA)-offspring tended to bury fewer marbles compared to saline-offspring, though the difference was not significant. **(B)** Female MIA-offspring buried fewer marbles compared to saline-offspring. Error bars depict SEM, $***p < 0.001$.

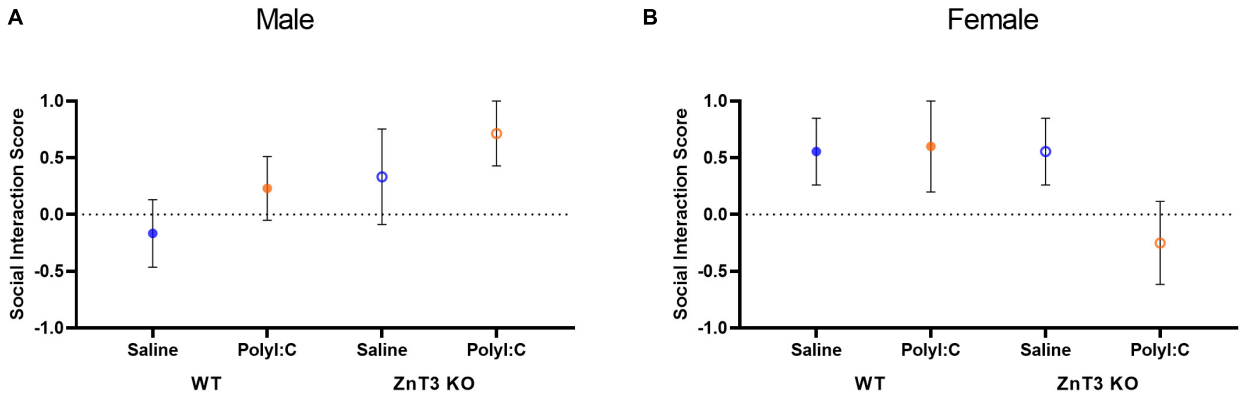

FIGURE 6 | Social interaction assessed in the three-chamber social test. **(A)** In males, no significant difference was observed between treatment or genotype. **(B)** In females, no significant difference was observed between treatment or genotype. Error bars depict SEM.

polyI:C administration at E9 and E17, and found opposite results, meaning that a deficit in one test at E9, was not significant at E17, and vice versa (Meyer et al., 2006b). This suggests that there may be different critical periods for the development of different brain systems/structures that underlie the different behaviors. Another study showed that polyI:C injection resulted in deficits in PPI and increased anxiety-like behavior at E12.5, but at E17, it resulted in decreased social interaction and time spent in the center of the open field (Meyer et al., 2006a,b; Ozawa et al., 2006; Hsiao et al., 2012; Reisinger et al., 2015).

Dosages of polyI:C used in previous studies vary from 2.5 to 20 mg/kg, and some studies have used multiple administrations rather than a single shot. The most commonly used dosage has been 20 mg/kg, and so far, it is the dose at which more differences have been observed in offspring. However, some studies have shown that lower doses produce more severe ASD- and SZ-like features in some, but not all, key symptoms. For example, 5 mg/kg of polyI:C administered three times during pregnancy produced extreme repetitive-like behavior and significantly lower sociability (Malkova et al., 2012). Aside from differences observed in behavioral outcomes in offspring, a dose-dependency has been observed in the maternal immune response, in which different doses activated different levels of pro-inflammatory cytokines (Meyer et al., 2005). This could influence the impact MIA has on the fetal brain and, consequently, the different behavioral outcomes that develop in adult offspring.

Although many studies have looked at MIA using different times, dosages, and number of polyI:C administrations, it is unclear how those differences truly impact the behavioral outcome of the offspring. This is due to a lack of replication in studies of MIA models (Kentner et al., 2018). Most studies use different assays, different timelines, different mouse models, and different purposes. One way that we could improve this is by increasing the transparency of the experimental design. For instance, in our experiment, many of the female breeders that did not deliver live pups were from the polyI:C-injected group. Furthermore, 72% of the dams from the saline group gave birth to viable litters, but only 49% of polyI:C-injected dams gave birth to viable litters. This could mean that the dosage of polyI:C we administered could have been too high for our mice. However, most studies of MIA models do not report losses. Many studies do not explain the reasoning for choosing a specific timeline. Doing so would allow others to better understand the approach and be better able to replicate the study. Providing exact measurements of the equipment or techniques used would also improve behavior testing in general.

Another important variable to consider is that many studies have tested offspring at different ages, which could explain the contrast between our results and what has been reported previously. Many of the published studies have used adolescent mice instead of adult mice. It is important to consider which symptoms we are evaluating to determine at what age we should test mice. For instance, if we are assessing ASD symptoms, we should test in early life stage and in adulthood. However, if we are testing SZ symptoms, which is diagnosed in adolescence and early adulthood in humans, we should test in adolescence and early adulthood to align with the clinical diagnosis we are trying to model (Kentner et al., 2018). The behavior tests should also align with the main purpose being studied. There is evidence that MIA models induce behavioral outcomes that are relevant across different neurological disorders, such as ASDs, SZ, depression, ADHD, bipolar disorder, and cerebral palsy (Canetta and Brown, 2012; Reisinger et al., 2015; Scola and Duong, 2017; Spann et al., 2018).

Zinc Transporter 3 Knockout Mice Do Not Show Enhanced Autism Spectrum Disorder- and Schizophrenia-Like Phenotype

Contrary to what we had hypothesized, ZnT3 KO mice did not show a more severe ASD- and SZ-like phenotype than WT mice. A lack of differences between genotype could, perhaps, indicate that the ZnT3 KO mice compensated for the lack of zinc during development. It is also possible that the behavior tests used in this experiment did not require vesicular zinc signaling. Besides the study by Yoo et al. (2016), most studies that have looked at ZnT3 KO mice have found no difference or only mild differences in

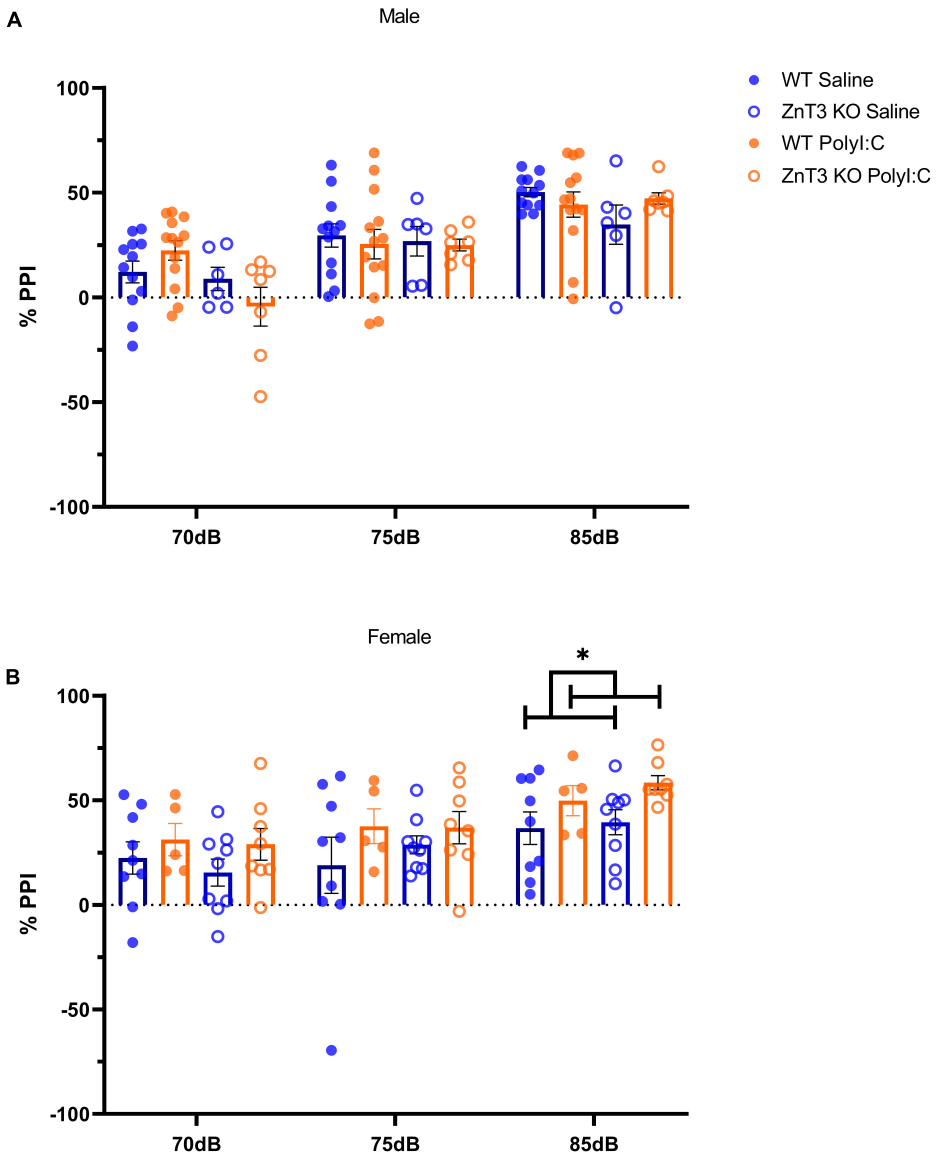

FIGURE 7 | Sensory-motor gating assessment in the pre-pulse inhibition (PPI) test. **(A)** In males, no significant difference was observed between treatment or genotype. **(B)** Female wild-type (WT) maternal immune activation (MIA) offsprinxg showed greater inhibition and zinc transporter 3 knockout (ZnT3 KO) MIA offspring showed impaired inhibition compared to both WT and ZnT3 KO offspring of saline-injected mothers at 85 dB (PPI 20). Error bars depict SEM, $*p < 0.05$.

behavior tests between KO and WT genotypes (Cole et al., 2001; Martel et al., 2011; Thackray et al., 2017).

In the introduction, we mentioned a study that found an ASD-like phenotype in male ZnT3 KO mice (Yoo et al., 2016). Since we did not see the same results they did, it is worth exploring what was different in our experimental approaches. The first possibility that could explain the differing results, is that Yoo et al. (2016) ran behavior tests in mice that were 4–5 weeks of age. However, we ran our behavior tests between 60 and 75 days of age (8–9 weeks of age), except for USVs, which we measured at P9. This would suggest that ZnT3 KO offspring show big differences at a younger age, but these effects are short-lasting and go away a few weeks later. Another possible explanation would be that we tested behaviors in a different order than they did and used different tasks; they started with the three-chamber social test, then marble burying, with the open field or reciprocal social interaction tests last. It also appears that they conducted the three-chamber social test slightly differently: one side chamber had a conspecific and the other side (and middle chamber) were empty, then they placed the same conspecific in one side and a new conspecific on the other side, leaving the middle chamber empty (Yoo et al., 2016). Having two conspecifics, rather than one conspecific and a novel inanimate object, such as the one presented here, could possibly be evaluating different features. The approach used by Yoo et al. (2016) is testing the curiosity-like behavior of a mouse to a novel conspecific compared to how much time it spends

TABLE 1 | Summary of behavioral statistically significant ($p < 0.05$) and marginally significant [#] ($0.05 < p < 0.100$) test results.

	Male	Female
USVs		
Number of calls (n)	Increased calls in MIA-offspring Increased calls in ZnT3 KO offspring	No difference
Length of calls (s)	No difference	No difference
Latency of call (s)	ZnT3 KO offspring were faster to emit their first call	No difference
USV min 1	Increased calls in ZnT3 KO offspring	No difference
USV min 2	Increased calls in MIA-offspring	No difference
USV min 3	Increased calls in MIA-offspring[#]	No difference
USV min 4	Increased calls in MIA-offspring[#]	No difference
Open field		
Distance traveled(m)	Decreased distance traveled in MIA- offspring[#]	Increased distance traveled in MIA- offspring
Total time spent in center (s)	No difference	No difference
Marble burying	Decreased marble burying in MIA- offspring[#]	Decreased marble burying in MIA- offspring
		Decreased marble burying in ZnT3 KO offspring
3-Chamber social test	No difference	No difference
PPI		
PPI5	Deficit in ZnT3 KO MIA-offspring	No difference
PPI10	No difference	No difference
PPI20	No difference	Greater inhibition in WT MIA- offspring
		Deficit in ZnT3 KO MIA-offspring

exploring the other conspecific that is no longer new. In our approach, we were looking at the preference between socializing with a novel conspecific or exploring a novel inanimate object.

Previous studies that used the ZnT3 mouse model to study behavioral outcomes, observed no difference between WT and ZnT3 KO mice for the time spent in the center or the distance traveled in the open field test (Cole et al., 2001; Martel et al., 2010; Thackray et al., 2017). ZnT3 KO mice also did not show social or PPI deficits compared to WT mice (Cole et al., 2001; Martel et al., 2011; Thackray et al., 2017). Based on these observations, our results are similar in that no differences were observed between ZnT3 KO mice and WT mice.

Autism Spectrum Disorder- and Schizophrenia-Like Phenotype Is Not More Pronounced in Male Zinc Transporter 3 Knockout Compared to Female Zinc Transporter 3 Knockout

We observed no significant difference between male and female ZnT3 KO mice. However, male offspring had more statistically significant differences than females did in the behavior assays.

It is possible that MIA, in general, affects females differently than males. Most studies have investigated male offspring and the studies that looked at both sexes either found no sex differences or only males showed significant ASD- and SZ-like phenotype (Grabrucker et al., 2016; Hui et al., 2018; Coiro and Pollak, 2019; Lins et al., 2019; Gogos et al., 2020). It is well known that clinical diagnosis of ASD and SZ is more common in males than females (Public Health Agency of Canada, 2018, 2020). This could potentially be due to differences in the severity of symptoms related to ASD and SZ, where female symptoms are more subtle than in males. Therefore, it is not surprising that our results show differences in key features of ASD and SZ in males, but not females. A reason we may be seeing sex differences in our results, namely, how males show more ASD- and SZ-like features than females, could be due to estrogen. A relationship between estrogen and ZnT3 has been shown, in which case, higher levels of estrogen reduce ZnT3 levels (Lee et al., 2004). Estrogen has also been shown to influence inflammatory response; that is, lower levels of estrogen increase inflammation (Monteiro et al., 2014).

Autism Spectrum Disorder- and Schizophrenia-Like Phenotype Is Not More Pronounced in Zinc Transporter 3 Knockout Offspring of Polyinosinic:Polycytidylic Acid Exposed Dams

We hypothesized that MIA prenatal exposure would affect the brain in the fetus causing important alterations that could interact with loss of ZnT3 later in life. Our results suggest that there is no interaction between MIA exposure and loss of vesicular zinc. Since we observe deficits in some PPI results, it is possible that the interaction between MIA and ZnT3 KO is more relevant to a schizophrenic model. This could be further explored by testing other symptoms of SZ-like behavior such as locomotor activity in response to psychotomimetic drugs (e.g., ketamine), working memory (e.g., T-maze working memory task), and spatial navigation (e.g., Morris water task) (Powell and Miyakawa, 2006).

Previous studies have shown that prenatal injection of polyI:C affect maternal care behavior, and an aspect we did not measure in this study (Ronovsky et al., 2017; Berger et al., 2018). Consequently, changes in maternal care have been shown to greatly impact offspring behavior (Champagne et al., 2008;

Ronovsky et al., 2017; Berger et al., 2018). A possible way to control for maternal care effect would be to use cross-fostering design and to include maternal care measures in the experimental design, such as nest building, licking of pups, and pup retrieval (Richetto et al., 2013; Ronovsky et al., 2017; Berger et al., 2018).

CONCLUSION

For this study, a lack of vesicular zinc did not produce offspring that were more susceptible to developing ASD- and SZ-like features in all the behavior assays performed in this experiment. We observed an ASD- and SZ-like phenotype in male offspring of polyI:C-injected dams, but not in female offspring.

It is important to keep in mind that environmental stressors and genetic mutations do not lead to NDDs. Rather, these events increase the risk of changes in brain morphology and behavior. Not all offspring exposed to MIA will develop an NDD later in life.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the Life and Environmental Sciences Animal Research Committee, University of Calgary.

AUTHOR CONTRIBUTIONS

KS collected and analyzed the data and wrote the original draft of the manuscript. ST, AW, and NN helped to carry out the experiment. CC and SR completed the initial and pilot experiments. KS, ST, and RD helped to supervise the project and design the experiment. RD reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.769322/full#supplementary-material

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