Impact of Neurodevelopmental Genes on the Trajectory of ADHD Severity: A Pilot Study

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IMPACT OF NEURODEVELOPMENTAL GENES ON THE TRAJECTORY OF ADHD SEVERITY: A PILOT STUDY

by

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ABSTRACT

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Attention-deficit/Hyperactivity Disorder (ADHD) is a chronic neurodevelopmental disorder characterized by symptoms of inattention and hyperactivity, and is associated with delays in neural development. To assess the association of genes involved in neurodevelopment with symptoms and trajectory of ADHD, saliva was collected and genotyped from 145 participants from a longitudinal study of preschoolers who were followed annually for 7 years. We examined four single nucleotide polymorphisms (SNPs) in genes associated with neurodevelopment: neuregulin-1 (NRG-1; SNP rs3924999), neurotropin-3 (NT-3; SNP rs6489630), brain-derived neurotrophic factor (BDNF; rs6265), and regulator of G protein signaling 4 (RGS4; rs951439). Hierarchical linear modeling revealed that neuregulin-1 and neurotropin-3 were associated with symptoms of inattention and hyperactivity at age 3-4 and throughout early childhood; however, these genes did not impact the trajectory of the inattentive and hyperactive symptoms over development. Early environmental factors such as maternal diabetes, maternal substance use (tobacco, alcohol, illicit drug) were also analyzed along with each of the genetic factors. Maternal gestational diabetes and NRG-1 risk allele, maternal gestational diabetes and BDNF, alcohol and NRG-1, and illicit drug use and NT-3 were all associated with greater inattentive and hyperactive symptoms. Although the sample size is small for a genetics study, these findings can inform future investigations in understanding the neurodevelopment and heritability of ADHD.
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Attention-Deficit Hyperactivity Disorder (ADHD) is a neurodevelopmental disorder characterized by inattention and/or hyperactivity/impulsivity above and beyond expected developmental and situational levels (American Psychiatric Association [APA], 2013). ADHD is among the most common diagnoses in childhood, affecting approximately 5% of school age children (APA, 2013). Symptoms of ADHD emerge in early childhood and for some individuals continue through adolescence and adulthood. Epidemiological studies indicate a higher proportion of boys diagnosed with ADHD than girls (Lahey, Miller, Gordon, & Riley, 1999). In clinical settings, boys are six to ten times more likely to be referred for clinical assessment (Willcutt & Pennington, 2000) and are three to four times more likely to meet diagnostic criteria for the disorder compared to girls (Cantwell, 1996). Despite clear operational diagnostic criteria for ADHD, children with the disorder are highly heterogeneous with regard to core symptoms, comorbidity, associated features, and life-span trajectories, and this heterogeneity has contributed to hindering research and understanding the underlying pathophysiology of the disorder.

**ADHD is a heterogeneous condition**

The ADHD diagnosis is divided into three presentations, predominantly inattentive, predominantly hyperactive/impulsive, and combined (APA, 2013). To meet diagnostic criteria for the inattentive or hyperactive/impulsive presentation, children need to display at least six out of nine possible symptoms from each category, respectively. To meet criteria for the combined presentation, children need at least six symptoms in both categories. Symptoms also need to be present in multiple settings, most commonly at home and school, and result in functional impairment. Notably, girls who are diagnosed with ADHD are more likely to exhibit inattentive symptoms while boys are more likely to exhibit symptoms of hyperactivity and impulsivity (Levy, Hay, Bennett, & McStephen, 2005; Biederman et al., 2002). The varying presentations and possible variation in the number and types of symptoms results in a clinically heterogeneous presentation of the disorder across individuals.
While ADHD most commonly emerges during the preschool and early school-age years, the trajectory of the disorder and type of presentation over time is also highly variable. Some individuals continue to meet criteria for the diagnosis through adolescence and adulthood, while other individuals show a remittance of the disorder (Campbell, 1995; Connor, 2002). Approximately 30-50% of childhood cases of ADHD remit by adolescence (Halperin, Trampush, Miller, & Newcorn, 2008) while remittance increases to as high as 85% by the age of 25 years (Faraone, Biederman, & Mick, 2006). Some data indicate an overall decline in symptoms of hyperactivity/impulsivity and inattention with age (Biederman, Mick, & Faraone, 2000), while other data suggest that symptoms of hyperactivity/impulsivity decrease over time while symptoms of inattention persist (DuPaul & Stoner, 2004; Wolraich et al., 2005). During preschool, children are more likely to be diagnosed with ADHD-Hyperactive/Impulsive Type, and as they reach school-age they are more likely to be diagnosed with ADHD - Combined Type (Lahey, Pelham, Loney, Lee, & Willcutt, 2005). Furthermore, children who meet criteria for ADHD – Combined Type are more likely to continue to meet criteria the disorder in later development compared to those that meet for Inattentive or Hyperactive/Impulsive Subtype (Hart, Lahey, Loeber, Applegate, & Frick, 1995). The lack of stability of symptom presentation and varying developmental trajectories contribute to the heterogeneity of the disorder.

In addition, children with ADHD are likely to display a wide range of comorbid psychiatric disorders. Among children with ADHD, as many as 70 – 80% meet diagnostic criteria for least one other psychiatric disorder, with many meeting criteria for multiple disorders, including other externalizing/behavioral as well as mood and anxiety disorders (Biederman, Newcorn & Sprich, 1991). Although children with ADHD are likely to exhibit many psychiatric disorders, most comorbidity is with other behavioral disorders. As many as half of children with ADHD meet criteria for Oppositional Defiant Disorder (ODD) or Conduct Disorder (CD) (Swanson et al., 2001), with boys being more likely to exhibit these comorbid externalizing behaviors (Abikoff et al., 2002; Gaub & Carlson, 1997). Yet
internalizing mood and anxiety disorders are not uncommon, reaching a 25% prevalence rate, often in combination with comorbid externalizing disorders (Schatz & Rostain, 2006).

In addition to psychiatric comorbidities, compared to their typically-developing peers, children with ADHD have difficulties with social adjustment and social functioning (DuPaul, McGoey, Eckert & VanBrakle, 2001; Pfiffner, Calzada, & McBurnett, 2000), elevated rates of language (Helland, Posserud, Helland, Heimann, & Lundervold, 2012) and motor impairments (Fliers et al., 2008; Buitelaar, 2008), and are more likely to underachieve academically (Marshall, Hynd, Handwerk & Hall, 1997). A recent meta-analysis of comorbid learning disability and ADHD revealed a mean comorbidity rate of 45.1%, which included writing disorders, reading and/or math disabilities (DuPaul, Gormley, & Laracy, 2013). The extremely high rates of comorbid disorders and functional difficulties further contribute to the heterogeneous profile of symptoms and patterns of functioning among children with ADHD.

*Biological Factors and ADHD*

Research attempting to elucidate the etiology of ADHD has focused largely on genetic and neurodevelopmental factors, although it is clear that environmental influences are also important. Family studies have shown that biologically related family members of hyperactive children were more likely to have hyperactivity than adoptive relatives (Morrison & Stewart, 1973), and rates of ADHD are higher among biological relatives of adopted ADHD children compared to adopted relatives (Sprich, Biederman, Crawford, Mundy & Faraone, 2000). Family studies rely on concordance of disease presentation from parents to offspring, and presence of disease among related siblings. A compilation of twin studies from the United States and Europe indicated that the mean heritability estimate of ADHD was .76, identifying ADHD as among the most heritable psychiatric disorders (Faraone et al., 2005). Although family and twin studies have indicated high heritability, as discussed in detail below, molecular
genetics studies have had very limited success in identifying specific genes that contribute to the expression of the phenotype.

From a neurodevelopmental perspective, ADHD is associated with abnormalities in brain anatomy, connectivity, and neurotransmitter function. Neuroimaging studies have indicated that children with ADHD have a delay in cortical maturation in frontal, striatal, parietal and cerebellar regions, with the most prominent delays in the prefrontal cortices (Castellanos et al., 2002a; Shaw et al., 2007). Individuals with ADHD have deficits in cortical gray matter, and decreases in volumes and cortical thickness of many brain regions, such as the superior frontal and orbital frontal cortex, anterior cingulate and inferior frontal cortex (Amico, Stauber, Koutsouleris & Frodl, 2011). Childhood ADHD and the persistence of ADHD in adulthood is associated with under activation of the lateral inferior fronto-striatal and ventromedial frontostriatal networks involved in attention and motivation (Cubillo, Halari, Smith, Taylor & Rubia, 2012; Konrad et al., 2010).

Based largely on the pharmacological effects of stimulant medications, it has been suggested that catecholaminergic neurotransmitters, namely dopamine (DA) and noradrenalin (NA), are involved in the etiology of ADHD (Noble, 2003). DA and NA activity in the prefrontal cortex appears to modulate higher cognitive executive functions, such as working memory, which is the ability to transiently hold and manipulate information necessary for generating forthcoming action (Durstewitz & Seamans, 2002). As children with ADHD have often been shown to be deficient in these cognitive functions (Willcutt, Doyle, Nigg, Faraone & Pennington, 2005), brain architecture and connectivity in these regions have been posited to play a role in the etiology of ADHD.

ADHD is associated with areas of neuropsychological impairment which have also been posited to be a contributing factor to the disorder’s etiology. Individuals with ADHD show deficiencies in executive functioning (EF) including effortful attention (Douglas, 1983), inhibitory control (Barkley, Grodzinsky, & DuPaul, 1992; Nigg, 2001; Schachar & Logan, 1990; Sergeant & Scholten, 1985), working
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memory (Karatekin & Asarnow, 1998; Mariani & Barkley, 1997; Tannock, 1998), planning or set shifting (Harrier & DeOrnellas, 2005; Nigg, Hinshaw Carte, & Treuting, 1998), and delay aversion (Sonuga-Barke, 2003). Poor inhibitory control, the ability to completely and suddenly stop an activity or planned course of action (Logan & Cowan, 1984), has been linked to difficulties in working memory, self-regulation, internalization of speech and behavioral analysis and synthesis (Barkley, 1997). However, only about half of children with ADHD have EF deficits and any specific type of EF deficit (e.g., working memory, inhibitory control, etc.) is present in even a smaller proportion of diagnosed children (Nigg, Willcutt, Doyle & Sonuga-Barke, 2005). Thus, differences in profiles of neuropsychological functioning are an additional source of heterogeneity in ADHD.

Genetics of ADHD

Despite compelling twin, adoption and family study evidence for the high heritability of ADHD, molecular studies have not been very successful in identifying specific genes that account for this heritability. Although limited and inconsistent, significant results in candidate gene research have implicated several genes involved in neurophysiological processes which are associated with ADHD. The following will be a brief review of findings.

Dopamine System Genes

DA is a neurotransmitter, which has been posited to be associated with the etiology of ADHD, along with an array of other psychiatric and neurological disorders (e.g., alcohol use, nicotine use, substance use, mood disorders, posttraumatic stress disorder, schizophrenia, bipolar disorder, movement disorders, migraines) (Noble, 2003). Interest in DA’s purported role in the pathophysiology of ADHD initially emerged from the mechanism of action of pharmacological treatment of ADHD. First-line treatment for ADHD often involves psychostimulants, such as methylphenidate and amphetamine, which have been found to be efficacious for most individuals for the short-term reduction of ADHD.
symptoms (Castellanos & Tannock, 2002b). Both methylphenidate and amphetamine increase the amount of dopamine in the synapse through somewhat different mechanisms. Methylphenidate blocks the reuptake of DA by the DA transporter, while amphetamine causes the DA transporter to be internalized (Saunders et al., 2000). Blocking the DA transporter and decreasing the number of DA transporters results in the increase of dopamine within the synapse (Kuczenski & Segal, 1997; Segal & Kuczenski, 1999; Volkow et al., 2002). Stimulants differentially increase dopamine release in the prefrontal cortex with minimal effects on subcortical brain regions (Berridge et al., 2006). This increase in synaptic levels of DA, in turn, is believed to enhance executive functioning by way of frontostriatal pathways (Swanson, Kinshourne, Nigg, Lanphear, Stefanatos, et al., 2007). The use of stimulant medication in the psychopharmacological treatment of ADHD and the involvement of the DA pathway in the mechanism of action of stimulants implicate DA as a possible source of ADHD pathophysiology, and has led to considerable interest in studying DA system genes as candidates for explaining the genetic basis of ADHD.

There are multiple points of interest along the DA pathway which can be implicated in the etiology of ADHD, and research has focused on several genes involved in the regulation of DA. One often studied candidate gene is the dopamine 4 receptor gene (DRD4). The DRD4 gene has several alleles, which present in distinct variable number tandem repeat (VNTR) polymorphisms. The most prevalent are the 2, 4 and 7-repeat alleles. Research has primarily focused on the 7-repeat allele of DRD4, which has been hypothesized to increase the risk for ADHD (Faraone, Doyle, Mick & Biederman, 2001). Functionally, the 7-repeat allele results in a receptor, which is less sensitive to DA, reducing the formation of cyclic AMP (Asghari et al., 1995). Results of studies examining the associations between the 7-repeat allele and ADHD have not been consistent, and no single study has indicated a significant association between the 7-repeat allele and ADHD. Nevertheless, when results of multiple studies were pooled, an overall significant, but small effect, was noted. The odds ratio (OR) for case-control studies
was 1.9 ($p < 0.001$), and for family-based studies was 1.4 ($p = 0.02$) (Faraone et al., 2005). Other DRD4 polymorphisms, the 2 and 4-repeat allele, have not demonstrated conclusive results (Kustanovich et al., 2003). Although far from conclusive, results implicate the DRD4 7-repeat allele as a possible contributor to the etiology of ADHD.

Also of potential interest is the dopamine receptor 5 (DRD5) gene. This receptor stimulates adenyl cyclase activity and is expressed in the limbic system of the brain. The limbic system is involved in motivation and emotion, and is regulated by the prefrontal cortex (Kustanovich, et al., 2003). Results from studies examining the association between DRD5 and ADHD have been inconsistent. Among three alleles of interest, 148-bp, 136-bp, and 146-bp alleles, one study revealed an association with the 148-bp allele, but not the 136-bp and 146-bp alleles (Barr et al., 2000). Similarly, two family-based studies of association identified the 148-bp to be associated with ADHD (Payton et al., 2001). Four meta-analyses indicated inconsistent results. Two indicated an association with the 148-bp allele and ADHD with an (OR)=1.3 (Li, Sham, Owen, & He, 2006a; Lowe et al., 2004). The remaining two meta-analytic studies did not find a relationship between the 148-bp allele and ADHD (Mill et al., 2005; Loo et al., 2003). Taken together, one allele of the DRD5, the 148-bp allele, may be a source of possible association with ADHD, but further investigation is needed.

Studies exploring the relations between the dopamine D2 receptor gene (DRD2) and ADHD have focused on the A1 allele. Imaging results indicate that individuals with the DRD2 A1 allele compared to those who do not have the allele, have a reduction in the number of D2 dopamine receptors and altered binding characteristics (Thompson et al., 1997). The reduction in the number of D2 dopamine receptors results in a decrease in various complex cognitive functions (Noble, 2003). One study supported the association between the A1 allele and ADHD, with a positive relationship such that increased frequency of the A1 allele was linked to greater severity of ADHD (Comings et al., 1996). In contrast, another study found that a higher number of ADHD symptoms were associated with a decrease in the frequency of the
A1 allele, while a positive correlation was found between the A2 allele and symptoms of hyperactivity and impulsivity (Rowe, 1999). Although a possible source of association with ADHD, findings involving DRD2 are inconsistent.

The Dopamine D1 Receptor (DRD1) is expressed in multiple brain areas with high expression in the prefrontal cortex and striatum (Jin, Wang & Friedman, 2001). DRD1 is implicated in multiple physiological functions such as regulation of neural growth and development (Arnsten, 2006; Paul, Graybiel, David, & Roberston, 1992) in addition to regulating hyperactivity (Crawford, Drago, Watson, & Levine, 1997) and attention (Arnsten, 2006). Two different case control samples found an association with DRD1 in childhood combined type ADHD, but not in adulthood ADHD (Ribasés et al., 2012). Bobb and colleagues (2005) found only two SNP alleles of the DRD1 gene to be significantly associated with ADHD (rs4532, rs265981).

Other genes in the DA pathway have also been investigated as possible sources of ADHD heritability. The dopamine transporter gene (DAT1) has been identified as a possible candidate due to stimulant medication’s mechanism of action of blocking the dopamine transporter (Spencer et al., 2012). Although one study found a significant association between DAT1 and the severity of ADHD symptomology, most studies have failed to show association of DAT1 and ADHD, (Faraone et al., 2005; Waldman et al., 1998; Franke et al., 2009; Spencer et al., 2012). Dopamine beta hydroxylase (DBH) is the enzyme in the dopamine pathway, which converts dopamine to norepinephrine. The A1 and A2 alleles of the Taq1 polymorphism of DBH were weakly but significantly associated with ADHD (Daly et al., 1999; Roman et al., 2002; Smith et al., 2003). A small number of studies investigating the association of Tyrosine Hydroxylase (TH), the enzyme involved in the synthesis of DA, with ADHD have not observed any associations (Faraone et al., 2005). Additionally, the dopamine D3 receptor (DRD3) has not been associated with ADHD (Brookes et al., 2006; Faraone et al., 2005; Guan et al., 2008). Overall, although theoretically linked to ADHD via the mechanism of action of stimulant medications, studies of genes
involved in the DA pathway have not been successful in consistently identifying specific genes in the DA pathway, which are implicated in the heritability and etiology of ADHD.

Other genes involved in the catecholaminergic system have also been studied in relation to ADHD. Catechol-O-methyltransferase (COMT) is involved in the degradation of catecholamines, such as dopamine, epinephrine, and norepinephrine. Association with ADHD has been inconsistent, with some studies showing significant association with the Val108Met polymorphism while other studies did not show significant associations (Faraone et al., 2005). Monoamine Oxidase A (MAO-A) is an enzyme involved in the degradation of norepinephrine, dopamine, and serotonin. Studies have shown association with ADHD for the 30-bp tandem repeat of the promoter region of the gene in two ethnic populations (Manor et al., 2001). Similar to DA genes, results of studies focusing on the associations between other catecholaminergic system genes and ADHD are weak and inconsistent.

**Noradrenergic (NA) System Genes**

In addition to the suggestion of a role for the DA pathway in the etiology of ADHD, pharmacological and neuroimaging studies also suggest the influence of the NA system. Besides acting on DA, pharmacological interventions for ADHD also influence the noradrenergic system. Not only do the stimulant medications have direct effects on central NA transmission that are analogous to their effects on DA, but atomoxetine, an effective nonstimulant, inhibits the presynaptic NA transporter, causing an increase in the release of monamines (Popper, 2000). Further, guanfacine, another non-stimulant preparation approved for the treatment of ADHD, is a highly selective Alpha-2a agonist. Thus, all medications that are effective for the treatment of ADHD have direct effects on the central NA system. In addition to the pharmacological evidence, the NA system has important influences on the prefrontal cortex (Asghari, et al., 1995; Berridge, et al., 2006), and the ascending NA pathways from the brainstem to the prefrontal cortex play a role in executive functioning (Arnsten, 2006) and have been
demonstrated to be responsible for maintaining alertness and attentional control (Aston-Jones, Rajkowski & Cohen, 1999).

The three most investigated NA genes associated with ADHD are the noradrenaline transporter (SLC6A2/NET1) and the three adrenergic receptors (ADRA1A, ADRA1B, and ADRA2B). An international study of 51 candidate genes associated with ADHD reported a positive association with three NA system genes: SLC6A2/NET1, ADRA1A and ADRA2B (Brookes et al., 2006). A subsequent meta-analysis did not find the same associations (Gizer, Ficks, & Waldman, 2009). Most recently, a 91 SNP investigation by Hawi and colleagues (2013a) did not find a significant association with SLC6A2/NET1, ADRA1A, ADRA1B or ADRA2B genes; however significant associations with multi SNP haplotypes of SLC6A2/NET1 and ADRA1B were found. These significant haplotype findings suggest a contribution of the NA system to the etiology of ADHD, with a call for further investigation.

Studies investigating NA transporter and receptor SNPs in relation to ADHD have revealed inconsistent results. Two studies found an association of a single SLC6A2 SNP (rs3785157), while only one study found an additional SNP association (rs998424) (Xu et al., 2005a; Bobb et al., 2005). However, Brookes and colleagues (2006) failed to replicate the same positive results; in contrast two additional SNPs were found to be significant (rs3785143 and rs11568324). While one study found a significant association between the G-1291C allele at the promoter region of the ADRA2A and ADHD (Comings et al., 2003), other studies failed to find the same associations (Xu et al., 2001; Roman et al., 2003; Park et al., 2004; Stevenson et al., 2005; Wang et al., 2006; Deupree et al., 2006). Similarly, results are inconsistent when examining the relations with ADHD symptom dimensions. However, Schmitz and colleagues (2006) found an association with the G-1291C allele exclusively in individuals with the Inattentive Subtype of ADHD.
Serotonergic System Genes

Although not as extensively investigated in the etiology of ADHD as catecholaminergic neurotransmitter systems, evidence suggests that serotonergic activity is associated with impulsivity, a key component of ADHD symptomatology (Oades, 2007). In addition, evidence suggests an interaction between the serotonin and DA pathways, which regulates levels of both serotonin and DA (Oades, 2007). Therefore, a strong theoretical case can be made for a role for serotonin in the etiology of ADHD, with a particular association with impulsivity.

Research on the role of serotonin in the genetic etiology of ADHD has largely focused on four receptors (HTR1B, HTR2A, HTR1E and HTR3B). Three studies found a significant association of the HTR1B receptor G861C allele with ADHD (Hawi et al., 2002; Quist et al., 2003; Smoller et al., 2006). A later study did not find the same association (Heiser et al., 2007). The T102C and G1438A polymorphisms of the HTR2A receptor have not been found to be significantly associated with ADHD (Zoroğlu et al., 2002). Results of two family-based studies revealed conflicting findings, one study found an association for only the His452Tyr allele and not the T102C allele (Quist et al., 2000) while another study did not find an association with either of these alleles (Hawi et al., 2002). No significant associations were found with the HTR1E and HTR3B receptors (Brookes et al., 2006).

The serotonin transporter has also been investigated as a source of ADHD etiology. The 5-HTTLPR polymorphism located in the promoter region of the serotonin transporter gene (SLC6A4) has been associated with ADHD in three case-control studies (Seeger, Schloss & Schmidt, 2001; Retz, Thome, Blocher, Badder & Rösler, 2002; Zoroğlu et al., 2002), with one study not finding an association (Beitchman et al., 2003). One family-based study found an association between the 5-HTTLPR polymorphism and ADHD (Kent et al., 2002), while another study found an association with only the combined type of ADHD (Manor et al., 2001). A third study did not find any association (Cadoret et al., 2003). Family based studies failed to identify an association with the promoter allele (Banerjee et
al., 2006; Kim et al., 2005; Li et al., 2007b; Xu et al., 2005b). In addition to the investigation of the 5-HTTLPR allele of the promoter region, an intron polymorphism has also been investigated. One study found a negative association with the allele (Banerjee et al., 2006), six studies found no association (Brookes et al., 2006; Heiser et al., 2007; Kim et al., 2005; Wigg et al., 2006; Xu et al., 2005b), and Li and colleagues (2007b) found a positive association.

The last area of interest in the serotonin pathway is tryptophan hydroxylase (TPH). TPH is a rate-limiting enzyme, which synthesizes serotonin from tryptophan. Polymorphisms of TPH have been associated with aggression and impulsivity (Manuck et al., 1999). Although two family-based studies did not find an association between ADHD and the TPH gene, one study found an under transmission of a haplotype consisting of two alleles in ADHD (Li et al., 2006b; Tang et al., 2001). A family-based study found significant positive associations with three alleles; the rs1843809 T-allele, rs1386497 A-allele, and rs1386493 C-allele (Sheehan et al., 2005). Brookes and colleagues (2006) also found positive associations with ADHD, although for rs1843809 A-allele and rs1386493 G-allele. An association with rs1007023 was not found by Sheehan et al. (2005). Overall, findings of associations between serotonin system genes and ADHD suggest potential sources of genetic etiology, but further investigation is warranted to clarify the findings.

**Genes involved in neural plasticity and development**

Due to evidence that ADHD is related to delayed brain growth and development (Shaw et al., 2007), genes involved in neuronal growth and development have been theorized to play a role in the pathophysiology of ADHD.

Synaptosomal-associated protein 25, kDa (SNAP25) is a protein found in the presynaptic plasma membrane that is involved in the regulation of vesicular neurotransmitter release. SNAP25, in combination with other proteins, forms a protein complex that is responsible for vesicle docking and adhesion at the presynaptic membrane which triggers neurotransmitter exocytosis from the presynaptic
membrane (Söllner et al., 1993). SNAP25 is also important for axonal growth and synaptic plasticity (Osen-Sand et al., 1993). A mouse model with a deletion in the gene that encodes SNAP25 leads to hyperactivity in the mice, along with compromised Ca^{++} dependent DA release in the dorsal striatum (Wilson, 2000). Four family-based studies have examined two SNPs (T1069C and T1065G) in relation to ADHD. Barr et al. (2000) found an association with the haplotype of the two alleles, while Kustanovich and colleagues (2003) were not able to find the same association. Two studies with overlapping samples found marginal associations (Mill et al., 2002; Mill et al., 2004). Pooled analysis for the T1065G SNP showed significant evidence for an association with ADHD (Mick & Faraone, 2008). Analysis of two family-based studies, indicated association with four out of 12 of SNPs (rs66039806-C, rs362549-A, rs362987-A, and the rs362998-C alleles) with one cohort, but not another cohort (Feng et al., 2005). Although Brookes and colleagues (2006) did not evaluate the same SNPs as the previous study, association with ADHD was found with rs363020 and rs362567. Analysis of the previously associated SNPs in addition to five additional SNPs (rs6077699, rs363006, rs362549, rs362987, rs362998) found no evidence of association with ADHD for any of the alleles (Kim et al., 2007). Moreover, pooled data indicated modest association with two SNPs (rs3746544-T, rs6077690-T) (Kim et al., 2007). Expression analysis indicated a significant association with one SNP (rs362990-A) and a haplotype of three SNPs (rs6108461, rs362990 and rs362998) (Hawi et al., 2013b). Evidence implicated specific SNPs from SNAP25 as being associated with ADHD.

One area of investigation has been the gene for brain-derived neurotrophic factor (BDNF), a protein that is involved in the survival and differentiation of dopaminergic neurons in the developing brain in addition to the stimulation of neuronal growth and differentiation. The Val66Met functional polymorphism of the BDNF gene impacts intracellular trafficking and is involved in the activity-dependent secretion of BDNF (Egan et al., 2003). Kent and colleagues (2005) found an association between ADHD and paternal transmission of the Val66 allele of BDNF but not maternal transmission of
the same allele. Xu and colleagues (2007) were not able to find the same results; in two family-based
studies they found an over transmission of a different allele, the C720T allele, in only one family cohort.
In contrast to the positive findings of the previous investigations, Brookes and colleagues (2006) failed
to find significant associations in 20 BDNF SNPs. Theoretically BDNF-related genes appear to be a
promising direction of research, although supportive evidence at this point is lacking.

*Glutamate-related genes*

Another area of investigation has involved glutamate receptors. Glutamate and N-methyl-
D-aspartate (NMDA) receptors have been associated with cognition. A SNP of the glutamate receptor,
ionotropic, N-methyl D-aspartate 2A (GRIN2A) gene has been associated with ADHD in a family-based
study (Turic et al., 2004). Haplotype associations with additional SNPs in the same study were weak. A
subsequent family-based study found no association with the GRIN2A SNP or two novel SNPs (Adams et
al., 2004). Similar to the results of BDNF, this area is theoretically promising, but results are inconsistent
and need further investigation.

*Genome-wide Association Studies*

In contrast to hypothesis-driven studies of candidate genes, Genome-Wide Association Studies
(GWAS) investigate common genetic variants in the entire genome of individuals and relate those
variants to a specific trait. Investigations utilizing the GWAS approach focus on associations between
individual SNPs and ADHD. Neale and colleagues (2010) were the first to conduct a case-control GWAS
study on children and adolescents with ADHD. Of the over one million SNPs, which were analyzed in the
study, none met statistical significance. One of the SNPs located in the Protein Kinase, CGMP-
Dependent 1 (PRKG1) gene was the closest to reaching significance. Results from a case-control GWAS
study conducted by Hinney et al. (2011) were similar, no SNP reached genome wide significance. A SNP
located on the Metabotropic Glutamate Receptor 5 (GRM5) gene was the best candidate for association.
A subsequent GWAS again did not find any significant associations, but the Cholinergic Receptor,
Nicotinic, Alpha 7 (CHRNA7) receptor, was found to be the most promising (Stergiakouli et al., 2012). Three family-based GWAS studies found no significant genome results (Neale et al., 2008; Lasky-Su et al., 2008; Mick et al., 2010). Although results did not reach significance, both Lasky-Su et al. and Mick et al. found SNPs associated with SLC9A9, an endosomal membrane protein gene involved in synaptic transmission and plasticity, to be the closest to reaching significance. A large combined case-control and family-based study also failed to find genome wide significance (Neale et al., 2010). Ebejer and colleagues (2013) performed a meta-analysis of GWAS studies using the inattention and hyperactivity symptom domains as continuous measures in the analysis. Although the methodological approach was novel, results were similar to previous the GWAS studies, failing to show significant associations.

More recent GWAS studies have taken different approaches to find an association with ADHD. In a departure from a typical GWAS approach that is not hypothesis driven, Bralten and colleges (2013) showed an association between the three pathways of dopamine/norepinephrine, serotonin, and neuritic out-growth genes and hyperactive/impulsive symptoms and not inattentive symptoms. Findings were not significant for multiple single gene SNPs, suggesting that the combined small effects of multiple gene variants. Due to limited data from genome association studies it has been suggested that biological processes such as, cell division, cell adhesion, neuronal migration, neuro plasticity, and transcription, and the genetic processes that are involved in these processes are likely to be involved in ADHD, in contrast to or in addition to dopaminergic, noradrenergic and serotonergic neurotransmitter (Franke, Neale & Faraone, 2009). Like prior studies, a recent GWAS did not find genome-wide significant SNPs associated with ADHD symptoms, however, there were three genes with high linkage detectability, LMOD2, ASB15, and WASL (Middeldorp et al., 2016). One of the genes, WASL, is involved in cytoskeletal organization during neuronal development, including long spine formation and neurite extension, which seems to be the likely gene associated with ADHD. To increase the number of subjects Faraone (2016) combined data sets from multiple studies using only participants of European decent. Analysis indicated
significance with FOXP2, which has been shown to be associated with cognitive functioning, development of language and speech, and expressed in the basal ganglia and inferior frontal cortex. A GWAS study of glutamate genes indicated an association with hyperactivity/impulsivity severity, and GABA genes were associated with inhibition, but only when corrected for genome-wide association levels. It is important to note that a single gene was not found to be significant but rather gene sets in tandem (Naaijen, et al., 2017).

An analysis of copy number variants (CNV) associated with biological pathways involved in neurodevelopment were significant for respiratory electron transport, organonitrogen compound catabolic process, transmembrane transporter activity, carbohydrate derivative catabolic process, ligand-gated ion channel activity, ligand-gated ion channel activity, transmembrane transport, and ion-gated channel activity (Thapar et al., 2016). This further supports evidence for continued investigation into neurodevelopmental pathways.

Summary of Genetic Findings and Conclusions

There are several possible explanations for the lack of positive and consistent molecular genetic results in ADHD studies. One possibility is that ADHD is caused by single rare gene alleles, which have a major determining effect on the likelihood of ADHD psychopathology. This possibility seems unlikely given the prevalence of ADHD. Conversely, ADHD could be the result of multiple genes of very small effect size with ADHD being the result of multiplicative gene effects or gene x gene interactions. Were either of these the case, conventional approaches to genetic analysis would be unlikely to detect specific genes related to the etiology of the disorder. Alternatively, it seems increasingly likely that ADHD is the result of multiple etiologic pathways (Nigg, Willcutt, Doyle & Sonuga-Barke, 2005; Nigg, Nikolas, Knottnerus, Cavanagh & Friderici, 2010; Sonuga-Barke, 2005). If this is the case and ADHD is etiologically heterogeneous, attempting to gain an understanding of specific genes involved in the
etiology of the disorder poses analytical difficulties and requires novel approaches to gene identification. Summary of genetic findings can be located on Table 1 in the Appendix.

In contrast to conceptualizing ADHD as a unitary disorder based solely on meeting diagnostic criteria for the disorder (yes/no), or even as distinct symptom dimensions of inattention and hyperactivity/impulsivity, an alternative approach is to examine potential endophenotypes, which are likely to be less genetically complex. Endophenotypes, the quantitative traits that impact an individual’s risk to develop a disorder, can be meaningful sources of ADHD genetic etiology (Almasy & Blangero, 1998). Individuals with ADHD demonstrate phenotypic heterogeneity in terms of symptom severity, mixture of symptoms, developmental course, comorbidity and cognitive performance. This phenotypic variability may be the result of different genetic risks and etiological pathways. Moreover, research focusing on endophenotypic refinement, such as cognitive performance, instead of more complex dichotomous diagnoses or behavioral symptom dimensions, may lead to more precise findings in more homogeneous subgroupings of the disorder.

Potential endophenotypes of ADHD based on aspects of executive functioning may mediate the relations between the genetic risk and ADHD symptomatology. The literature suggests that between 30-50% of children with ADHD have an executive function deficit (Loo et al., 2007; Nigg, Willcutt, Doyle, & Sonuga-Barke, 2005). Executive functioning can include such neuropsychological constructs as working memory, attention, planning, organization, inhibitory control, mental flexibility, initiation and self-monitoring (Arnsten, 2006). Evidence of dysfunction in frontostriatal pathways, which play a key role in executive functioning, has been reported in neuroimaging studies, eletrophysiological studies, and neuropsychological assessment (Seidman, Valera & Makris, 2005; Chabot & Serfotentin, 1996; Willcutt et al., 2005).

In addition to its symptomatic and neurocognitive heterogeneity, ADHD also has tremendous heterogeneity regarding the developmental course and outcome. One longitudinal study found that
approximately half of 3- and 4-year-old children who presented with behavioral difficulties no longer had symptoms at age 6 (Campbell, 1987). Others who used more rigorous diagnostic criteria for ADHD found greater persistence from preschool to the school-age years, yet outcomes during childhood were highly variable (Lahey, Pelham, Loney, Lee & Willcutt, 2005). This heterogeneity of course persists throughout the lifespan with anywhere from 22 – 65% of children with ADHD having persistence of symptoms into adolescence and adulthood (Barkley, Murphy, DuPaul & Bush, 2002; Biederman, Petty, Evans, Small & Faraone, 2010; Halperin, Trampush, Miller, Marks & Newcorn, 2008; Klein et al., 2012; Mannuzza et al., 2002).

While most genetic studies of ADHD have focused on the onset (presence vs. absence) of the disorder or its associated symptoms, it has been hypothesized that the onset of ADHD and its subsequent developmental trajectory, in particular as related to persistence and remittance, involve distinct neural processes (Halperin & Schulz, 2006). Consistent with this idea, preliminary data suggest that gene regulation might play a role in the divergent developmental trajectories of ADHD and that genetic risk for ADHD can contribute to the developmental stability of the disorder. For example, Kuntsi et al. (2005) found that children with ADHD who also had the DRD4 7-repeat allele were less likely to persist with ADHD into middle childhood as compared to those without that allele. Further, children with the 7-repeat allele had greater normalization of cortical thinning, higher IQ scores, and better global functioning compared to children with ADHD who did not have the 7-repeat allele (Kuntsi et al., 2005).

A British twin study found inter-individual differences in the developmental course of ADHD. Hyperactive/Impulsive symptom severity was mostly explained by strong additive genetic influences, while inattentive symptoms were associated with nonadditive genetic influence (dominant genetic influence) (Pingault et al., 2015). This suggests that large genetic influences on the trajectory of ADHD
symptoms are mostly specific and independent of those that account for variation in the baseline level of symptoms.

Further, the developmental trajectory of ADHD may also be linked to gene expression. DNA methylation, histone acetylation or methylation can regulate gene expression, which in turn influences transcription activity and ultimately downstream expression of the gene product (Li et al., 2007a). Thus, it is possible for gene expression to have a developmental course, with gene transcription varying across development (Doyle et al., 2005). In addition to transcription factors, posttranscriptional factors such as microRNAs are also involved in gene silencing (He & Hannon, 2004). MicroRNA’s have been shown to regulate various developmental and physiological processes. The differential trajectory of ADHD across development suggests that the role of gene regulation through external factors can play a role in the remittance or persistence of ADHD.

Current voids in the understanding of ADHD and the plethora of inconsistent and largely negative findings call for novel approaches to investigating the role of genes in ADHD. Further, the dearth of studies that have considered the phenotypic heterogeneity or the role of development in this highly heterogeneous neurodevelopmental disorder is striking. Clearly, further longitudinal research focusing on the relations between genes and the developmental course/trajectory of ADHD is warranted. Considering the heterogeneity of this disorder, the assessment of mediators and moderators of the relations between genes and trajectory might yield particularly fruitful results. Focusing on genes regulating monoamines closely associated with ADHD and frontostriatal systems in the brain, such as the catecholamines, as well as brain growth and development might be a good place to start.

**Alternative Genetic Direction: Genes Associated with Neurodevelopment**

As a developmental disorder, it is expected that genes associated with early neurodevelopment would have a substantial role in the etiology and/or the trajectory of ADHD. Due to the absence of
strong molecular genetic associations in the presence of strong heredity a genetic proposal of alternative genes and SNPs is proposed. Given that ADHD is most likely related to many genes of small effect size, it is likely that few or none of these genes alone will demonstrate a significant association with the disorder. Rather, the combination of several genes might be more promising.

**Neuregulin-1**

Neuregulin-1 (NRG1), a pleotropic growth and differentiation factor, contributes to the increase in the phosphorylation of tyrosine kinase (Falls, 2003). Studies on the functionally of NRG1 have observed that the gene is involved in the establishment and fine-tuning of cortical circuitry, and synaptic efficacy. More specifically, in vitro studies demonstrated NRG1 to prompt the transcription of mRNAs encoding neurotransmitter receptors (Ozaki et al., 1997), to modulate glutamatergic, GABAergic, cholinergic, and dopaminergic neurotransmission (Gu et al., 2005; Ting et al., 2011; Woo et al., 2007), and to promote dendritic spine growth (Cahill et al., 2013). Furthermore, dysregulation of expression impaired synaptic plasticity, and overexpression of Ig-NRG1 in mice lead to synaptic dysfunction and behavioral deficits, which included hyperactivity (Yin et al., 2013). Both increased and decreased expression of the NRG1 has been associated with susceptibility to schizophrenia (Bertram et al., 2007; Law et al., 2006, Yang, et al., 2003). NRG1 has been shown to be involved in multiple aspects of efficient and effective neuronal formation, and is associated behavioral outcomes.

**Neurotrophic factors: Neurotrophin-3 and Brain Derived Neurotrophic Factor**

Neurotrophin-3 (NT-3) and Brain Derived Neurotrophic Factor (BDNF) are both essential for neural growth, survival, and differentiation, and are therefore crucial for brain development. NT-3 and BDNF both play a neuroprotective role against glutamate toxicity and glucose deprivation and neuronal calcium homeostasis and protect neurons against metabolic/excitotoxic insults (Cheng & Mattson, 1994). Animal studies demonstrated that NT-3 and BDNF are expressed in the embryonic cortex, with NT-3 expression decreasing as maturation progresses. In contrast BDNF is low in developing regions of
the cortex and increases with maturation of the region (Maisonpierre, et al., 1990). Evidence shows that both BDNF and NT-3 are involved in regulating the dendritic growth of pyramidal neurons in layer 4 of the cerebral cortex, and NT-3 inhibits the dendritic growth stimulated by BDNF. In contrast, in layer 6 of the cerebral cortex, BDNF inhibited dendritic growth stimulated by NT-3 (McAllister, Katz, & Lo, 1997).

Neurotrophins have also been investigated as mechanisms of synaptic plasticity, and long-term functional and structural modification of synaptic connections (Poo, 2001). Primary alterations in the activity of neurotrophins could lead to inappropriate alterations in cortical circuitry and synaptic transmission in the developing brain, which could then lead into the neuronal dysfunction which underlying psychiatric disorders such as schizophrenia (Favalli, Belmonte-de-Abreu, Wong, & Daskalakis, 2012).

NT-3 has been demonstrated to be involved in regulating central nervous system neurogenesis, more precisely regulating aspects of early CNS development. In vitro studies corroborate the effects of NT-3 on cell proliferation and differentiation, which are key regulators of neurogenesis in the CNS, which can result in neuronal differentiation (Ghosh & Greenburg, 1995). A haplotype of NT-3 (rs4074967-rs6332-rs6489630-rs7956189) was shown to be more associated with childhood ADHD compared to adulthood ADHD and control (Ribasés, et al., 2008). NT-3 SNP rs6489630 has shown to be a protective factor for Alzheimer’s Disease (Liu et al., 2016).

BDNF is involved in neurodevelopment, synapse regulation, and synaptic plasticity, it has been proposed as a candidate to explain part of the pathogenesis of neurodevelopmental disease. BDNF has been shown to modulate synaptic transmission (Levine, Dreyfus, Black, & Plummer, 1995). Adult BDNF knockout mice have impaired hippocampal-dependent learning and long-term potentiation. In early development, BDNF knockout mice exhibited hyperactivity and hippocampal learning deficits (Monteggia, et al., 2004). BDNF SNP rs6265 has been shown to be involved in the pathophysiology of
mood disorders and schizophrenia (Fernandes et al., 2014), and associated with parental reports of ADHD symptoms (Gadow, et al., 2009).

*Regulator of G protein signaling 4*

Although less investigated than the prior genes, *Regulator of G protein signaling 4* (*RGS4*), has been implicated in dopamine expression and psychiatric illness. Specially, *RGS4* SNP (rs951439) is one of several SNPS being implicated in the clinical symptoms and antipsychotic treatment response of schizophrenia, identifying *RGS4* as a gene that contributes to the susceptibility of the disorder (Ding, Styblo, Drobna, & Hegde, 2016). It is posited that one of the ways that *RGS4* contributes to the etiology is by influencing dopamine signaling, with *RGS4* expression associated with cortical dopamine signaling with *COMT* Val158Met genotype being associated with prefrontal along with hippocampal *RGS4* expression (Lipska, 2006). *RGS4* expression in the dorsolateral prefrontal cortex can also inhibit dopamine D2 and D3 receptor signaling, while a decrease in *RGS4* can increase D2 and D3 receptor signaling (Min, 2012). Although there is not currently a direct link to suggest that *RGS4* has a role in the etiology of ADHD, there is a theoretical associated between similar neurotransmitter pathways.

*Environmental Factors*

In addition to genetic factors, several prenatal environmental factors have been shown to influence neurodevelopment with some factors being associated with the development of ADHD.

*Nicotine*

Several studies have implicated prenatal nicotine exposure in the etiology of ADHD. A meta-analysis linked maternal and environmental nicotine exposure to signs of stress and nicotine withdrawal following birth, presence of ADHD, and other externalizing outcomes, such as rule breaking and aggressive behavior (Manzano et al., 2016). A retrospective study determined that children with ADHD
were 2.1 times as likely to be exposed to nicotine during pregnancy compared to non-ADHD controls (Mick, Biederman, Faraone, Sayer, & Kleinman, 2002). Additionally, a large Finish study revealed a significant increase in ADHD in individuals exposed to maternal tobacco smoking (Joelsson et al., 2016). Similarly, a large South Korean study of children born to mothers that smoked during pregnancy were 2.64 times more likely to be diagnosed with ADHD (Han et al., 2015). An investigation into the neurocognitive and behavioral outcomes of children exposed to maternal smoking during pregnancy showed lower verbal IQ, sluggish cognitive tempo on a measure of visual sustained attention (CPT), more externalizing behaviors, emotional lability, restlessness/impulsivity, and visual working memory deficiencies during school age (Thakur et al., 2013). The level of neuropsychological impairment had a dose dependent impact. A recent Western Australian study found maternal smoking to a be a risk factor for ADHD (Silva, Colvin, Hagemann, & Bower, 2013), and a large Danish study found that both maternal and paternal smoking was associated with ADHD risk, with maternal smoking posing a higher risk, even when the mother used nicotine replacement during pregnancy (Zhu et al., 2014). In contrast to these studies Langley and colleges (2012) observed that both maternal and paternal smoking during pregnancy was associated with ADHD symptoms, suggesting that the association with ADHD might be more associated with heredity than prenatal environment.

**Alcohol**

Excessive maternal alcohol intake during pregnancy is associated with Fetal Alcohol Syndrome, facial dysmorphology, growth restriction, and central nervous system/neurodevelopmental abnormalities, which present as cognitive and social difficulties (Sokol, Delaney-Black, & Nordstrom, 2008). A Ukrainian study indicated that maternal alcohol consumption was associated with neurodevelopmental deficits at 6 and 12 months (Bandoli, et al., 2016). A meta-analysis investigating mild, moderate, and binge alcohol exposure during pregnancy found that binge consumption had a
negative impact on cognition, and a moderate association with child behavior (Flack, 2013). Maternal alcohol use during pregnancy was associated with increased ADHD risk 1.55 times, compared to mothers that did not use alcohol during pregnancy (Han, et al., 2014). Children diagnosed with ADHD were 2.5 times as likely to be exposed to alcohol during pregnancy compared to non-ADHD controls (Mick, Biederman, Faraone, Sayer, & Kleinman, 2002).

**Illicit Drug Use**

Research into maternal illicit drug use during pregnancy is limited, and due to the nature of the substances used, are likely to be underreported. Nevertheless, literature on cannabis use during pregnancy indicates adverse effects on neurodevelopment, especially during critical periods of brain growth during fetal and adolescent maturation, such that children have long-term difficulties with visual memory, executive functioning progressing through adolescence with aggression and attention problems reported in preschool age children (Jaques et al., 2014). Children ages 1 and 3 with prenatal exposure to methamphetamine had lower gross motor functioning, but not lower cognitive functioning (Wouldes et al., 2014). Cocaine exposure during pregnancy has been associated with attention difficulties in preschool and school age children (Leech, Richardson, Goldschmidt & Day, 1999; Bandstra, Morrow, Anthony, Accornero, & Fried, 2001; Arendt, et. al, 2004; Savage, Brodsky, Malmud, Giannetta, & Hurt, 2005). Illicit drug use in pregnancy has many confounds associated with the research. Nevertheless, current literature does suggest long-term cognitive and behavioral effects associated with exposure.

**Maternal Gestational Diabetes**

Maternal gestational diabetes has also been shown to be associated with ADHD. A computerized measure of inattention, vigilance and perseveration (Conners CPT-II), did not show differences. However, individuals born to women with gestational diabetes, were shown to have more
psychopharmacological medication use for ADHD (Byhoff et al., 2017). A study following children born to mothers with gestational diabetes, at 18-months had lower gross motor and expressive language functioning (Torres-Espinola et al., 2015). A study of early infant development indicated that at 6 and 18-months there was greater inattention as indicated using an ERP paradigm (Cai et al., 2016). School age children born to mothers with gestational diabetes showed fine and gross motor impairments and difficulties with attention (Ornoy, Ratzon, Greenbaum, Wolf & Dulitzky, 2001). However, findings are not consistent where one study showed that maternal obesity was associated with increases risk for ADHD in preschool, maternal gestational diabetes was not (Daraki et al., 2017). Nomura and colleagues demonstrated that maternal gestational diabetes and low SES accounted for a 2-fold increase in ADHD diagnosis at 6 years (2012).

The Present Study

The aim of the current study is to examine the moderating effects of gene polymorphism on the developmental trajectory of ADHD using hierarchical linear modeling. Participants in the current study are a part of a larger established longitudinal study which has followed and evaluated children annually from preschool to school age. During these annual evaluations, beginning at age 3-4 and continuing until age 10, a parent diagnostic interview was conducted to determine ADHD symptom severity. The moderating effects of four single nucleotide polymorphisms (SNPs) associated with neurodevelopment and/or psychopathology were examined: neuregulin-1 (NRG-1; rs3924999), neurotropin-3 (NT-3; rs6489630), brain-derived neurotrophic factor (BDNF; rs6265), and regulator of G protein signaling 4 (RGS4; rs951439). By using a novel longitudinal approach, rather than a single time point, allowed for a more complete understand of the heterogeneity in the developmental trajectory of the disorder.

Taking reference from Rutter (1987) and the impact of psychosocial risk factors and the interactive process of these factors on development, the current study examined the impact of prenatal
environmental risk factors on ADHD symptom severity with the moderation of the chosen gene polymorphisms and the prenatal environmental risk factors of maternal gestational diabetes, maternal tobacco, alcohol and illicit drug use during pregnancy were examined.

Hypotheses

It was hypothesized that at risk-alleles of *NRG-1, NT-3, BDNF*, and *RGS4*, would be associated with ADHD symptoms at preschool age. Additionally, it was hypothesized that the risk-alleles of *NRG-1, NT-3, BDNF*, and *RGS4* would be associated with persistence of ADHD symptoms until school age. Finally, prenatal environmental factors (maternal gestational diabetes, maternal nicotine, alcohol and illicit drug use) were explored as moderating factors of ADHD symptom severity at preschool age and at school age.

METHOD

Participants

As part of larger longitudinal study examining factors associated with the persistence of ADHD symptoms in preschoolers, a community-based sample of 3- and 4-year-old children were recruited. At the initial recruitment permission form principals of local preschools was obtained to screen the school for children with attention and behavior problems. Additional referrals from schools and clinicians were also accepted. Entry into the study was based on parent and teacher reports on the home and school version of the Attention-Deficit/Hyperactivity Disorder Rating Scale, Fourth Edition (ADHD-RS-IV, McGoey, DuPaul, Haley, & Shelton, 2007). Children who received 6 or more symptoms rated as “Often” or “Very Often” on the Hyperactivity/Impulsivity and/or Inattention subscale(s) either by parent or teacher were considered hyperactive/inattentive. Children who received fewer than 3 items rated as “Often or Very Often” by both parents and teachers were expected into the study as Typically Developing. Exclusion criteria were: Full Scale IQ < 80, as measured by the Wechsler Preschool and
Primary Scale of Intelligence-Third Edition (WPPSI-III; Wechsler, 2002); evidence of a Pervasive Developmental Disorder, Post-traumatic Stress Disorder or a diagnosed neurological disorder; non-English-speaking parent or child; child not attending school; and treatment with systemic medication for a chronic medical condition (including ADHD). The final sample for the longitudinal study was 216. Among those, genetic data were successfully collected for the participants in this study, which consisted of 145 children (109 boys) ranging in age from 3.00 to 4.92 years (M=4.22, SD=0.49) at the initial evaluation (T1) and ranging in age from 10.37 to 12.51 years (M=11.43, SD=0.51) at the final evaluation (T8). Of the 71 that were not included in the analysis, 48 were boys ranging in age from 3.19 to 5.00 (M=4.41, SD=0.44) at the first initial evaluation.

The sample was racially and ethnically diverse, comprising of 16 black (11.0%), 18 Asian (12.4%), 25 Hispanic (17.2%), 25 more than one race (17.2%), and 61 (42.1%) white children. Socioeconomic status was assessed by the Occupational Prestige Scale (Nakao & Treas, 1994); the sample was largely middle class (M=63.86, SD=17.39) although a wide range was represented (range=20-97). (Sample characteristic Table 2).

This study was approved by the Institutional Review Board of the university in which the research was conducted. Following a complete description of the study, a parent of each child provided written informed consent for participation.

Measures

Primary caregivers were interviewed at the baseline assessment (T1) and then annually to the final measurement (T8) using the Kiddie Schedule for Affective Disorders and Schizophrenia – Present and Lifetime Version (Kiddie-SADS-PL; Kaufman, Birmaher, Brent, Rao, & Ryan, 1996). The Kiddie-SADS-PL is a commonly used diagnostic semi-structured interview that, when administered by trained evaluators, has been shown to have strong psychometric properties (Kaufman et al., 1999). Diagnostic
questions on the K-SADS-PL correspond to the 18 ADHD symptoms of ADHD. Each symptom was rated as either 0 (symptom was not present), 1 (subthreshold symptom) to 2 (symptom present at diagnostic level). The total score from all 18 symptoms provided the value for Total ADHD symptom severity. The score from the 9 inattentive symptoms provided the value for the Inattentive symptom severity. The score for the remaining 9 hyperactive/impulsive symptoms provided the value for the Hyperactive/Impulsive symptom severity. When available, teacher rating on the school version of the ADHD-RS-IV were incorporated to reach the final determination about each symptom at each time point.

At the time of the baseline evaluation the caregiver completed an early developmental history questionnaire that assessed prenatal risk factors. The caregiver indicated the presence of the following during gestation: maternal gestational diabetes, maternal substance use (alcohol, tobacco, illicit drug use).

**Genotyping procedure**

Genomic DNA was extracted from saliva (oragene•DNA, DNA Genotek, Inc, Ottawa, Ontario, Canada) quantified on a spectrophotometer (NanoDrop ND-1000, NanoDrop, Wilmington, DE). A 1 ng DNA was amplified in duplicate with a pre-designed TaqMan assay (assay ID C___1877332_20) according to the manufacturer’s instructions (Thermo Scientific, Waltham, MA) and identified by measuring end-point fluorescence on a LightCycler 480 II (Roche, Indianapolis, IN). Genotypes were determined for four genes *NRG1* (rs392499), *NT-3* (rs6489630), *BDNF* (rs6265) and *RGS4* (rs951439). The number of risk alleles was calculated for each gene, and participants were categorized as either having no risk alleles or at least one risk allele (1 or 2 risk alleles). A secondary analysis for the total number of risk gene alleles was completed for *NRG1, NT-3 and BDNF*. 
Data Analysis

Due to the longitudinal nature of the data, varied ages of participants at the initial time point, and missing values, Hierarchical linear modeling (HLM) was used at the primary statistical analysis. The advantage of HLM is that it takes into account initial values beginning at different time points and missing values. Therefore, in the current study, HLM can describe the growth curve of each individual’s trajectory of ADHD symptom severity, collected yearly, across an 8-year period. HLM allows modeling both within-and between-individual variations. At level-1, each individual’s data of ADHD symptom severity was fitted into a regression line. Level-1 coefficients are empirical Bayesian estimates, which are optimal estimates based on data from the individual and the entire sample. At level-2, the individual genetic risk factors were entered to explain the between-subject variation in the intercept and the linear slope.

To evaluate the longitudinal trajectory of ADHD symptom severity, the level-1 model was defined, and then evaluated with level-2 predictors. At level-1, the linear model was tested for ADHD symptom severity for three values: total, inattentive and hyperactive/impulsive symptoms. After the level-1 model was determined, the genetic risk factors (for NRG-1, NTF3, BDNF, RGS4) were entered into the model as level-2 variables, to determine if they explained a significant amount of the variance in the mean intercept or slope. The intercept corresponded to the level of ADHD symptoms at the initial time point, while the slope corresponded to change in ADHD symptom trajectory over the 8 time points.

To evaluate the impact of early prenatal environmental factors, a secondary analysis employed independent samples t-tests to explore significant differences on ADHD Total symptom severity at the final time point (T8). Lastly, regression analyses were used for the early environmental risk factors (maternal gestational diabetes, maternal substance use (alcohol, tobacco, illicit drug use) in conjunction with NRG-1, NTF3, and BDNF alleles.
Table 2: Race and Ethnicity Characteristics

<table>
<thead>
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</tr>
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<tbody>
<tr>
<td>White</td>
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<tr>
<td>Asian</td>
<td>18 (12%)</td>
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<tr>
<td>Black</td>
<td>16 (10%)</td>
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<tr>
<td>Other/Mixed</td>
<td>25 (17%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>145</strong></td>
</tr>
<tr>
<td>Not Hispanic</td>
<td>104 (72%)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>41 (28%)</td>
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</table>

RESULTS

I. Gene Main Effects

Change in Total ADHD Symptom Severity

As shown in Table 3 and depicted in Figure 1, the estimated mean Total ADHD Symptom Severity at 3-4 years was 17.08 (SE=0.94). The average slope or rate of change in ADHD severity over time was -0.10 (SE=0.10). There was significant estimated variation around the intercept ($\chi^2 = 1107.99$, df=144, $p<0.001$) and the slope ($\chi^2 = 369.94$, df=144, $p<0.001$). This indicates that the children varied significantly in their Total ADHD Symptom Severity at age 3-4 and in their rate of change in Total ADHD Symptom Severity over time.
TABLE 3: Change in Total ADHD Symptom Severity over time

<table>
<thead>
<tr>
<th>Results</th>
<th>Analysis</th>
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<tr>
<td>Fixed Effects</td>
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<tr>
<td>Level 1 intercept</td>
<td>Intercept</td>
</tr>
<tr>
<td>Level 1 linear slope</td>
<td>Intercept</td>
</tr>
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</table>

<table>
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<th>χ²</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level 1 intercept</td>
<td>112.22</td>
<td>144</td>
<td>1107.99</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Level 1 linear slope</td>
<td>0.98</td>
<td>144</td>
<td>369.94</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

FIGURE 1

Model 1: Trajectories of Total ADHD Symptom Severity from ages 3 to 11 years.

Figure 1: The model is centered at the baseline age, 3-4 years, which is indicated as 0 on the x-axis. The y-axis indicates total ADHD symptom severity.
Change in Inattentive Symptom Severity

As shown in Table 4 and depicted in Figure 2, the estimated mean Inattentive Symptom Severity at 3-4 years was 7.24 (SE=0.46). The average slope or rate of change in Inattentive Symptom severity was 0.28 (SE=0.06). There was significant estimated variation around the intercept ($\chi^2 = 727.26$, df=144, $p<0.01$) and the slope ($\chi^2 = 338.81$, df=144, $p<0.001$). This indicates that the children varied significantly in their Inattentive Symptom severity at age 3-4 and in their rate of change in Inattentive Symptom severity over time.

TABLE 4: Change in Inattentive Symptom Severity over time

<table>
<thead>
<tr>
<th>Results</th>
<th>Coefficient</th>
<th>SE</th>
<th>T ratio</th>
<th>p</th>
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<tr>
<td>Fixed Effects</td>
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<tr>
<td>Level 1 intercept</td>
<td></td>
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<tr>
<td>Intercept</td>
<td>7.24</td>
<td>0.46</td>
<td>15.844</td>
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<tr>
<td>Level 1 linear slope</td>
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<tr>
<td>Intercept</td>
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<td>4.69</td>
<td>&lt;0.001</td>
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<td>Level 1 intercept</td>
<td>24.47</td>
<td>144</td>
<td>727.26</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Level 1 linear slope</td>
<td>0.54</td>
<td>144</td>
<td>338.81</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
FIGURE 2:
Model 1: Trajectories of Inattentive Symptom Severity from ages 3 to 11 years.

Figure 2: The model is centered at the baseline age, 3-4 years, which is indicated as 0 on the x-axis. The y-axis indicates Inattentive Symptom Severity.

Change in Hyperactive/Impulsive Symptom Severity

As shown in Table 5 and depicted in Figure 3, the estimated mean Hyperactive/Impulsive Symptom Severity at 3-4 years was 9.82 (SE=0.53). The average slope or rate of change in Hyperactive/Impulsive Symptom Severity over time was -0.38 (SE=0.06). There was significant estimated variation around the intercept ($\chi^2 = 1165.16$, df=144, $p<0.01$) and the slope ($\chi^2 = 393.09$, df=144, $p<0.001$). This indicates that the children varied significantly in their Hyperactive/Impulsive Symptom Severity at age 3-4 and in their rate of change in Hyperactive/Impulsive Symptom Severity over time.
TABLE 5: Change in Hyperactive/Impulsive symptom severity over time

<table>
<thead>
<tr>
<th>Results</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed Effects</td>
<td></td>
</tr>
<tr>
<td>Level 1 intercept</td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>9.82</td>
</tr>
<tr>
<td>Level 1 linear slope</td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.38</td>
</tr>
</tbody>
</table>

| Random Effects                 |            |
| Variance Component df χ² p      |            |
| Level 1 intercept              | 36.32      | 144        | 1165.16 | <0.001 |
| Level 1 linear slope           | 0.57       | 144        | 393.09  | <0.001 |

FIGURE 3:

Model 1: Trajectories of Hyperactive/Impulsive Symptom Severity from ages 3 to 11 years.

Figure 3: The model is centered at the baseline age, 3-4 years, which is indicated as age 0 on the x-axis. The y-axis indicates Hyperactive/Impulsive Symptom Severity.
**Neuroregulin-1 (NRG1)**

**Association of NRG1 risk allele with Total ADHD symptom severity over time**

As shown in Table 6 and depicted in Figure 4, the estimated mean of Total ADHD symptom severity at age 3-4 years in children without the NRG1 risk allele was 11.08 (SE=2.00), with a significant increase of 7.31 (SE=2.25) with at least one NRG1 risk allele (p<0.01). The average slope or rate of change in Total ADHD symptom severity did not differ by NRG1 risk allele from age 3-4 years to age 11 years (p=0.518). There was significant variation around the average intercept ($\chi^2=1030.20$, df=143, p<0.001) and the average slope ($\chi^2=368.45$, df=143, p<0.001) that was not accounted for by the model.

**TABLE 6: Association of NRG1 risk allele with Total ADHD symptom severity over time**

<table>
<thead>
<tr>
<th>Results</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed Effects</td>
<td>Coefficient</td>
</tr>
<tr>
<td>Level 1 intercept</td>
<td>Intercept</td>
</tr>
<tr>
<td></td>
<td>NRG1</td>
</tr>
<tr>
<td>Level 1 linear slope</td>
<td>Intercept</td>
</tr>
<tr>
<td></td>
<td>NRG1</td>
</tr>
<tr>
<td>Random Effects</td>
<td>Variance Component</td>
</tr>
<tr>
<td>Level 1 intercept</td>
<td>105.34</td>
</tr>
<tr>
<td>Level 1 linear slope</td>
<td>0.98</td>
</tr>
</tbody>
</table>
Figure 4: Association of \textit{NRG1} risk allele with Total ADHD symptom severity over time

![Graph showing the association between NRG1 risk allele and Total ADHD symptom severity over time]

Figure 4: The model is centered at the baseline age, 3-4 years, which is indicated as age 0 on the x-axis. The y-axis indicates total ADHD Symptom Severity. Red line indicates at least one risk allele for \textit{NRG-1}, while the blue is no risk alleles for \textit{NRG-1}.

**Association of \textit{NRG1} risk allele with Inattentive symptom severity over time**

As shown in Table 7 and depicted in Figure 5, the estimated mean of Inattentive symptom severity at age 3-4 years in children without the \textit{NRG1} risk allele was 4.82 (SE=0.94), with a significant increase of 2.95 (SE=1.06) with at least one \textit{NRG1} risk allele ($p<0.01$). The average slope or rate of change in Inattentive symptom severity did not differ by \textit{NRG1} risk allele from age 3-4 years to age 11 years ($p=0.250$). There was significant variation around the average intercept ($\chi^2=689.620$, df=143, $p<0.001$) and the average slope ($\chi^2=333.40$, df=143, $p<0.001$) that was not accounted for by the model.

**TABLE 7: Association of \textit{NRG1} risk allele with Inattentive symptom severity over time**

<table>
<thead>
<tr>
<th>Results</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed Effects</td>
<td>Coefficient</td>
</tr>
<tr>
<td>Level 1 intercept</td>
<td>Intercept</td>
</tr>
</tbody>
</table>
NEURODEVELOPMENTAL GENES ADHD

<table>
<thead>
<tr>
<th>Gene</th>
<th>Estimate</th>
<th>SE</th>
<th>t-stat</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRG1</td>
<td>2.96</td>
<td>1.06</td>
<td>2.79</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Level 1 linear slope

| Intercept | 0.11 | 0.17 | 0.61 | 0.544 |
| NRG1      | 0.21 | 0.18 | 1.65 | 0.250 |

Random Effects

<table>
<thead>
<tr>
<th>Variance Component</th>
<th>df</th>
<th>( \chi^2 )</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>Level 1 intercept</td>
<td>4.84</td>
<td>689.61</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Level 1 slope</td>
<td>0.54</td>
<td>333.40</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Figure 5: Association of *NRG1* risk allele with Inattentive symptom severity over time

![Figure 5](image)

Figure 5: The model is centered at the baseline age, 3-4 years, which is indicated as age 0 on the x-axis. The y-axis indicates Inattentive Symptom Severity. Red line indicates at least one risk allele for *NRG-1*, while the blue is no risk alleles for *NRG-1*.

**Association of NRG1 risk allele with Hyperactive/Impulsive symptom severity over time**

As shown in Table 8 and depicted in Figure 6, the estimated mean of Hyperactive/Impulsive symptom severity at age 3-4 years in children without the *NRG1* risk allele was 6.25 (SE=1.11), with a significant increase of 4.35 (SE=1.26) with at least one *NRG1* risk allele (p<0.01). The average slope or rate of change in Hyperactive/Impulsive symptom severity did not differ by *NRG1* risk allele from age 3-4.
years to age 11 years ($p=0.958$). There was significant variation around the average intercept ($\chi^2=1078.79$, df=143, $p<0.001$) and the average slope ($\chi^2=392.99$, df=143, $p<0.001$) that was not accounted for by the model.

**TABLE 8: Association of NRG1 risk allele with Hyperactive/Impulsive symptom severity over time**

<table>
<thead>
<tr>
<th>Results</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed Effects</td>
<td></td>
</tr>
<tr>
<td>Level 1 intercept</td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>6.25</td>
</tr>
<tr>
<td>NRG1</td>
<td>4.35</td>
</tr>
<tr>
<td>Level 1 linear slope</td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.38</td>
</tr>
<tr>
<td>NRG1</td>
<td>-0.01</td>
</tr>
<tr>
<td>Random Effects</td>
<td></td>
</tr>
<tr>
<td>Level 1 intercept</td>
<td>5.82</td>
</tr>
<tr>
<td>Level 1 linear slope</td>
<td>0.57</td>
</tr>
</tbody>
</table>
Figure 6: Association of NRG1 risk allele with Hyperactive/Impulsive symptom severity over time

The model is centered at the baseline age, 3-4 years, which is indicated as age 0 on the x-axis. The y-axis indicates Hyperactive/Impulsive Symptom Severity. Red line indicates at least one risk allele for NRG-1, while the blue is no risk alleles for NRG-1.

**Neurotropin-3 (NT-3)**

Association of NT-3 risk allele with Total ADHD symptom severity over time

As shown in Table 9 and depicted in Figure 7, the estimated mean of Total ADHD symptom severity at age 3-4 years in children without the NT-3 risk allele was 16.57 (SE=1.17) with a nonsignificant increase of 1.45 (SE=1.97) in children with the at least one NT-3 risk allele (p=0.462). The average slope or rate of change in Total ADHD symptom severity did not differ significantly by NT-3 risk allele from age 3-4 years to age 11 years. However, there was a trend for rate of change as a function of NT-3 allele (p=0.104) that can be seen in Figure 7. To better understand the trend in the change of ADHD over time a secondary analysis indicated significantly higher total ADHD symptom severity for children with at least one NT-3 risk allele compared to children with no risk NT-3 allele at the final time point [t(114)=−3.270, p<0.01], but this difference was not evident at baseline. There was significant variation around the average intercept ($\chi^2=1103.96$, df=143, $p<0.001$) and the average slope ($\chi^2=361.32$, df=143, $p<0.001$) that was not accounted for by the model.
### TABLE 9: Association of NT-3 risk allele with Total ADHD symptom severity over time

<table>
<thead>
<tr>
<th>Results</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed Effects</td>
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</tr>
<tr>
<td></td>
<td>Coefficient</td>
</tr>
<tr>
<td><strong>Level 1 intercept</strong></td>
<td>16.57</td>
</tr>
<tr>
<td>Intercept</td>
<td>1.45</td>
</tr>
<tr>
<td><strong>Level 1 linear slope</strong></td>
<td>-0.22</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.34</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Random Effects</th>
<th>Variance Component</th>
<th>df</th>
<th>χ²</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Level 1 intercept</strong></td>
<td>10.61</td>
<td>143</td>
<td>1103.96</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Level 1 linear slope</strong></td>
<td>0.97</td>
<td>143</td>
<td>361.32</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Figure 7: Association of NT-3 risk allele with Total ADHD symptom severity over time
Figure 7: The model is centered at the baseline age, 3-4 years, which is indicated as age 0 on the x-axis. The y-axis indicates total ADHD Symptom Severity. Red line indicates at least one risk allele for NT-3, while the blue is no risk alleles for NT-3.

Association of NT-3 risk allele with Inattentive symptom severity over time

As shown in Table 10 and depicted in Figure 8, the estimated mean of Inattentive symptom severity at age 3-4 years in children without the NT-3 risk allele was 7.11 (SE=0.57) with a nonsignificant increase of 0.36 (SE=0.95) in children with at least one NT-3 risk allele (p=0.703) and the average slope or rate of change in Inattentive symptom severity did not differ by NT-3 risk allele from age 3-4 years to age 11 years (p=0.297). There was significant variation around the average intercept ($\chi^2=726.01, df=143, p<0.001$) and the average slope ($\chi^2=334.96, df=143, p<0.001$) that was not accounted for by the model.

TABLE 10: Association of NT-3 risk allele with Inattentive symptom severity over time

<table>
<thead>
<tr>
<th>Results</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed Effects</td>
<td>Coefficient</td>
</tr>
<tr>
<td>Level 1 intercept</td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>7.11</td>
</tr>
<tr>
<td>NT-3</td>
<td>0.36</td>
</tr>
<tr>
<td>Level 1 linear slope</td>
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</tr>
<tr>
<td>Intercept</td>
<td>0.24</td>
</tr>
<tr>
<td>NT-3</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Random Effects | Variance Component | df | $\chi^2$ | p |
| Level 1 intercept | 24.64 | 143 | 726.01 | <0.001 |
| Level 1 linear slope | 0.54 | 143 | 334.96 | <0.001 |
Figure 8: Association of NT-3 risk allele with Inattentive symptom severity over time

Figure 8: The model is centered at the baseline age 3-4 years, which is indicated as age 0 on the x-axis. The y-axis indicates total Inattentive Symptom Severity. Red line indicates at least one risk allele for NT-3, while the blue is no risk alleles for NT-3.

Association of NT-3 risk allele with Hyperactive/Impulsive symptom severity over time

As shown in Table 11 and depicted in Figure 9, the estimated mean of Hyperactive/Impulsive symptom severity at age 3-4 years in children without the NT-3 risk allele was 9.44 (SE=0.65). There was a small non-significant increase of 1.07 (SE=1.12) in children with at least one NT-3 risk allele (p=0.342). The average slope or rate of change in Hyperactive/Impulsive symptom severity did not differ by NT-3 risk allele from age 3-4 years to age 11 years. However, there was a trend for rate of change as a function of NT-3 allele (p=0.063) that can be seen in Figure 9. To better understand the trend in the change of ADHD over time a secondary analysis indicated significantly higher total Hyperactive/Impulsive symptom severity for children with at least one NT-3 risk allele compared to children with no risk NT-3 allele at the final time point [t(114)=-3.865, p<0.01]. This difference was not significant at baseline. There was significant variation around the average intercept (\(\chi^2=1159.49, \text{df}=143, p<0.001\)) and the average slope (\(\chi^2=382.78, \text{df}=143, p<0.001\)) that was not accounted for by the model.
### TABLE 11: Association of NT-3 risk allele with Hyperactive/Impulsive symptom severity over time

<table>
<thead>
<tr>
<th></th>
<th>Analysis</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fixed Effects</strong></td>
<td><strong>Coefficient</strong></td>
<td><strong>SE</strong></td>
<td><strong>T ratio</strong></td>
<td><strong>p</strong></td>
<td></td>
</tr>
<tr>
<td>Level</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>9.44</td>
<td>0.65</td>
<td>14.418</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>NT-3</td>
<td>1.07</td>
<td>1.12</td>
<td>0.95</td>
<td>0.342</td>
<td></td>
</tr>
<tr>
<td>Level 1 linear slope</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.46</td>
<td>0.08</td>
<td>-5.80</td>
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</tr>
<tr>
<td>NT-3</td>
<td>0.21</td>
<td>0.12</td>
<td>1.88</td>
<td>0.063</td>
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<td><strong>Random Effects</strong></td>
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<td><strong>χ²</strong></td>
<td><strong>p</strong></td>
<td></td>
</tr>
<tr>
<td>Level 1 intercept</td>
<td>6.03</td>
<td>143</td>
<td>1159.49</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Level 1 linear slope</td>
<td>0.56</td>
<td>143</td>
<td>382.78</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

Figure 9: Association of NT-3 risk allele in Hyperactive/Impulsive symptom severity over time
Figure 9: The model is centered at the baseline age, 3-4 years, which is indicated as age 0 on the x-axis. The y-axis indicates total Hyperactive/Impulsive Symptom Severity. Red line indicates at least one risk allele for NT-3, while the blue is no risk alleles for NT-3.

**Brain-derived Neurotrophic Factor (BDNF)**

**Association of BDNF risk allele with Total ADHD symptom severity over time**

As shown in Table 12 and depicted in Figure 10, the estimated mean of Total ADHD symptom severity at age 3-4 years in children without the BDNF risk allele was 11.36 (SE=2.58) with a significant increase of 6.42 (SE=2.76) in children with at least one BDNF risk allele ($p=0.021$). The average slope or rate of change in Total ADHD symptom severity did not differ by BDNF risk allele from age 3-4 years to age 11 years ($p=0.418$). There was significant variation around the average intercept ($\chi^2=1071.85$, df=143, $p<0.001$) and the average slope ($\chi^2=370.21$, df=143, $p<0.001$) that was not accounted for by the model.

**TABLE 12: Association of BDNF risk allele with Total ADHD symptom severity over time**

<table>
<thead>
<tr>
<th>Results</th>
<th>Analysis</th>
<th>Coefficient</th>
<th>SE</th>
<th>T ratio</th>
<th>p</th>
</tr>
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<tbody>
<tr>
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<td></td>
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<tr>
<td>Level 1 intercept</td>
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<td>Intercept</td>
<td>11.36</td>
<td>2.58</td>
<td>4.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BDNF</td>
<td>6.42</td>
<td>2.76</td>
<td>2.33</td>
</tr>
<tr>
<td>Level 1 linear slope</td>
<td></td>
<td>Intercept</td>
<td>0.18</td>
<td>0.38</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BDNF</td>
<td>-0.32</td>
<td>0.39</td>
<td>-0.81</td>
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<tr>
<td>Random Effects</td>
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<td>Variance Component</td>
<td>df</td>
<td>$\chi^2$</td>
<td>p</td>
</tr>
<tr>
<td>Level 1 intercept</td>
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<td>109.09</td>
<td>143</td>
<td>1071.85</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Level 1 linear slope</td>
<td></td>
<td>0.99</td>
<td>143</td>
<td>370.21</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
FIGURE 10: Association of *BDNF* risk allele with Total ADHD symptom severity over time

Figure 10: The model is centered at the baseline age, 3-4 years, which is indicated as age 0 on the x-axis. The y-axis indicates Total ADHD Symptom Severity. Red line indicates at least one risk allele for *BDNF*, while the blue is no risk alleles for *BDNF*.

**Association of *BDNF* risk allele with Inattentive symptom severity over time**

As shown in Table 13 and depicted in Figure 11, the estimated mean of Inattentive symptom severity at age 3-4 years in children without the *BDNF* risk allele was 4.83 (SE=1.25) with a significant increase of 2.72 (SE=1.34) in children with at least one *BDNF* risk allele (*p*=0.044). The average slope or rate of change in Inattentive symptom severity did not differ by *BDNF* risk allele from age 3-4 years to age 11 years (*p*=0.782). There was significant variation around the average intercept ($\chi^2=706.97$, df=143, *p*<0.001) and the average slope ($\chi^2=339.50$, df=143, *p*<0.001) that was not accounted for by the model.
TABLE 13: Association of BDNF risk allele with ADHD Inattentive symptom severity over time

<table>
<thead>
<tr>
<th>Results</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
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<td>Fixed Effects</td>
<td>Coefficient</td>
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<tr>
<td>Level 1 intercept</td>
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<tr>
<td>Intercept</td>
<td>4.82</td>
</tr>
<tr>
<td>BDNF</td>
<td>2.72</td>
</tr>
<tr>
<td>Level 1 linear slope</td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>0.34</td>
</tr>
<tr>
<td>BDNF</td>
<td>-0.06</td>
</tr>
<tr>
<td>Random Effects</td>
<td>Variance Component</td>
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<tr>
<td>Level 1 intercept</td>
<td>23.95</td>
</tr>
<tr>
<td>Level 1 linear slope</td>
<td></td>
</tr>
</tbody>
</table>

FIGURE 11: Association of BDNF risk allele with Inattentive symptom severity over time

Figure 11: The model is centered at the baseline age, 3-4 years, which is indicated as age 0 on the x-axis. The y-axis indicates total Inattentive Symptom Severity. Red line indicates at least one risk allele for BDNF, while the blue is no risk alleles for BDNF.
Association of BDNF risk allele with Hyperactive/Impulsive symptom severity over time

As shown in Table 14 and depicted in Figure 12, the estimated mean of Hyperactive/Impulsive symptom severity at age 3-4 years in children without the presence of the BDNF risk allele was 6.53 (SE=1.40) with a significant increase of 3.69 (SE=2.44) in children with the presence of at least one BDNF risk allele (p=0.016). The average slope or rate of change in Hyperactive/Impulsive symptom severity did not differ by BDNF risk allele from age 3-4 years to age 11 years (p=0.417). There was significant variation around the average intercept ($\chi^2=1128.72$, df=143, p<0.001) and the average slope ($\chi^2= 390.73$, df=143, p<0.001) that was not accounted for by the model.

TABLE 14: Association of BDNF risk allele with in Hyperactive/ Impulsive severity over time

<table>
<thead>
<tr>
<th>Results</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
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<td><strong>Fixed Effects</strong></td>
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<tr>
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<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>6.53</td>
</tr>
<tr>
<td>BDNF</td>
<td>3.69</td>
</tr>
<tr>
<td>Level 1 linear slope</td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.16</td>
</tr>
<tr>
<td>BDNF</td>
<td>-0.25</td>
</tr>
<tr>
<td><strong>Random Effects</strong></td>
<td>Variance Component</td>
</tr>
<tr>
<td>Level 1 intercept</td>
<td>35.28</td>
</tr>
<tr>
<td>Level 1 linear slope</td>
<td>0.32</td>
</tr>
</tbody>
</table>
Figure 12: The model is centered at the baseline age, 3-4 years, which is indicated as age 0 on the x-axis. The y-axis indicates total Hyperactive/Impulsive Symptom Severity. Red line indicates at least one risk allele for BDNF, while the blue is no risk alleles for BDNF.

**Regulator of G protein signaling 4 (RGS4)**

Association of RGS4 risk allele with Total ADHD symptom severity over time

As shown in Table 15 and depicted in Figure 13, the estimated mean of Total ADHD symptom severity at age 3-4 years in children without the RGS4 risk allele was 15.61 (SE=1.58), with an increase of 2.39 (SE=1.96) with at least one RGS4 risk allele ($p=0.223$). The average slope or rate of change in Total ADHD symptom severity did not differ by RGS4 risk allele from age 3-4 years to age 11 years ($p=0.378$). There was significant variation around the average intercept ($\chi^2=1101.34$, df=143, $p<0.001$) and the average slope ($\chi^2=368.43$, df=143, $p<0.001$) that was not accounted for by the model.
TABLE 15: Association of $RGS4$ risk allele with Total ADHD symptom severity

<table>
<thead>
<tr>
<th>Results</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed Effects</td>
<td></td>
</tr>
<tr>
<td>Level 1 intercept</td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>$15.61$</td>
</tr>
<tr>
<td>$RGS4$</td>
<td>$2.39$</td>
</tr>
<tr>
<td>Level 1 linear slope</td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>$0.02$</td>
</tr>
<tr>
<td>$RGS4$</td>
<td>$-0.19$</td>
</tr>
</tbody>
</table>

| Random Effects                |          |
| Variance Component            |          |
| Level 1 intercept             | $111.91$ | $143$  | $1101.34$ | $<0.001$ |
| Level 1 linear slope          | $0.97$   | $143$  | $368.43$  | $<0.001$ |

FIGURE 13: Association of $RGS4$ risk allele with Total ADHD symptom severity over time
Figure 13: The model is centered at the baseline age, 3-4 years, which is indicated as age 0 on the x-axis. The y-axis indicates Total ADHD Symptom Severity. Red line indicates at least one risk allele for RGS4, while the blue is no risk alleles for RGS4.

Association of RGS4 risk allele with Inattentive symptom severity over time

As shown in Table 16 and depicted in Figure 14, the estimated mean Inattentive symptom severity at age 3-4 years in children without the RGS4 risk allele was 6.71 (SE=0.73), with a nonsignificant increase of 0.87 (SE=0.94) with the least one RGS4 risk allele (p=0.354). The average slope or rate of change in Inattentive symptom severity did not differ by RGS4 risk allele from age 3-4 years to age 11 years (p=0.330). There was significant variation around the average intercept ($\chi^2$=724.61, df=143, p<0.001) and the average slope ($\chi^2$= 336.94, df=143, p<0.001) that was not accounted for by the model.

TABLE 16: Association of RGS4 risk allele with change in Inattentive symptom severity over time

<table>
<thead>
<tr>
<th>Results</th>
<th>Coefficient</th>
<th>SE</th>
<th>T ratio</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fixed Effects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level 1 intercept</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>6.71</td>
<td>0.73</td>
<td>9.17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RGS4</td>
<td>0.87</td>
<td>0.94</td>
<td>0.93</td>
<td>0.354</td>
</tr>
<tr>
<td>Level 1 linear slope</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>0.35</td>
<td>0.09</td>
<td>3.945</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RGS4</td>
<td>-0.12</td>
<td>0.12</td>
<td>-0.98</td>
<td>0.330</td>
</tr>
<tr>
<td><strong>Random Effects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Level 1 intercept</td>
<td>25.54</td>
<td>143</td>
<td>724.61</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Level 1 linear slope</td>
<td>0.30</td>
<td>143</td>
<td>336.94</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
FIGURE 14: Association of *RGS4* risk allele with Inattentive symptom severity over time

Figure 14: The model is centered at the baseline age, 3-4 years, which is indicated as age 0 on the x-axis. The y-axis indicates total Inattentive Symptom Severity. Red line indicates at least one risk allele for *RGS4*, while the blue is no risk alleles for *RGS4*.

**Association of *RGS4* risk allele with Hyperactive/Impulsive symptom severity over time**

As shown in Table 17 and depicted in Figure 15, the estimated mean of Hyperactive/Impulsive symptom severity at age 3-4 years in children without the *RGS4* risk allele was 8.87 (SE=0.91), with an increase of 1.55 (SE=1.12) with at least one *RGS4* risk allele (*p*=0.166). The average slope or rate of change in Hyperactive/Impulsive symptom severity did not differ by *RGS4* risk allele from age 3-4 years to age 11 years (*p*=0.533). There was significant variation around the average intercept ($\chi^2=1156.25$, df=143, *p*<0.001) and the average slope ($\chi^2=392.51$, df=143, *p*<0.001) that was not accounted for by the model.
### TABLE 17: Association of RGS4 risk allele with Hyperactive/Impulsive symptom severity over time

<table>
<thead>
<tr>
<th>Results</th>
<th>Analysis</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fixed Effects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Level 1 intercept</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>8.87</td>
<td>0.91</td>
<td>9.73</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RGS4</td>
<td>1.55</td>
<td>1.12</td>
<td>1.39</td>
<td>0.166</td>
</tr>
<tr>
<td><strong>Level 1 linear slope</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.33</td>
<td>0.10</td>
<td>-3.263</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>RGS4</td>
<td>-0.08</td>
<td>0.12</td>
<td>-0.62</td>
<td>0.533</td>
</tr>
<tr>
<td><strong>Random Effects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Variance Component</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Level 1 intercept</strong></td>
<td>36.05</td>
<td>143</td>
<td>1156.25</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Level 1 linear slope</strong></td>
<td>0.57</td>
<td>143</td>
<td>392.51</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

### FIGURE 15: Association of RGS4 risk allele with Hyperactive/Impulsive symptom severity over time
Figure 15: The model is centered at the baseline age, 3-4 years, which is indicated as age 0 on the x-axis. The y-axis indicates total Hyperactive/Impulsive Symptom Severity. Red line indicates at least one risk allele for RGS4, while the blue is no risk alleles for RGS4.

**Total Risk Genes**

Three out of the four genes showed some evidence of gene effects with NRG-1 and BDNF, having significant associations with Total ADHD symptoms severity, and NT-3 showing marginal evidence of a trend toward allele-related differences. To assess the additive role of the risk alleles for the association with Total ADHD symptom severity, the number of risk alleles for the three genes were summed to arrive at a sum gene risk variable (possible range 0 -6). Due to low frequencies for risk values 0 (n=3) and 6 (n=2), those with 0 and 1 risk alleles were combined as were those with 5 and 6 risk alleles. Table 18 shows the frequency distribution for sum number of risk alleles.

**TABLE 18: Distribution of Sum Number of Risk Alleles**

<table>
<thead>
<tr>
<th>Sum Number of Risk Alleles</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
</tr>
<tr>
<td>3</td>
<td>41</td>
</tr>
<tr>
<td>4</td>
<td>44</td>
</tr>
<tr>
<td>5-6</td>
<td>21</td>
</tr>
</tbody>
</table>

Note: The 0-1 group is comprised of 3 children with 0 risk alleles and 13 with 1 risk allele. The 5-6 group is comprised of 19 and 2 children with 5 and 6 risk alleles, respectively.

To determine the association between the sum of risk alleles and Total ADHD, Inattentive and Hyperactive/Impulsive symptom severity Pearson correlations revealed significant positive associations between symptom severity and total number of risk alleles at each time-point (T1-T8) with increasing r values as children got older (Figure 16). Figures 16 A-H show symptom levels as a function of number of
risk alleles at each annual time point. Notably, by the latter time points (T7 and T8) there is a clear linear relation between number of risk alleles and ADHD symptom severity.

FIGURE 16: Correlation of Number of Risk Alleles with Total ADHD symptoms, Inattentive symptoms and Hyperactive/Impulsive Symptoms at annual time points.

\* = all three p<0.01, ** = all three p<0.001
Figure 17: Total ADHD symptoms as a function of number of risk alleles over time

A. Time 1

B. Time 2

C. Time 3

D. Time 4

E. Time 5

F. Time 6
Gene x Environment Interaction

To determine whether environmental risk factors for ADHD interacted with gene effects, the following risk factors were examined using exploratory moderation analyses: maternal gestational diabetes, maternal substance use (alcohol, tobacco, illicit drug use) during pregnancy and low SES (SES less than the 10%ile for SES for the sample). Table 19 displays the frequencies for the environmental risk factors. The following are the only significant results that were obtained in this exploratory analysis.

TABLE 19: Distribution of Sum Number of Environmental risk alleles.

<table>
<thead>
<tr>
<th>Prenatal Risk Factor</th>
<th>No</th>
<th>Yes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Gestational Diabetes</td>
<td>130</td>
<td>15</td>
</tr>
<tr>
<td>Maternal Tobacco Use</td>
<td>119</td>
<td>26</td>
</tr>
<tr>
<td>Maternal Alcohol Use</td>
<td>113</td>
<td>32</td>
</tr>
<tr>
<td>Maternal Illicit Drug Use</td>
<td>135</td>
<td>10</td>
</tr>
<tr>
<td>Low SES (&lt;10%)</td>
<td>133</td>
<td>12</td>
</tr>
<tr>
<td>Any Prenatal Risk Factor</td>
<td>80</td>
<td>65</td>
</tr>
</tbody>
</table>
NRG-1 and Early Environmental Risk Factor of Maternal gestational diabetes

Maternal gestational diabetes was examined as a moderator of the relation between NRG-1 and Total ADHD symptom severity at Time 1. NRG-1 risk alleles and presence of maternal gestational diabetes were entered in the first step of the regression analysis. In the second step of the regression analysis, the interaction term between NRG-1 risk alleles and maternal gestational diabetes was entered, and it explained a significant increase in Total ADHD symptom severity [$\Delta R^2 = 0.084$, $F(3,141)=4.314$, $p<0.01$]. Maternal gestational diabetes was a significant moderator of the relationship between NRG-1 and Total ADHD symptom severity, such that having at least one NRG-1 risk allele and maternal gestational diabetes increases Total ADHD Symptom severity compared to maternal gestational diabetes without a NRG-1 risk allele; however, Total ADHD symptom severity was lower without maternal gestational diabetes and at least one risk allele for NRG-1 compared to both maternal gestational diabetes risk allele and NRG-1 allele risk and no maternal gestational diabetes and no NRG-1 risk allele (See Figure 18).

Figure 18: Moderation of NRG-1 and Maternal Gestational Diabetes.
NRG-1 and Early Environmental Risk Factor of Maternal Alcohol Use

Maternal alcohol use during pregnancy was examined as a moderator of the relation between *NRG-1* and Total ADHD symptom severity at Time 1. *NRG-1* risk alleles and presence of maternal alcohol use were entered in the first step of the regression analysis. In the second step of the regression analysis, the interaction term between *NRG-1* risk alleles and maternal alcohol use was entered, and it explained a significant increase in Total ADHD symptom severity ($\Delta R^2 = 0.087, F(3,141)=4.497, \ p<0.01$). Maternal alcohol use was a significant moderator of the relationship between *NRG-1* and Total ADHD symptom severity, such that having no *NRG-1* risk allele and no maternal alcohol use had lowest Total ADHD severity compared to maternal alcohol use with and without the *NRG-1* risk allele and maternal alcohol use and at least one *NRG-1* risk factor (See Figure 19).

Figure 19: Moderation of *NRG-1* and Maternal Alcohol Use.

![Graph showing moderation of NRG-1 and Maternal Alcohol Use](image)

NTF3 and Early Environmental Risk Factor of Maternal Tobacco Use

Maternal tobacco use during pregnancy was examined as a moderator of the relation between *NTF3* and Total ADHD symptom severity at Time 1. *NTF3* risk alleles and presence of maternal tobacco use were entered in the first step of the regression analysis. In the second step of the regression
analysis, the interaction term between *NTF3* risk alleles and maternal tobacco use was entered, and it explained a significant increase in Total ADHD symptom severity \[\Delta R^2 = 0.08, F(3,141)=3.852, p<0.05\]. Maternal tobacco use was a significant moderator of the relationship between *NTF3* and Total ADHD symptom severity, such that having at no *NTF3* risk alleles and no maternal tobacco use resulted in the lower Total ADHD severity compared to maternal tobacco use and no *NTF3*; however, having at least one *NTF3* risk allele and maternal tobacco use was lower compared to no maternal tobacco use and at least one *NTF3* risk allele (See Figure 20).

Figure 20: Moderation of *NTF3* and Maternal Tobacco Use.

*BDNF* and Early Environmental Risk Factor of Maternal Gestational Diabetes

Maternal gestational diabetes during pregnancy was examined as a moderator of the relation between *BDNF* and Total ADHD symptom severity at Time 1. *BDNF* risk alleles and presence of maternal gestational diabetes were entered in the first step of the regression analysis. In the second step of the regression analysis, the interaction term between *BDNF* risk alleles and maternal gestational diabetes was entered, and it explained a significant increase in Total ADHD symptom severity \[\Delta R^2 = 0.06, F(3,141)=3.060, p<0.05\]. Maternal gestational diabetes was a significant moderator of the relationship
between *BDNF* and Total ADHD symptom severity, such that having no *BDNF* risk alleles and no maternal tobacco use resulted in the high Total ADHD severity compared to maternal gestational diabetes and no *BDNF* risk alleles; however, having at least one *BDNF* risk allele and maternal gestational diabetes was higher compared to no maternal tobacco use and at least one *BDNF* risk allele. (See Figure 21).

Figure 21: Moderation of *BDNF* and Maternal Gestational Diabetes.

![Graph showing moderation of BDNF and Maternal Gestational Diabetes](image)

**DISCUSSION**

ADHD is a neurodevelopmental disorder that is typically first identified in early childhood and often continues through adulthood (APA, 2013). It is a highly heritable disorder with heritability estimates in the range of .76 (Faraone et al., 2005). ADHD has been associated with abnormalities in both neuroanatomical and molecular functioning (Castellanos et al., 2002a; Shaw et al., 2007; Amico et al., 2001). Driven largely by the effectiveness of psychostimulant medication for treating symptoms of ADHD, the majority of genetic studies have focused on catecholaminergic and noradrenergic systems...
with weak and inconsistent results (Kustanovich et al., 2003; Mill et al., 2005; Loo et al., 2003; Ribasés et al., 2012; Faraone et al., 2005; Gizer, Ficks, & Waldman, 2009). Similarly, serotonin system genes necessitate further investigation. Beyond genes affecting neurotransmitter systems associated with ADHD, genes involved in brain growth and development have indicated that some promising areas that also suggest greater investigation (Mill et al., 2002; Mill et al., 2004; Mick & Faraone, 2008; Hawi et al., 2013b). Findings from genome-wide association studies have not yet yielded definitive results in identifying a gene or genes responsible for ADHD (Neale et al., 2010; Hinney et al., 2011; Ebejer et al., 2013). Theoretically BDNF and glutamate-related genes suggest an association with ADHD, however, again, findings have been mixed (Turic et al., 2004; Kent et al., 2005).

In response to the limited success of previous research to identify specific genes related to the presence of ADHD, the current study undertook a novel neurodevelopmental approach in an attempt to elucidate the genetic basis of ADHD. The study followed a longitudinal sample of preschool-age children that was evaluated for the presence of ADHD symptoms annually for 8 years. Four genes involved in neurodevelopment were selected for this exploratory study; *NRG-1, NT-3, BDNF, RGS4*. Additionally, information on early environmental risk factors (i.e., maternal gestational diabetes, maternal substance use - alcohol, tobacco, illicit substance-) was collected to explore moderating relations between genetic risk and early environmental risk.

The initial modeling of ADHD symptoms over time, independent of other factors, revealed little group-level change in total ADHD symptom severity with age. However, there was significant individual variability at the initial preschool age time-point as well as in change in severity over time. In contrast to total symptom levels, there was a significant increase in inattentive symptom severity with increasing age and a significant decrease in hyperactive/impulsive symptoms over time. Nevertheless, again, there was significant inter-individual variability in both the initial level and the slope of change over time in
both symptom domains. These results were largely expected as the typical course of ADHD is such that the high prevalence of ADHD symptoms in early childhood tends to decrease through adolescence and adulthood (Campbell, 1995; Connor, 2002, Halperin, Trampush, Miller, & Newcorn, 2008, Faraone, Biederman, & Mick, 2006). Symptoms of hyperactivity/impulsivity decrease over time, while symptoms of inattention are more likely to persist (Biederman, Mick, & Faraone, 2000; DuPaul & Stoner, 2004; Wolraich et al., 2005).

Among the four genes that were evaluated, three were found to have a notable association with ADHD symptomatology. Individuals with at least one risk allele of NRG-1 or BDNF had more total ADHD, inattentive, and hyperactive/impulsive symptoms at preschool age, although the rate of change in symptom severity over time did not vary as a function of NRG-1 or BDNF genotype. These findings are consistent with the hypothesis that NRG-1 and BDNF influence the onset of ADHD, but not the trajectory of ADHD over development from the preschool to school-age years. NRG-1 is associated with fine-tuning of cortical circuitry and synaptic efficacy, and has an influence on the transcription of multiple classes of neurotransmitter receptors. These changes in neuronal formation have been associated with behavioral outcomes (Arnsten, 2006; Yin et al., 2013). BDNF is involved in neurodevelopment, synapse regulation and synaptic plasticity, modulates synaptic transmission, and is associated with behavioral disorders, such mood disorders and schizophrenia (Fernandes et al., 2014). BDNF also demonstrates a protective role with neuronal homeostasis and is protective against neuronal insults. Both are associated with synaptic plasticity and long-term functionality. Changes in neurotropic activity during brain development can results in neuronal dysfunction which can lead to disordered behavior. It was hypothesized that both NRG-1 and BDNF would be associated with ADHD symptoms at preschool and associated with the persistence of ADHD symptoms until school age. Our results supported the onset of ADHD symptoms being associated with NRG-1 and BDNF, but not the persistence of ADHD, at least prior to puberty.
In contrast, NT-3 genotype did not significantly relate to ADHD onset or trajectory. However, a trend emerged suggesting that NT-3 might influence the trajectory of total ADHD symptoms and hyperactive/impulsive symptoms in particular. Although NT-3 has been identified as regulating CNS neurogenesis during early development, results suggest that NT-3 may impact ADHD symptoms later in development. Finally, RGS4 risk alleles did not impact ADHD onset or trajectory. Although, we hypothesized that RGS4 allelic variability would relate to ADHD symptoms onset and trajectory, out of the four genes evaluated it was the one with the least substantial theoretical support.

Collectively this information suggests a role for three of the neurodevelopmental genes examined, with their effects primarily on the onset of ADHD symptoms. Although, this was an exploratory study, it was hypothesized that these genes would have an impact on the trajectory of ADHD. Apart from, NT-3, which showed a trend such that it impacted ADHD later in development, the other two genes only related to early onset at preschool age. One possible explanation is that these neurodevelopmental genes have their largest impact on the early onset ADHD symptoms and other genes have greater contribution to influencing ADHD symptoms in late school age (Pingault et al., 2015). Alternatively, individuals with NT-3 risk allele are more likely to grow into their genes in late school age and early adolescence.

Although the results indicated an association such that individual genes related to ADHD symptomatology, it is highly likely that ADHD is not a result of a few genes, but rather a collection of genes with small effect size that contribute to the onset and/or the persistence of the disorder. To examine the collective nature of multiple genetic risks, the next analysis combined total number of genetic risk alleles. It was expected that with more genetic risk one would be more likely to have an increase in ADHD symptoms (Rutter, 1987). As proposed, the number of genetic risk alleles was associated with increased total ADHD, inattentive, and hyperactive/impulsive symptoms at each time
point, such that the more risk alleles an individual had the more ADHD symptoms. Notably, the strength of the association between number of risk alleles and ADHD symptoms increased with increasing age. This suggests an additive effect of genetic risk on ADHD symptoms at onset and over time, and raise the possibility that as children get older, genetic influences on ADHD severity get stronger as environmental influences diminish. Although the combination of risk alleles from the three genes assessed was associated with ADHD, it is very likely that many more genes, not only the ones selected for this study, are involved as there continues to be significant unaccounted variability.

**Exploratory Moderation Analyses**

Exploratory analyses into early environmental factors were conducted to evaluate gene-by-environment interactions and to explore the unaccounted-for variability. The influence of maternal gestational diabetes and maternal substance use during pregnancy was mixed. It was hypothesized that early environmental risk factors would moderate ADHD symptoms and provide further explanation for individual variability in symptomatology. Only a few of several analyses conducted yielded significant findings, and some are difficult to interpret.

Significant moderation was found between *NRG-1* and two maternal risk factors; gestational diabetes and maternal alcohol use during pregnancy. Yet, the pattern of the interaction differed between these two environmental risk factors. Maternal gestational diabetes was unrelated to ADHD symptom severity in those without an *NRG-1* risk allele. However, among individuals with an *NRG-1* risk allele, those with and without maternal gestational diabetes had the highest and lowest levels of ADHD symptoms, respectively. As stated above, the nature of the interaction was different for maternal alcohol use during pregnancy. Children of mothers who drank alcohol during pregnancy had elevated levels of ADHD symptoms irrespective of *NRG-1* risk, as did those with an *NRG-1* risk allele irrespective of maternal alcohol use. Those without the genetic (i.e., *NRG-1* risk allele) and environmental (i.e.,
maternal alcohol use during pregnancy) risk factors had significantly lower levels of ADHD symptoms as compared to the other three groups. Thus, it appears that effect of gestational diabetes on the offspring’s neurodevelopment is dependent upon NRG-1 risk status. In contrast, maternal alcohol use during pregnancy has negative effects irrespective of genetic status.

A significant interaction also emerged between NT-3 risk status and maternal tobacco use during pregnancy, although the nature of the interaction was surprising and interpretation of this finding is less clear. NT-3 risk and maternal tobacco use resulted in lower levels of ADHD symptoms compared to those with the genetic risk but no maternal tobacco use. Overall, the nature of this interaction suggests that NT-3 risk is reduced in those whose mothers smoked during pregnancy.

Finally, significant moderation was found between BDNF and maternal gestational diabetes. Here the nature of the interaction was such that BDNF risk had a minimal impact when gestational diabetes was not present. However, among those with gestational diabetes, elevated ADHD symptom levels were seen only in those children who had a BDNF risk allele.

Taken together it appears that maternal gestations diabetes is associated with elevated levels of ADHD symptoms only in youth at elevated genetic risk (via NRG-1 and/or BDNF), consistent with a gene-by-environment interaction. In contrast, maternal alcohol use during pregnancy may moderate the influence of NRG-1 on ADHD symptoms. Although the environmental risk analysis was exploratory, it was expected that environmental risk factors would moderate genetic risk. In contrast, the interaction involving NT-3 and maternal tobacco use is more difficult to explain and understand. It appears that maternal smoking may mitigate the risk associated with NT-3. While the results for the impact of environmental factors are mixed, these analyses are exploratory and may reflect chance findings given the large number of analyses conducted. Even though the analyses are exploratory and limited these
areas of investigation could yield valuable information in understanding the impact of suboptimal early prenatal development brain.

**Limitations**

As this was a small pilot study there were several limitations. The most apparent limitation is the small sample size. As stated previously, the current sample was selected from a slightly larger longitudinal study. Compared to contemporary genetic studies the sample size is substantially smaller. However, despite the small sample size, there were some significant findings with three out of the four genes studied. Although this does not exclude the chance of random findings, it does suggest that a potential avenue of further research. Environmental risk factors information was collected at the initial evaluation time point, but was still dependent on retrospective recall. Further, the information that was used in the analysis was binary (i.e., endorsement or lack of endorsement). Greater sensitivity might have emerged had dimensional measures of environmental risk been employed. Additionally, the binary nature could lead to uncaptured variability between individuals. For example, there could be a difference in outcome depending on the prenatal developmental time point the substance was used, or the frequency and extent of the substance that was used. With maternal gestational diabetes, there could be variability in management of the disease and ultimately variability on the impact on the developing fetus and in turn impacting neurological development. Additionally, the low frequency of endorsement of maternal substance use limited the use of some of the analysis. A sample with greater proportion of maternal substance use including more detailed information on the timing of exposure during pregnancy, and the impact of varying amounts of fetal exposure, would allow more depth of analysis such as time sensitivity and dose dependent moderation. Finally, it is possible that respondents were not completely forthcoming with information, particularly as related to more sensitive topics such as substance use during pregnancy.
Despite clear evidence from behavioral genetic studies for the importance of genes in the etiology of ADHD, the elucidation of specific genes has not been as fruitful or rewarding as expected. Much of the field has moved in the direction of genome-wide analyses, and some genetic markers are beginning to emerge (Franke et al., 2012; Elia et al., 2012; Sánchez-Mora et al., 2015). However, such analyses require extremely large samples and are generally not helpful for identifying gene-by-environment interactions because it is difficult, if not impossible to acquire individualized environmental data from such large samples (Moore, 2017). This study offers a somewhat different approach to studying and understanding the etiology of this neurodevelopmental disorder by focusing on genes involved in neurodevelopment.

The current study was limited in both the breadth and depth of possible genes involved in the onset and the persistence of ADHD. There are many more genes that are involved in neurodevelopmental processes that affect aspects of neural development, which impact neural pathways, and ultimately influence behavior that suggest additional avenues of investigation. Even among the genes evaluated, only single nucleotide polymorphisms were evaluated and yet there are possible alternative gene variants even in the genes evaluated that can impact ADHD symptomatology. One clear area of further research is to explore the cumulative nature of neurodevelopmental risk alleles of additional neurodevelopmental genes. Another area to explore is the impact of these genes on ADHD symptoms in later adolescence and early adulthood, where genes involved in the maturation of the cortex exert the most impact and can differentiate those that continue to experience functionally impactful symptoms of ADHD. Further, the current study did not have the power to properly evaluate the impact on males versus females. Finally, and possibly most importantly, due to the exploratory nature of the study, these findings need to be replicated. Genetic findings, especially with a limited sample require further analyses.
Future Directions

Additional routes of further research can include environmental factors. The current study looked at the prenatal environmental risk factors to identify additional areas that can impact individual symptomatology of ADHD. Maternal alcohol use, tobacco use and maternal gestational diabetes were identified as possible factors. In addition to these factors, physical abuse, child neglect, maternal exposure to stress, trauma, poor nutrition, premature birth, perinatal complications, very low birth weight, early hypoxic episodes, and exposure to environmental toxins can also be explored as risks to early neurologic development. Early environmental factors can also have a role in the expression of these genes. Further exploration of gene production will provide better understanding of gene and environmental interactions. More accurate measurement and assessment of environmental factors can contribute to the understanding of cumulative risk on neurodevelopment.

With the understanding that the developmental trajectory of ADHD varies in presentation and persistence over development; better understanding of the factors that impact ADHD symptomatology can give direction to those treating individuals with the disorder in a more personalized way. It is possible that in the future genetic profiles could identify individuals at risk for the disorder prior to preschool age and provide information on the possible severity and persistence over development. This information can be used to structure early optimal behavioral treatment and symptom management. Genetic profiles may also suggest optimal pharmacological interventions.

Conclusions

The findings of this study suggest a possible fruitful new direction into both the understanding of the etiology of ADHD across development and an alternative approach into exploring neurodevelopmental genetic risk factors. Going forward this study suggests an approach that considers the onset and persistence of ADHD symptoms and identifies those genes that undermine optimal
neurodevelopment. Lastly, the study also suggests incorporating early environmental factors that can negatively impact brain development and how these factors, in coordination with genetic factors, manifest in psychopathology. Although the current study focused on ADHD symptomatology, investigations into other neurodevelopmental disorders, such as Autism Spectrum Disorder, may also benefit from this approach.
### Table 1: Summary of genetic results

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type of Study</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRD4</td>
<td>Case control</td>
<td>7 –repeat – inconsistent findings, no single significant findings</td>
</tr>
<tr>
<td></td>
<td>Case control</td>
<td>7 –repeat - OR = 1.9 ($p &lt; 0.001$)</td>
</tr>
<tr>
<td></td>
<td>Meta-analysis</td>
<td>7 –repeat - OR = 1.4 ($p = 0.02$)</td>
</tr>
<tr>
<td></td>
<td>Case control</td>
<td>2 and 4-repeat allele – not significant</td>
</tr>
<tr>
<td>DRD5</td>
<td>Case control</td>
<td>148-bp allele – association</td>
</tr>
<tr>
<td></td>
<td>Family based</td>
<td>136-bp and 146-bp alleles – no association</td>
</tr>
<tr>
<td></td>
<td>Meta-analysis</td>
<td>2/4 studies found 148-bp allele – association</td>
</tr>
<tr>
<td>DRD2</td>
<td>Case control</td>
<td>A1 allele – inconsistent association</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A2 allele – association with hyperactivity and impulsivity</td>
</tr>
<tr>
<td>DRD1</td>
<td>Case control</td>
<td>association with DRD1 in childhood combined type ADHD, but not in adulthood ADHD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>two SNPs associated with ADHD (rs4532, rs265981)</td>
</tr>
<tr>
<td>DAT1</td>
<td>Case control</td>
<td>No association</td>
</tr>
<tr>
<td>DBH</td>
<td>Case control</td>
<td>A1 and A2 allele – significant but weak association</td>
</tr>
<tr>
<td>TH</td>
<td>Case control</td>
<td>No association</td>
</tr>
<tr>
<td>DRD3</td>
<td>Case control</td>
<td>No association</td>
</tr>
<tr>
<td>COMT</td>
<td>Case control</td>
<td>Inconsistent association (Val108Met polymorphism)</td>
</tr>
<tr>
<td>MAO-A</td>
<td>Case control</td>
<td>30-bp tandem repeat of the promoter region of the gene in two ethnic populations</td>
</tr>
<tr>
<td>SLC6A2/NET1</td>
<td>Case control</td>
<td>Association</td>
</tr>
<tr>
<td></td>
<td>Meta-analysis</td>
<td>No association</td>
</tr>
<tr>
<td></td>
<td>SNP studies</td>
<td>rs3785157 – association in two studies, not replicated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs998424 – association in one study, not replicated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs3785143 and rs11568324 – association in one study</td>
</tr>
<tr>
<td>ADRA1A</td>
<td>Case control</td>
<td>Association</td>
</tr>
<tr>
<td></td>
<td>Meta-analysis</td>
<td>No association</td>
</tr>
<tr>
<td>ADRA1B</td>
<td>Case control</td>
<td>Association</td>
</tr>
<tr>
<td></td>
<td>Meta-analysis</td>
<td>No association</td>
</tr>
<tr>
<td>Genes</td>
<td>Study Type</td>
<td>Findings</td>
</tr>
<tr>
<td>-------------------------------------</td>
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<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>SLC6A2/NET1 and ADRA1B</td>
<td>Case control</td>
<td>multi SNP haplotypes association</td>
</tr>
<tr>
<td>ADRA2A</td>
<td>Case control</td>
<td>G-1291C allele at the promoter region, not replicated G-1291C association with inattentive subtype</td>
</tr>
<tr>
<td>Serotonin Receptor Genes</td>
<td>Case control study</td>
<td>HTR1B receptor G861C allele with ADHD – association, not replicated HTR2B receptor T102C and G1438A – not associated HTR1E receptor – no association HTR3B receptor – no association</td>
</tr>
<tr>
<td>Serotonin transporter</td>
<td>Case control</td>
<td>5-HTTLPR polymorphism - 2/4 found an association</td>
</tr>
<tr>
<td>Family based</td>
<td>5-HTTLPR polymorphism – one study found an association Another study found an association with combined type Third study found no association</td>
<td></td>
</tr>
<tr>
<td>TPH</td>
<td>Family based</td>
<td>2 studies did not find an association 1 study - rs1843809 T-allele, rs1386497 A-allele, and rs1386493 C-allele – association 2nd study - rs1843809 A-allele and rs1386493 G-allele – association</td>
</tr>
<tr>
<td>SNAP25</td>
<td>Family based Meta-analysis</td>
<td>T1069C and T1065G – association, not replicated T1065G association with ADHD rs66039806-C, rs362549-A, rs362987-A, rs362998-C alleles, rs363020, rs362567- association, not all replicated</td>
</tr>
<tr>
<td>BDNF</td>
<td>Case control</td>
<td>Val66 allele parental transmission and association of ADHD, not replicated No association with 20 SNPs</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Family based</td>
<td>GRIN2A , Association, not replicated Haplotype – weak association</td>
</tr>
</tbody>
</table>
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