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Gene-Environment Interactions in Attention Deficit Hyperactivity Disorder

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GENE-ENVIRONMENT INTERACTIONS IN ATTENTION DEFICIT HYPERACTIVITY DISORDER

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Abstract

The overall goal of this project is to advance our understanding of the multifactorial etiology of Attention Deficit Hyperactivity Disorder (ADHD) by testing a diathesis-stress model of gene x environment (g x e) interactions. Although the literature increasingly supports g x e interactions in the manifestation of ADHD, few studies have investigated multiple genetic and environmental risk factors, included direct tests of gene – environment correlations (rG-Es), explored the specificity of interactions to symptom dimensions, or attempted to minimize comparisons. Therefore, utilizing both within-family (FBAT/PBAT) and case-control methodology, this study sought to (1) explore main effects of polymorphisms in the DRD2, DRD4, DRD5, DAT1, 5HTT, ADRA2C and DBH genes on ADHD symptoms in a community sample, (2) explore main effects of environmental risk factors on ADHD symptoms (including direct tests of gene – environment correlation), (3) test for g x e interaction effects between those environmental and genetic risk factors substantiated by main effects, and (4) investigate whether results were specific to particular symptom dimensions of ADHD. Analyses demonstrated a robust main effect of the DRD4 4-repeat allele (DRD4*4R) on ADHD symptoms rather than the DRD4 7-repeat allele (DRD4*7R), that had previously been implicated in ADHD. Analyses also revealed main effects of maternal smoking, prenatal alcohol exposure, season of birth, parental education, and television viewing habits on

ADHD symptoms. After considering rG-Es, results demonstrated significant diathesis-stress g x e interactions between DRD4*4R and season of birth, maternal smoking, and parental education that selectively exacerbated hyperactive-impulsive (HI) symptoms. Exploratory analyses demonstrated a main effect of the DAT1 10-repeat allele (DAT1*10R) on ADHD-Combined Type and HI symptoms, and revealed significant interactions between DAT1*10R and parental education and season of birth on HI behaviors. Taken together, these data are consistent with a diathesis-stress model for g x e interactions in ADHD, suggest a possible alternate risk factor in linkage disequilibrium with DRD4*4R and DRD4*7R that may be the true "risk" allele, provide evidence that DAT1*10R may play into a subtype-specific etiology for ADHD-C, and support the idea that polymorphisms in dopaminergic genes interact with parental education and season of birth to selectively exacerbate HI symptoms.

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Finally, this dissertation is dedicated to my mother, who is my best friend and talked me through many long nights, and to my father, who taught me to be smart, make a plan, and persevere.

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FIGURE 4: DRD4*4R X MATERNAL SMOKING 51

INTRODUCTION

The primary goal of this study is to test a diathesis-stress model of gene x environment (g x e) interactions in the etiology of Attention Deficit Hyperactivity Disorder (ADHD). Several risk alleles thought to be involved in ADHD have empirical support; however, their effects are small and much of the variance in ADHD symptom expression remains unexplained. A diathesis-stress model posits that some risk alleles may have bigger effects in certain risk environments. Although explicating g x e interactions in ADHD is critical to psychoeducation aimed at prevention, early identification, and intervention, to date, few studies testing for such g x e interactions in ADHD have been performed. Furthermore, very few have investigated more than one genetic or environmental risk factor, explored the specificity of interactions to ADHD dimensions, or taken care to minimize comparisons. By contrast, this study utilizes main effects to screen variables for inclusion in g x e analyses, and thereby minimizes comparisons while examining a wide array of genetic and environmental risk factors. Additionally, the present study rigorously examines gene – environment correlations (rG-Es), and tests for dimensional specificity of interactions. In the following pages, I will begin by giving a general overview of ADHD, as well as the existing literature on genetic and environmental contributants thereto. Secondly, I will explore the existing literature on g x e interactions in ADHD, highlighting and discussing in greater detail the literature's most relevant findings and most notable weaknesses. Finally, I will discuss

the analyses conducted over the course of this study and the results of these analyses in detail, underscoring how these investigations will make a contribution to the existing knowledge and understanding regarding the etiology of ADHD in children.

Overview of ADHD: Genes and Environments

Prevalence and Characteristics of ADHD

Attention Deficit Hyperactivity Disorder (ADHD) is one of the most common disorders of early childhood, with prevalence rates in the United States ranging from 3% to 10% (APA, 1994; Satcher, 1999) and it is associated with important social consequences (Mannuzza, Klein, Bessler, & LaPadula, 1993) and significant health care costs (Leibson, Katusic, Barbaresi, Ransom, & O'Brien, 2001). ADHD is characterized by a history of inattentive and hyperactive-impulsive symptoms with onset before the age of 7 (APA, 1994). However, the disorder often persists through adolescence and into early adulthood (Wilens, Biederman, & Spencer, 2002), disrupting many of the tasks necessary for adult development due to the centrality of sustained effort, planning, and organization in adult responsibilities. Although a subset of children with ADHD grow out of their diagnosis (Hill & Schoener, 1996), most children with ADHD symptomatology are at increased risk for later problems (Rasmussen & Gillberg, 2000).

Despite considerable research, the field has yet to find an environmental factor strongly associated with the disorder, or to identify most of the genetic loci underlying its high heritability. Rather, multiple studies have nominated a number of potential psychosocial and bioenvironmental risk factors and weakly associated loci. These data are consistent with a multifactorial model of the disorder that incorporates multiple genetic and environmental risk factors (Pennington, 2006) interacting to manifest

different levels of hyperactive-impulsive and inattentive symptomatology. G x e interactions, specifically diathesis-stress models, are a cornerstone of the conceptualization of psychopathology (O'Connor, Caspi, DeFries, & Plomin, 2003). These models predict that a diathesis, or genetic vulnerability, coupled with an environmental stress, leads to disordered behavior (Durand & Barlow, 2000). Despite the fact that these models have been applied widely in psychopathology, only recently have they been applied in samples presenting with hyperactive-impulsive and/or inattentive symptoms. The goal of the current study is to test a diathesis-stress model in the etiology of ADHD by examining a variety of genetic and environmental risk factors in a community sample, exploring g x e interactions between these risk factors, and investigating the specificity of interactions to ADHD symptom dimensions. Identifying these genetic and environmental risk factors and their interactions is vital to early identification and intervention in ADHD, and may thereby reduce the severity and costs associated with the disorder.

GENETIC CONTRIBUTIONS TO ADHD

The familiality (e.g., Biederman, Faraone, Keenan, Knee, & Tusang, 1990; Faraone, Biedermand, Keenan & Tsuang, 1991) and heritability (e.g., Gillis, Gilger, Pennington, & DeFries, 1992; Levy, Hay, McStephen, Wood, & Waldman, 1997; Willcutt, Pennington, & DeFries, 2000a; Willerman, 1973) of ADHD have been firmly established, with large-scale twin studies consistently producing high heritability estimates (h^2 & $h^2g > 0.7$). Heritability has been demonstrated for both dimensions of the DSM-IV ADHD diagnosis; however, the heritability of the hyperactive-impulsive subtype (HI) is negligible once the correlation between the two dimensions is accounted

for (Willcutt, Pennington, & DeFries, 2000b), emphasizing the need for research into specific risk loci and/or environmental risk factors that may underlie different aspects of the disorder. To date, the following candidate genes (and associated risk alleles/polymorphisms) have provided the most compelling and replicable associations with ADHD:

- The 9-repeat (DAT1*9R) and 10-repeat (DAT1*10R) alleles of a 40-base pair variable number tandem repeat (VNTR) polymorphism in the 3'-untranslated region (UTR) of DAT1 (Chen et al., 2003; Cook et al., 1995; Curran et al., 2001; Daly, Hawi, Fitzgerald, & Gill, 1999; Gill, Daly, Heron, Hawi, & Fitzgerald, 1997; Leventhal, 1995; Waldman et al., 1996; Waldman et al., 1998).
- The 7-repeat allele of a VNTR polymorphism in exon 3 of DRD4 (DRD4*7R; for meta-analysis, see Faraone, Doyle, Mick, & Biederman, 2001).
- A 44-bp insertion/deletion in the promoter region of 5-HTT that yields long (5HTT*Long) and short alleles (Kent et al., 2002; Manor et al., 2001; Seeger, Schloss, & Schmidt, 2001; Zoroglu et al., 2002).
- The A1 and A2 alleles of a TaqI polymorphism in intron 5 of DBH as well as a dinucleotide repeat that lies 5' of the gene (Daly et al., 1999; Hawi et al., 2003; Muller-Smith et al., 2003; Roman et al., 2002).
- The A1 and A2 alleles of a TaqI polymorphism of DRD2 (Comings et al., 1991; Comings et al., 1996; Rowe et al., 1999; Sery et al., 2006).
- The 148-bp dinucleotide repeat that lies 18.5 kb from DRD5 (Daly et al., 1999; for joint analysis, see Lowe et al., 2004).
- A dinucleotide repeat that lies approximately 6 kb away from ADRA2C

(Comings et al., 1999).

- A promoter region (MspI) of ADRA2A.
- The 861G allele of HTR1B (Hawi et al., 2002; Ouist et al., 2003).
- SNPs at positions 1065 and 1069 of SNAP-25 (Barr et al., 2000; Brophy, Hawi, Kirley, Fitzgerald, & Gill, 2002).

The present study focuses primarily on the majority of these genes (all but HTR1B, ADRA2A and SNAP-25). It is important to note that these risk alleles, most of which have been replicated in several independent association studies of ADHD, confer little genetic risk, indicating that these genes account for relatively little of the heritability of the disorder (Bobb, Castellanos, Addignton, & Rappoport, 2004). In addition to the wealth of association studies of ADHD, linkage studies have broadened the search, but as of yet have produced largely inconsistent findings. Whole-genome scans of ADHD samples using the affected sib-pair method have suggested possible target regions. However, these studies converged on only one locus: 5p13 (Bakker et al., 2003; Fisher et al., 2002; Ogdie et al., 2004). Model-based and model-free linkage analyses coupled with the pedigree disequilibrium test found significant linkage at 4q13.2, 5q33.3, 11q22, and 17p11 (Arcos-Burgos et al., 2004). Another study using fine mapping demonstrated significant linkage at 5p13, 6q12, and 16p13, and supported linkage findings at 17p11 (Ogdie et al., 2004). In summary, given the lack of convergent evidence across studies, it is possible that individual genes conferring moderate to large genetic risk do not exist.

ENVIRONMENTAL CONTRIBUTIONS TO ADHD

The environmental influences on ADHD that have received the most research attention can be broadly divided into two categories: psychosocial and bioenvironmental.

Associations have been demonstrated between ADHD and early television exposure (Christakis, Zimmerman, DiGiuseppe, & McCarty, 2004), environmental adversity (e.g. family conflict, social class, family size etc.; Biederman, Faraone, & Monuteaux, 2002a, 2002b; Biederman et al., 1995a; Biederman et al., 1995b) and exposure to adult ADHD (potentially a genetically-mediated effect; Biederman, Faraone, & Monuteaux, 2002b). Although all of these studies statistically controlled for some potential confounding factors (e.g., gestational age, prenatal substance use/abuse, socioeconomic status), it is important to note that these were not genetically-sensitive designs and none of the reported associations was very strong. It is therefore not surprising that in studies examining family-genetic and psychosocial risk factors for ADHD, only genetic influences appeared to be responsible for the familiality of the disorder (Biederman, Faraone, Keenan, Knee, & Tsuang, 1990).

Bioenvironmental correlates of ADHD include environmental lead exposure and pediatric head injury (for review, see Barkley, 1996), but these factors only account for a small number of cases. Therefore research has focused on more common pre- and peri-natal environmental risk factors that may primarily impact prenatal development of dopaminergic systems. Some of the risk factors implicated in ADHD are: obstetric complications (Biederman & Faraone, 2005; Milberger, Biederman, Faraone, Guite, & Tsuang, 1997), older maternal age at birth (Linnet et al., 2003), drug/alcohol exposure (Knopik et al., 2005; Linnet et al., 2003; Mick, Biederman, Faraone, Sayer, & Kleinman, 2002; Nigg, 2006; Thapar et al., 2005), and spring/summer season of birth (Brookes et al., 2005). The most consistently replicated environmental associations to date have been between ADHD and low birth weight (Bhutta, Cleves, Casey, Cradock, & Anand, 2002;

Claycomb, Ryan, Miller, Schnakenberg-tt, 2004; Milberger et al., 1997; Siegel, 1982; Thapar et al., 2005), and between ADHD and maternal smoking (Kotimaa et al, 2003; Langley et al., 2005; Mick et al., 2002; Thapar et al., 2003; Wakschlag, Leventhal, Pine, Pickett, & Carter, 2006). The latter association has been found to persist even when variance attributable to social adversity, birth weight, and antisocial symptom scores is removed (Claycomb et al., 2004). Although some psychosocial risk factors are considered, this study focuses primarily on bioenvironmental risks. Each of the aforementioned pre- and peri-natal risk factors is included in the current study.

In this study, information on psychosocial and bioenvironmental risk factors has been obtained primarily through parent-report measures. However, we use two objective measures of the home environment that are related to cognitive development: birth order and family size (Siegel, 1982). Additionally, three objective measures of bioenvironmental risk, birth weight, maternal age at birth, and season of birth, are included in the study.

GENE X ENVIRONMENT INTERACTIONS IN ADHD

Prior to this study, there had been four studies published addressing g x e interactions in ADHD. Three of these studies utilized case-control methodology, and one utilized family-based methods. Taken together, these studies provided some evidence for the interaction of well-replicated ADHD risk alleles and pre- and peri-natal environmental risk factors such as maternal smoking, prenatal alcohol exposure, and season of birth. Below I will summarize the main findings of each of these four studies and highlight any notable limitations. Summary statistics for these studies are presented in Table 1. More recent literature will be addressed in the DISCUSSION section.

Kahn and colleagues (2003) examined the role of the DAT1 10-repeat risk allele (DAT1*10R) and maternal smoking on the manifestation of inattentive, hyperactive-impulsive, and oppositional behaviors as measured by the Conners' Parent Rating Scale – Revised Long Version (CPRS-R:L) (Conners, Sitareneos, Parker, & Epstein, 1998). The authors found no main effect of DAT1 on the inattentive, hyperactive-impulsive, or oppositional scales; however, there was a main effect of smoking on the latter two scales ($p < .05$). Children with prenatal smoke exposure and two copies of DAT1*10R had significantly higher hyperactive-impulsive and oppositional scores than all other groups. Linear regression analyses provided support for an interaction between prenatal smoke exposure and DAT1*10R on the hyperactive-impulsive and oppositional, but not the inattentive, scales ($p < .01$).

Neuman and colleagues (2006) examined potential interactions between DAT1 and DRD4 polymorphisms and prenatal smoking or prenatal alcohol exposure in the

Table 1: G x E Interaction Studies of Common Risk Alleles/Environmental Risk Factors on ADHD

		Genetic Risk			
	Risk (Prevalence)	**DRD4*7R (31-40%)**	**DAT1*10R (57%)**	**DAT1*9R (43-54%)**	**DAT1 Haplotype (59-74%)**
Environmental Risk	**Smoke Exposure (21-24%)**	Neuman et al., (2007) p = .0003 ADHD Symptoms	Kahn et al., (2003) p = .01; HI p = .001; Opp	Neuman et al., (2007) p = .001 ADHD Symptoms	
	Alcohol Exposure (5-58%)				Brookes et al., (2006) p = .04 ADHD Symptoms
	Season of Birth (44-50%)	Seeger et al., (2004) p = .013 HD + CD			

manifestation of DSM-IV or population-defined (extrapolated by Latent Class Analysis (LCA)) ADHD subtypes. Their results demonstrated a main effect of maternal smoking ($p = .006$), but not prenatal alcohol exposure ($p = .34$), on DSM-IV ADHD symptoms; the latter variable was not included in further analyses. Logistic regression revealed that children who were exposed to prenatal smoking demonstrated significantly elevated odds ratios (ORs) for developing DSM-IV ADHD-C if they had inherited the DAT1 9-repeat risk allele (DAT1*9R; OR = 2.93, 95% CI = 1.2 - 7.1) or the DRD4 7-repeat allele (DRD4*7R; OR = 2.83, 95% CI = 1.1 - 7.4). Mean symptom counts were significantly greater in subjects with smoke exposure than without, and increased with an increasing number of DRD4*7R ($p = .003$) or DAT1*9R ($p = .001$) risk alleles. For those exposed children with both risk alleles, the ORs for any DSM-IV or population-defined ADHD-C were 3.2 (95% CI 1.1 – 9.6) and 9.0 (95% CI = 2.0 - 41.5), respectively, suggesting a potential gene x gene (g x g) interaction. Although the authors explored these interaction effects within a twin sample, they did not use family-based designs, which are robust to certain artifacts (e.g. population stratification, and for within-pair design, the effects of age and other pair-specific variables), and may therefore have provided a more powerful g x e test.

Brookes and colleagues (2006) introduced a novel genetic association with ADHD by examining main effects and possible interactions between a common DAT1 haplotype (a combination of the 3' UTR 40-bp VNTR and an intron 8 30-bp VNTR) and maternal smoking or prenatal alcohol exposure. Family-based association tests demonstrated a main effect of genotype on ADHD symptomatology ($p = .003$). The ORs for transmission of the risk haplotype to offspring differed significantly across alcohol

exposure groups (p = .04), and this finding replicated in a Taiwanese sample, providing compelling evidence for a g x e interaction. Limitations of this study included a trend towards a gene-environment correlation (rG-E) between offspring ADHD and prenatal alcohol exposure (p = .07) that was not fully addressed in the interpretation of findings, broad screening questions for environmental risk (e.g., maternal smoking defined as "yes" or "no" in response to the questions "Did you smoke at least 20 cigarettes a day for 3 months of pregnancy?", and "Did you give up alcohol during pregnancy?"), and no examination of the main effects of environmental risk.

Finally, a study conducted in Germany by Seeger and colleagues (2004) examined the interaction between DRD4*7R and season of birth on comorbid hyperkinetic disorder (HD; ICD-10 equivalent of ADHD) and conduct disorder (CD). Chi-square analyses demonstrated no main effect for either DRD4*7R or season of birth. Researchers demonstrated significant ORs for comorbid HD + CD in children with one copy of the DRD4*7R allele born in spring and summer (OR = 2.8, p = .013) and autumn and winter (OR = -5.4, p = .002). An increase in relative risk in one environment (spring and summer) juxtaposed with a decrease in relative risk in another environment (autumn and winter) is suggestive of a crossover interaction between season of birth and DRD4.

The pattern of results that emerges from these studies is a curious one: In most cases, examination of interaction effects between an ADHD risk alleles and a bioenvironmental risk factor yielded increased risk for hyperactive-impulsive and oppositional behaviors, while inattentive symptoms were not affected. Evidence from twin studies (Willcutt, 2008; Willcutt, Pennington, & DeFries, 2000), however, demonstrates that extreme inattention scores are highly heritable regardless of levels of

hyperactivity-impulsivity, whereas extreme hyperactive-impulsive scores are only heritable when accompanied by a concurrent elevation in inattention scores (such as in ADHD-C). If inattention is the dimension driving the heritability of ADHD, one would not necessarily expect g x e interactions to selectively exacerbate hyperactive-impulsive symptomatology. These data collectively support dimensional specificity, and further suggest that environmental risk factors may contribute differentially to symptom manifestation. As ADHD demonstrates significant comorbidity with other psychopathologies, such as Conduct Disorder (CD) (Souza, Pinheiro, Denardin, Mattos, & Rohde, 2004), it is possible that g x e interactions produce an increased relative risk for hyperactive-impulsive symptoms and oppositional or conduct problems rather than inattentive symptoms. Taken together, these studies highlight the need for further research into potential g x e interaction effects in ADHD utilizing a broader array of environmental risk factors and giving specific attention to individual ADHD symptom dimensions.

As Table 1 illustrates, while these four studies have provided some compelling evidence in support of g x e interactions in the manifestation of ADHD, there continue to be substantial gaps in the literature regarding even the most well-associated genetic and environmental risk factors. This study examines g x e interactions in our ADHD sample. It was modeled after recent within-family designs, as well as Caspi and colleagues' (Caspi et al., 2002; Caspi, et al., 2003) efforts to identify and understand g x e interactions in depression and conduct disorder. We examine multiple risk alleles and psychosocial and bioenvironmental risk factors, and further explore these interactions within symptom dimensions (and, when appropriate, subtypes) of ADHD. This study is

an application of a diathesis-stress model to ADHD in a population where g x e interactions are rarely studied (children).

When considering g x e interactions in the manifestation of behavior, it is important to note certain key differences between the animal and human literature. Specifically, when studying animal models of behavior, g x e interactions almost always occur in the presence of main effects of gene and/or environment (e.g., Crabbe, Walston, & Dudek, 1999; Valdar, 2006). However, in literature targeting human behavior (and specifically the manifestation of ADHD symptoms), such interactions are often demonstrated in the absence of significant main effects. It is possible that, in the manifestation of ADHD, either some interactions are "crossover" in nature – that is, a particular gene confers increased risk in a "risk" environment and decreased risk in a "no risk" environment – thus washing out any main effects, or that the noted interactions are not substantial enough to support main effects. However, this disparity from animal to human literature has led some to speculate that some published g x e interaction findings are the result of statistical "fishing expeditions". This assertion appears to be bolstered by inconsistent replication of such findings, and calls for a greater measure of methodological rigor in pursuing g x e interactions. As such, this study pursues main effects of both gene and environment, and utilizes its findings to inform subsequent g x e interaction analyses in the hopes of minimizing Type I error.

GENE-ENVIRONMENT CORRELATIONS

An additional complication when studying environmental risk factors is that genetic factors may be correlated with the environmental factors (Rutter et al., 1997). It is not unreasonable to anticipate a gene-environment correlation (rG-E) in the case of

ADHD, given the environmental variables that have been previously researched. For example, a predisposition to nicotine addiction may be the consequence of possessing unfavorable alleles for attention. Therefore, although it may appear that nicotine is impacting the fetus, it may be that mothers who smoke while pregnant are passing on these unfavorable alleles to their children. In summary, although research into psychosocial and bioenvironmental risk factors associated with ADHD often statistically controls for genotypic risk (e.g., Biederman et al., 1990; Biederman et al., 2002a, 2002b; Biederman et al., 1995a; Biederman et al., 1995b) and some designs are more genetically sensitive than others (e.g., Thapar et al., 2003), to date few studies on environmental risk factors associated with ADHD have included a direct measure of rG-E. This study directly tests for the existence of rG-Es and thereby demonstrates to what extent our "environmental" variables are truly independent. If rG-Es are found, they are taken into account in the interpretation of g x e interactions.

SPECIFIC AIMS

The overall goal of this study is to advance understanding of the multifactorial etiology of ADHD by testing a diathesis-stress model of g x e interactions. Furthermore, by focusing on g x e interactions using a wide array of risk alleles and psychosocial and bioenvironmental risk factors, this study has the potential to significantly expand the literature on the etiology of ADHD. The specific aims of this study are as follows: (1) to explore the main effects of genotype on ADHD symptomatology by conducting an association study between ADHD and polymorphisms in the DRD2, DRD4, DRD5, DAT1, 5HTT, ADRA2C and DBH genes within a community sample, (2) to explore the

main effects of specific bioenvironmental and psychosocial risk factors on the later manifestation of ADHD symptoms, (3) to test for g x e interaction effects between those environmental risk factors and risk alleles substantiated by main effects, and (4) to investigate whether these results are specific to particular symptom dimensions of ADHD.

Although the literature surrounding ADHD and genetic or environmental contributants thereto has become progressively suggestive of the presence of g x e interactions, to date, few studies testing for such interactions have been performed, and very few have investigated more than one genetic or environmental risk factor, explored the specificity of interactions to ADHD symptom dimensions, included direct tests of rG-E, and sought to minimize comparisons. As such, this study presents several advantages over previous research in this area, and should constitute a significant contribution to the literature concerning the etiology of ADHD.

METHOD

PARTICIPANTS

The present study is part of the ongoing Colorado Learning Disabilities Research Center Twin Project (CLDRC, DeFries et al., 1997), in which all twin pairs between the ages of 8 and 18 years are ascertained, without regard to ADHD status, through 22 different school districts in 928 different schools in metro Denver to create a population-based twin sample of children with reading disability, ADHD, comorbid disorders, and control subjects. After initial ascertainment, permission was sought to review the school records of both twins for evidence of academic difficulties or ADHD. If either member of the twin pair had a history of such problems, both members of the twin pair were invited to participate in the project. The zygosity of same-sex twin pairs was determined using selected items from the Nichols and Bilbro (1966) questionnaire, and, in ambiguous cases, was confirmed by genetic analysis. Whenever possible, biological siblings of the twin pair that were within the 8-18 age range were also tested. Exclusion criteria and study parameters have been described previously (DeFries et al., 1997). The overall sample was divided into two groups based on how the sample was originally ascertained. One group was comprised of multiple twin pairs (and their siblings) in which at least one twin presented with a history of academic difficulties or ADHD (children with ADHD did not necessarily demonstrate academic difficulties, although the two often co-occurred), and one group was comprised of control subjects. For the purposes of this

Table 2: Descriptives and Significance Tests for Overall Sample

Descriptives N = 1,473	**Affected N = 353**	**Unaffected N = 1,120**	**Sig. Tests**
Age	11.23	11.38	t = 0.951 p = 0.342
FSIQ	103.52	109.78	t = 8.706 p < 0.001
Gender	69.7% (male)	43.6% (male)	χ^2 = 73.232 p < 0.001

study, a sample of children that had provided genetic and/or symptom data were selected from the aforementioned population-based sample for case-control and within-family analyses. A description of the overall sample (from which all sub-samples were drawn for analyses) is presented in Table 2. Additionally, a sample of parents who had provided genetic data as part of their participation were selected to complete pedigrees for within-family analyses. Finally, a subset of families who had previously participated in the CLDRC were re-contacted by mail in an attempt to obtain supplemental information pertinent to peri-natal environmental risk and retrospective maternal ADHD symptomatology (See *PROCEDURE* for a full description of all data collection). In all cases, children were excluded from analyses if they failed to meet general CLDRC inclusion criteria (DeFries et al., 1997), presented with a Full Scale IQ (FSIQ) < 70, or carried a confounding medical diagnoses that would impact cognition and therefore influence results (e.g., seizure disorder). Samples sizes and additional inclusion/exclusion criteria will be discussed per analysis due to differential inclusion of participants across analytic approaches, as well as differential availability of genetic and environmental data across groups.

Within this sample, participants were considered to have an "affection status" (e.g., diagnosis) of ADHD if they met DSM-IV diagnostic criteria for ADHD during their

participation in the CLDRC Twin Project, meaning they demonstrated 6 or more symptoms or inattention, 6 or more symptoms of hyperactivity-impulsivity, or 6 or more in both domains. Affection status was assigned based on the OR rule, meaning that if either the parent OR the teacher endorsed a given symptom, it was counted in the assignment of affection status. Data on frequency, severity, and nature of symptoms was collected for dimensional analyses. Finally, in an effort to mirror DSM-IV diagnostic criteria as accurately as possible, information on level of impairment across settings was collected for confirmatory purposes; however, the scarcity of such data made its inclusion in initial diagnostic procedures unfeasible. However, significant correlations between level of impairment and ADHD symptomatology were evident in this sample ($r = 0.678$, $p < 0.001$), indicating that as ADHD symptoms increased, as did level of impairment.

MEASURES

Diagnostic ADHD Measure

1.) Attention Deficit Hyperactivity Disorder Rating Scale – IV (DuPaul, Power, Anastopoulos, & Reid, 1998): The ADHDRS-IV was administered to at least one parent and one teacher of each subject recruited for the study. In most instances, maternal reports were used in analyses, as more mothers were available to participate. The ADHDRS-IV is a questionnaire that implements DSM-IV criteria for ADHD. Children are diagnosed as ADHD if they demonstrated 6 or more inattentive symptoms, 6 or more hyperactive-impulsive symptoms, or 6 or more in both domains, rated by either a parent

or teacher. Participants were further classified as either ADHD-Combined Type (ADHD-C), ADHD-Inattentive Type (ADHD-I), or ADHD-Hyperactive/Impulsive Type (ADHD-HI) in accordance with DSM diagnostic criteria based on symptom endorsements.

Home Environment Measures

1.) Parent Education: Parental education is often used as a marker variable for SES (Smith, Brooks-Gunn, & Glebanov, 1997). The CLDRC collected information about education level for both the mother and father.

2.) Family Size: Parents provided dates of birth for all of the children in the family.

3.) Television Viewing Habits: Parents provided information regarding hours of television their children watched per week.

Pre- and Peri-natal Risk Factors

1.) Pregnancy and Birth Injury module from the Diagnostic Interview for Children and Adolescents – IV (PBIQ; DICA-IV) (Reich, Welner, & Herjanic, 1997): Mothers were either interviewed or completed a self-report questionnaire about pregnancy and birth for the twins and non-twin siblings included in the study. DICA-IV variables are described in Table 3.

2.) Retrospective ADHD Interview for Mothers (MSRADD): Mothers provided information regarding their own experiences of inattention and hyperactivity-impulsivity as a child before the age of 12 either via interview or self-report questionnaire. As the mother provides the peri-natal "environment" for the child, the decision was made to focus, in part, on maternal ADHD sypmtomatology (in addition to maternal and child genotype) in examining rG-Es.

3.) Maternal Age: Mothers provided their date of birth.

4.) Season of Birth: Derived from child's date of birth.

Table 3: DICA-IV PBIQ Variables Collected by the CLDRC

Categorical & Continuous Variables			**Categorical Variables**
Smoking	Weight Loss	Medication	Emotional Problems
Drinking	Infection	Quality of Nutrition	Breech
Substance Use/Abuse	High Blood Pressure	Premature Birth	Caesarean Section
Light Bleeding	Seizures/Convulsions	Incubator Stay	**Continuous Variable**
Heavy Bleeding	Accidents	Extended Hosp. Stay	Birth Weight
Severe Nausea	Illness		

As multiple variables included here fall under the rubric of obstetric complications, we explored data reduction methods in order to minimize the number of variables in analyses and maximize power to detect significant interactions (See DATA REDUCTION AND CLEANING).

PROCEDURE

Data collection/extraction took place at the University of Colorado, the University of Denver, and the University of Nebraska Medical Center. In some cases, supplemental environmental information was provided by mail (See *ENVIRONMENTAL DATA COLLECTION*). Research protocols were approved by the IRBs at the three universities.

INITIAL RECRUITMENT AND CLDRC DATA COLLECTION

Parents provided consent for their child to participate in the behavioral portion of this study, and children provided assent. The twins and siblings completed a psychoeducational battery of cognitive tasks at the University of Colorado and the University of Denver that included measures of general cognitive ability, executive

functioning, and other neuropsychological functioning relevant to ADHD. Teachers provided measures of child classroom performance and attention. The battery was administered by doctoral students in psychology or advanced undergraduates with experience working with young children. As incentive, children received rewards of up to $20 for completing tasks and $100 following the sessions for their participation. Parents received $20 for completing questionnaires regarding their child's medical and developmental history, environmental risk factors surrounding the pregnancy, birth, and delivery of their child, and any ADHD symptomatology. Environmental variables – both shared (shared by twin pairs) and nonshared (specific to individual twins) – collection site and measure utilized, and associated sample sizes are presented in Table 4.

Table 4: Environmental Risk Factors and Associated Sample Sizes

Environmental Risk Factor	Collection Site	Type	Method of Ascertainment	N
Prenatal Smoke Exposure	DU	Shared	PBIQ	1,062
Prenatal Alcohol Exposure	DU	Shared	PBIQ	1,050
Birth Weight	DU	Nonshared	PBIQ	1,047
Obstetric Complications	DU	Both	PBIQ	707
Maternal Age	CU	Shared	Mother's birth date	1,377
Season of Birth	CU	Shared	Child's birth date	1,473
Parental Education	DU	Shared	Parent report	1,031
Family Size	CU	Shared	Parent report	1,401
TV Viewing Habits	CU	Nonshared	Parent report	1,473

GENOTYPING

Following informed consent procedures, the children and their parents also gave blood samples or, alternatively, buccal samples that underwent genetic analysis at the University of Nebraska Medical Center (UNMC). DNA extraction from blood results in the maximum quantities of high quality DNA. Ten cc's of blood were requested by anticubital venipuncture using EDTA (purple top) vacutainer tubes. If a subject was unwilling to give blood, buccal cell samples were requested using either a cytobrush or

saliva collection using Oragene kits (DNA Genotek). Buccal brushing is variable since it is particularly dependent upon the vigor with which the subject brushes the mucosa, but we have developed specific instructions asking the subjects to use 4 brushes to sample each section of the mouth for at least 30 seconds, including the "gutter region" above the gums (Saftlas, Waldschmidt, Logsden-Sckett, Triche, & Field, 2004).

DNA was extracted from whole blood or buccal brush samples using the appropriate PUREGENE DNA Isolation Kit (Gentra Systems) with minor modifications of the manufacturer's protocol. DNA extraction from Oragene samples follows the manufacturer's protocol. For both types of buccal samples, the preamplification extension procedure GenomiPhi (Amersham Biosciences) was used immediately to amplify the amount of DNA if the DNA was of good quality. DNA was checked using DNA/RNA spectrophotometric ratios. Later amplification of buccal DNA has resulted in allele-dropping, presumably due to the more rapid degradation of DNA from these samples. This procedure was utilized after testing its fidelity with DNA samples from both blood and buccal sources, and published studies have also found it to be reliable (Lovmar, Fredriksson, Liljedahl, Sigurdsson, & Syvanen, 2003). Risk alleles, methods of ascertainment and sample sizes to date are presented in Table 5.

Table 5: Risk Alleles Typed at UNMC and Associated Sample Sizes

Candidate	Polymorphism	Method of ascertainment	N
DAT1	40-bp VNTR in the 3' UTR	agarose electrophoresis	512
DRD4	48-bp VNTR in exon 3	agarose electrophoresis	509
5HTT	44-bp insertion/deletion in promoter	agarose electrophoresis	496
DRD2	TaqI site	agarose electrophoresis	303
DBH	Dinucleotide repeat 5' of transcription site	automated capillary electrophoresis	164
ADRA2C	Dinucleotide repeat 6bp from coding region	automated capillary electrophoresis	137
DRD5	Dinucleotide repeat in the 5' UTR	automated capillary electrophoresis	129

ENVIRONMENTAL DATA COLLECTION

As the PBIQ and MSRADD were introduced into the CLDRC testing battery in the year 2000 (whereas data relevant to genotype and demographic information had been collected since as early as 1996), at the time of study inception, 413 children from 203 families had previously participated in CLDRC data collection and had provided a viable blood or buccal sample, but were missing PBIQ and/or MSRADD data (pertinent to peri-natal environmental risk and retrospective maternal ADHD symptomatology). Procedures specific to the collection of this missing data are outlined below:

Families missing data were examined and excluded from recruitment if they failed to meet the following criteria: 1) The presence of a biological maternal-child relationship, 2) ADHD ratings on at least one child within the family, 3) a Full Scale IQ (FSIQ) score greater than 70 for at least one child within the family, 4) no current or past confounding medical condition that would impact cognitive functioning (e.g., seizure disorder). Following this screening, 178 families were identified as candidates for re-contact. Within this sample, families were assigned a Group Number identifying which measures they were currently missing (i.e., PBIQ, MSRADD, or both).

Families eligible for re-contact were mailed a packet that included an introductory letter describing the study, two copies of a consent form, questionnaires appropriate to their Group Number, and a pre-paid envelope for return of materials. All families were contacted by phone within one week of the initial mailing in order to further explain the intent of the study, emphasize confidentiality, and to provide the opportunity to ask questions or refuse participation. If necessary, a secondary follow-up call was made

approximately one week later. Parents were given the option to either return one copy of the consent form and the questionnaires using the provided pre-paid envelope or to provide consent and fill out the questionnaires online (www.surveymonkey.com) using an identifying number provided in the mailing. Participants were offered $10 for the completion of the questionnaires, provided in the form of a gift certificate.

Of those 178 families re-contacted in the initial mailing, 86 families were unreachable by phone or mail (e.g., telephone number was disconnected, packet was returned to sender with no forwarding address). Of the remaining 92 families, 72 consented to participate in the study and completed questionnaires (representing a 40% overall response rate, and a 78% response rate for those families we were able to contact). In total, MSRADD data was collected on an additional N = 137 mothers and PBIQ data was collected on an additional N = 178 children for inclusion in analyses.

Primary Analyses

Our analyses focused on four primary areas of study: 1) main effects of genotype on ADHD symptomatology, 2) main effects of specific bioenvironmental and psychosocial risk factors on the later manifestation of ADHD symptoms, 3) g x e interaction effects between those environmental risk factors and risk alleles substantiated by main effects, and 4) specificity of interactions to particular symptom dimensions of ADHD. In the following, I will review the rationale behind our choice of association approaches targeting main effects and discuss the logic of the chosen approaches. Then, I will discuss in detail the g x e analytic strategies that were used, addressing power, efforts to minimize comparisons, and additional exploratory and supplementary analyses.

DATA CLEANING AND REDUCTION

All subjects from the CLDRC collective databases who had environmental or genetic data were selected for analyses, creating a total overall sample 1,473 children and 480 parents. Data were restricted to those environmental and genetic variables under consideration, as well as relevant covariates (e.g., age, sex) and exclusionary measures (e.g. FSIQ, measures of composite reading). Within this sample, environmental independent variables were dichotomized (when feasible) to accommodate the calculation of odds ratios for association analyses (e.g., birth weight, which is collected as a continuous variable, was recoded in accordance with medical standards to be "Weight < 2,500 grams = Low Birth Weight", "Weight ≥ 2,500 = Normal"). However, as continuity of data provides increased power to detect main effects and interactions, variables for which data was collected continuously were also included in within-family FBAT/PBAT (see *ANALYSES* section for further details on FBAT/PBAT) and case-control regression analyses. The distributions for all environmental variables were examined for normality, and variables falling outside of acceptable ranges for skewness and kurtosis (greater than -3 and less than +3) were appropriately transformed to fall within guidelines. Data were screened for outliers, and none were found.

Dependent variables for all analyses included affections status (e.g., a "diagnosis" of ADHD or an associated subtype, designated by the OR rule), overall ADHD symptom counts, or dimensional symptoms counts (e.g. symptoms of inattention or hyperactivity-impulsivity). As the manifestation of ADHD symptoms is often thought to differ across age and gender, continuous ADHD variables were examined for such differences.

ADHD symptom counts were then age- and gender-regressed in order to account for significant correlations. This process created a new variable (comprised of the standardized residuals produced via age- and gender-regression) which was subsequently used as the dependant variable in the majority of analyses.

It has been proposed that obstetric complications are associated with ADHD symptomatology (Biederman & Faraone, 2005; Milberger, Biederman, Faraone, Guite, & Tsuang, 1997). However, such complications are often defined differently from one study to the next. Additionally, some complications are thought to be less threatening to the fetus than others (e.g., light spotting vs. viral infection). Therefore, in the hopes of minimizing comparisons, we explored data reduction methods in order to consolidate the multitude of obstetric complications into a single, more powerful composite measure of obstetric risk. The main approach in the literature dealing with minor obstetric and perinatal complications has been the use of so-called optimality indices in which a mixed bag of complications are added together to provide a composite score of optimality. To date, the most used scales are those produced by Gillberg & Gillberg (1983), Rutter and colleagues (2003), and the Groningen group (Touwen et al., 1980).

As the present study was not designed to examine solely obstetric complications, we were limited by the scope of PBIQ in the creation of an optimality index. In order to maximize the variance of our final measure of optimality, items from the PBIQ were cross-referenced with items from the Gillberg, Rutter, and Groningen scales and any PBIQ variable appearing in any of the three optimality indices was identified as a candidate variables for inclusion. Obstetric variables that have independent associations with ADHD symptomatology (e.g., birth weight and maternal age) were removed from

the index and examined separately. In accordance with the literature, remaining variables were subsequently coded as "Optimal = 1", "Not optimal = 0" and summated to form an optimality index for the PBIQ. The final index contained the following variables: vaginal bleeding (heavy), infection, illness, serious psychiatric symptomatology, drugs prescribed, labor complications, Caesarean section, prematurity, and incubator stay or other special care for the infant post-labor.

Finally, prior to all case-control g x e analyses, variables were mean-centered in order to "break the matrix" and address issues of multicollinearity. For within-family analyses, variables were entered in their raw state, and an appropriate offset was specified (see *WITHIN FAMILY ANALYSES* in *MAIN EFFECTS OF GENOTYPE* below).

(1) MAIN EFFECTS OF GENOTYPE ON ADHD SYMPTOMATOLOGY

Although the primary aim of this study is to investigate g x e interaction effects in ADHD, it is important to attempt to replicate those associations between ADHD and risk alleles that have been noted in the literature. Furthermore, it is important to minimize number of comparisons and empirically inform the selection of those alleles most likely to participate in g x e interactions. Within the animal literature, interactions typically occur in the presence of main effects. Therefore, this study sought to screen risk alleles for inclusion in g x e analyses through examination of main effects. There are two primary methods utilized in the literature to explore main effects of genotype on psychopathology: within-family and case-control. Both approaches, and their methods of implementation for the purposes of this study, are elucidated below.

WITHIN-FAMILY ANALYSES

In order to examine main effects of genotype utilizing within-family methods (preferable, as such methods are robust to certain artifacts, such as population stratification), we utilized Family-Based Association Tests (FBAT). The unified approach to family-based tests of association (Laird, Horvath, & Xu, 2000; Rabinowitz and Laird, 2000), builds on the original transmission disequilibrium test (TDT) method (Spielman, McGinnis, & Ewens, 1993) in which alleles transmitted to affected offspring are compared with the expected distribution, derived using Mendel's Law of Segregation. Similar in spirit to a classical TDT test, the approach compares the genotype distribution observed in the 'cases' to its expected distribution under the null hypothesis, the null hypothesis being "no linkage and no association" or "no association, in the presence of linkage". Put another way, genotypes of 'cases' are compared to those of their parents to explore whether a specific allele, or marker, at a locus of interest appears to be transmitted in excess of what is expected on the basis of Mendelian inheritance. Excess transmission of a particular allele from parent to a child expressing a particular phenotype (e.g., ADHD) indicates that cases are being selected for that allele, thereby providing evidence that the allele is a risk factor for disease. Since the FBAT statistic is calculated within-family, this technique avoids confounding due to admixture or population stratification (Lazzeroni and Lange, 2001; Rabinowitz and Laird, 2000).

The general "FBAT" statistic *U* (Laird et al., 2000) is based on a linear combination of offspring genotypes and traits:

$\boldsymbol{U = S - E[S],}$ where $\boldsymbol{S = \Sigma_{ij} T_{ij} X_{ij}}$

The actual test results will differ depending upon how the user specifies T_{ij} and X_{ij}, and how the distribution of X_{ij} (hence of U) is determined. For the purposes of this study, X_{ij} denotes the allele status of the j-th offspring of family i for the gene being tested (As an example, for an analysis including 100 informative families, X_{ij} for the 2nd offspring in family #50 would be designated $X_{50\ 2}$, and would denote the child's allele status for the gene under examination). In this instance, T_{ij} is the coded trait, typically specified as $Y_{ij} - u_{ij}$. Here, Y_{ij} denotes the observed trait of the j-th offspring in family i, and u_{ij} is seen as an offset value. For dichotomous traits (such as ADHD affection status), the literature (Laird et al., 2000; Whittaker and Lewis, 1998) suggests assigning u_{ij} as the disease prevalence in the general population. For more common diseases (such as ADHD), taking $0< u <1$ can increase the power of the test (Lange & Laird, 2002), and allows both affected and unaffected children to contribute to the test statistic. In this case, a conservative offset value of 0.1 was used, indicating a *population* prevalence for ADHD of approximately 10%. When using a single quantitative trait (such as age- and gender- regressed ADHD symptoms), a common approach to ascertaining T_{ij} is to mean center Y_{ij}. Here, u is simply a (weighted) sample mean of the Y_{ij}s. Thus, when examining continuous ADHD symptomatology, the offset value was specified as the sample mean.

The expected value in the expression $E(S)$ for the general FBAT statistic U is calculated under the null hypothesis of no association, conditioning on T_{ij} and on parental genotypes. Under the same null hypothesis, U is unbiased since $E[U] = 0$. Using the distribution of the offspring genotypes (treating X_{ij} as random and T_{ij} as fixed), V =

$Var(U) = Var(S)$ is calculated under the null and used to standardize U. If X_{ij} is a scalar summary of an individual's genotype, then the large sample test statistic

$$Z = U/\sqrt{V}$$

is approximately $N = (0,1)$. If X_{ij} is a vector, then

$$\chi^2 = U' V^{-} U$$

has an approximate χ^2 distribution with degrees of freedom equal to the rank of V. Thus, ultimately the FBAT statistic, within our sample, is calculated as a χ^2 of observed versus expected allele distributions, similar to TDT.

CASE-CONTROL ANALYSES

It has been asserted that case-control association studies may have particular power with disorders with high heritabilities (Risch, 2001). For these analyses, risk allele frequencies among participants with an affection status of ADHD were compared with the allele frequencies from a control sample utilizing a Chi-square test of significance. We utilized this methodology to investigate associations between risk alleles (e.g., DRD4*7R) and ADHD affection status (e.g., affected vs. not affected), as well as to calculate Odds Ratios (ORs) associated with particular risk alleles. For continuous genetic risk (having 0, 1, or 2 risk alleles), regression analyses were used to determine whether an increasing number of risk alleles predicted increasing ADHD symptomatology in a linear fashion.

(2) MAIN EFFECTS OF ENVIRONMENT ON ADHD SYMPTOMATOLOGY

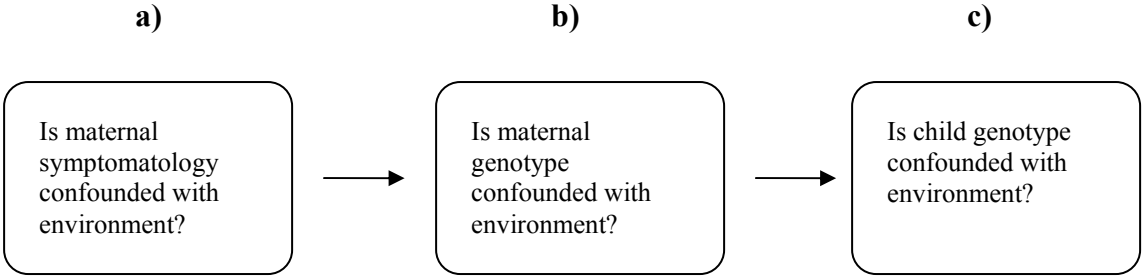
Utilizing a similar rationale to that presented in *MAIN EFFECTS OF GENOTYPE,* this study also sought to screen environmental risk factors for inclusion in g x e analyses

through examination of main effects. As it is not feasible to examine main effects of environment utilizing within-family methods (parents do not "transmit" environments to their children as they do alleles, and typically environments are generalized across children within a family, that is, most environmental variables are "shared"), Chi-square analyses were performed to investigate associations between environmental risk factors (e.g., maternal smoking) and ADHD affection status (e.g., affected vs. not affected), as well as to calculate Odds Ratios (ORs) associated with particular risk environments. For continuous environmental risk (e.g, frequency of smoking), regression analyses were used to determine whether level of environmental risk was predictive of ADHD symptomatology.

GENE-ENVIRONMENT CORRELATIONS

Prior to interpreting g x e interactions, it is important to consider that a particular gene may be confounded with environment (Rutter et al., 1997). Therefore, in order to better inform our interpretation of g x e interactions, analyses targeting potential gene – environment correlations (rG-Es) were conducted. These analyses proceeded systematically from the general to the specific, and addressed three questions (Figure 1):

Figure 1: Flow Chart for G – E Correlation Analyses



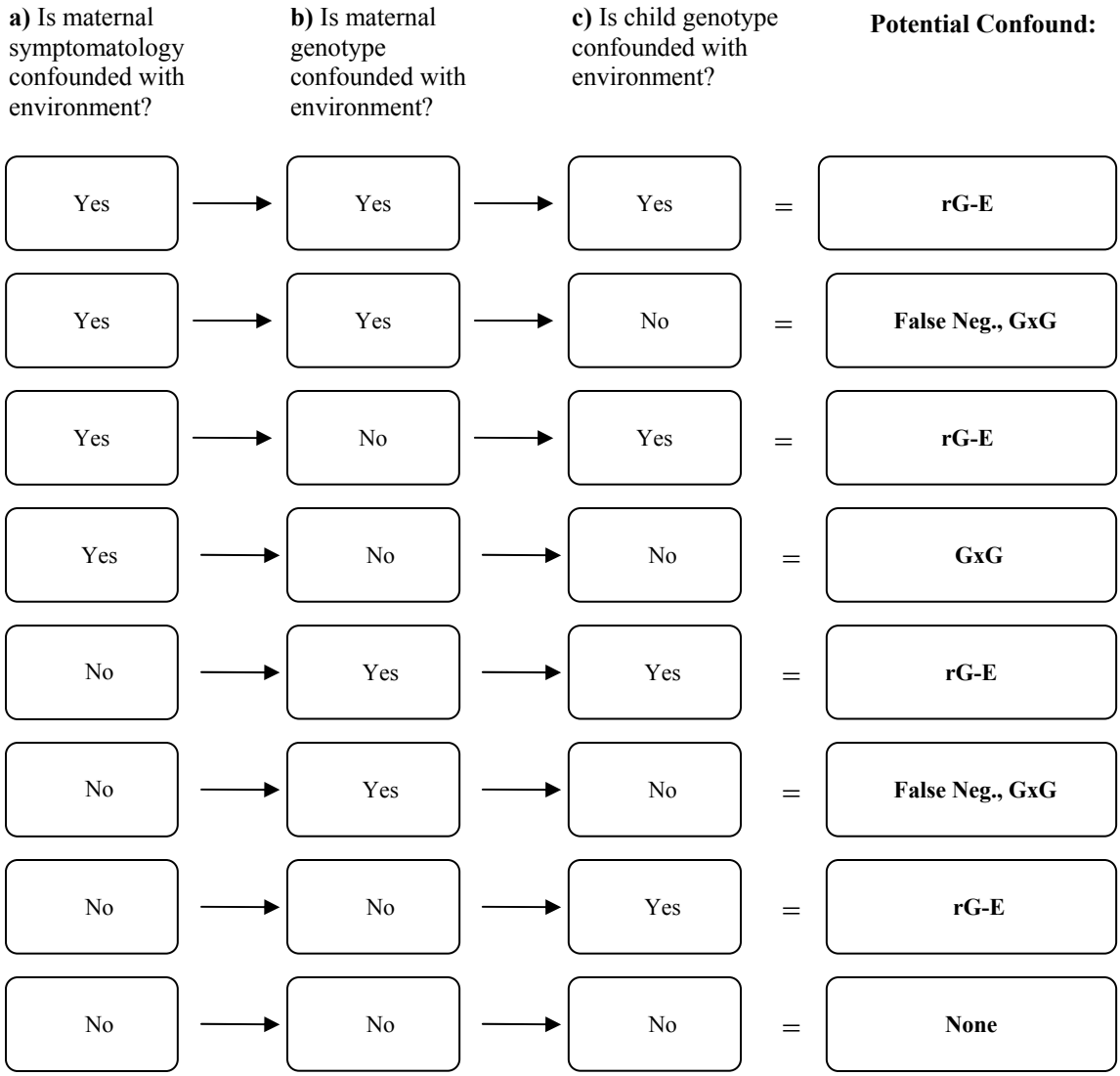
Question a) addresses whether maternal ADHD symptomatology (as a proxy for genetic risk) is correlated with environment, casting a wide net in order to examine the scope of potential rG-Es. Although these analyses give us a general idea of potential gene – environment confounds, they are insufficient for the accurate interpretation of particular g x e interactions. In order to accurately interpret possible findings, we must go further and explore whether b) maternal or c) child genotypes are confounded with environment. Thus, questions b) and c) attempt to narrow the focus of rG-E analyses by asking whether maternal risk genotype is correlated with environment, and finally whether that rG-E is present in children included in our analyses.

To address question a), we examined whether levels of environmental symptomatology differed across levels of mothers' retrospective self-report of ADHD symptomatology. For categorical environmental variables (such as smoking behavior), we employed independent t-tests in order to determine whether the severity of maternal symptomatology differed between "risk" and "no risk" environmental groups. A significant rG-E, for example, would indicate that ADHD symptomatology was substantially higher in mothers who smoked while pregnant. For continuous environmental variables, regression analyses were employed to predict mothers' retrospective self-report of ADHD symptoms with level of environmental risk. Again, if we found that environmental risk was predictive of maternal symptomatology, rG-E would be supported. To address question b), maternal genotype (no vs. any risk alleles) and environmental variables were subjected to chi-square analyses to determine whether the presence of a "risk" environment differed by allele status (in the case of TV viewing

habits, this analysis was conducted as an independent t-test). To address question c), the previous analyses were repeated substituting child for maternal genotype.

Given the stepwise methodology of this approach, there are multiple possible outcome patterns that might lead us to interpret g x e interactions differently. Therefore, it is important to address each in turn so as to provide a better context in which we may appropriately examine our g x e results. As we are targeting rG-Es through 3 distinct analyses, there are a total of 8 outcomes that could potentially emerge, each with its own potential confound (Figure 2). As is evident in this figure, in all cases where child genotype is significantly correlated with environment (denoted by "Yes" in response to question c), analyses would support the presence of rG-E. Such a finding would preclude a meaningful test of g x e interaction, as we would not be able to determine to what extent our g x e interaction results were driven by rG-E. Alternatively, analyses may demonstrate a maternal symptomatology – environment correlation (denoted by "Yes" in response to question a), and/or a maternal genotype – environment correlation (denoted by "Yes" in response to question b) in the absence of a child genotype – environment correlation. Were such a pattern to emerge, we may be concerned about potential gene x gene (g x g) interactions. In other words, it is possible that, even in absence of evidence in support of rG-Es for question c), mothers are conferring an unknown genetic risk to children that is correlated with the environment. This unknown genetic risk may be interacting with our targeted risk allele in order to exacerbate ADHD symptomatology. If maternal symptomatology (as a proxy for genotypic risk) is correlated with the environment in the absence of a specific maternal genotype – environment correlation, it

Figure 2: Possible rG-E Outcomes and Potential Confounds



might suggest either a systematic rater bias (mothers who report smoking also rate themselves more highly on retrospective maternal ADHD symptomatology – the presence of such a bias would not necessarily influence main effect and interaction analyses, as the inclusion of both parent *and* teacher ratings of a child's ADHD symptoms would protect, in part, against systematic bias), or that the positive correlation is not driven by our identified risk factors. Alternatively, given that ADHD is a

multifactorial disorder, it is possible that multiple genetic risk factors included here (as an example, dopamine genes) act synergistically to manifest a symptom – environment correlation, while failing to demonstrate a specific genotype – environment correlation. Conversely, if maternal genotype is correlated with environment in the absence of a symptom – environment correlation, or if both maternal symptomatology and genotype are correlated with environment in the absence of a child genotype – environment correlation, it may also be suggestive of false negative results for maternal symptomatology – environment or child genotype – environment analyses, respectively. Of course, were we to demonstrate negative results for questions a), b), and c), then we would be relatively confident that rG-Es were not influencing our g x e results.

(3) GENE X ENVIRONMENT INTERACTIONS

WITHIN-FAMILY ANALYSES

Our within-family approach to interaction analyses relied on a family-based association test of g x e interactions (FBAT-I, Lake and Laird, 2004), implemented in an integrated software package called PBAT (Lange, DeMeo, Silverman, Weiss, & Laird, 2004). FBAT-I uses a family trio design (examining, individually, trios comprised of two parents and one affected child), and is equivalent to the sum of the sample covariance among the affected offspring of the coded genotype and environmental exposure across parental mating types. Put another way, FBAT-I tests whether a particular risk allele and a particular risk environment are over- or under-transmitted *together* to affected children. This statistic is sensitive to a wide range of g x e interaction models, and presents advantages over other family-based methods in that it allows for continuous

environmental exposure. Additionally, FBAT-I stratifies the observed data by parental mating type, resulting in a test robust to confounding from differing subpopulation allele *and* exposure frequencies. Parental mating type is the combination of alleles at the disease locus for a given set of parents. As an example, for the 5HTT biallelic genetic polymorphism comprised of long (L) and short (s) alleles, the six parental mating types would be as follows: (LL x ss), (LL x Ls), (LL x LL), (Ls x Ls), (Ls x ss), (ss x ss).

Following the stratification of observed data, let us assume that there are I parental mating types, and F_i family trios within the i-th strata. For the affected offspring of the j-th trio within parental mating type I, $X(g_{ij})$ is the univariate coding of the g_{ij} genotype, and C_{ij} is the measure of environmental exposure. The FBAT-I test statistic T is the sum of the contributions from each parental mating type:

$$T = \Sigma_{i=1}^{I} T_i$$

Where T_i represents the contribution of the i-th parental mating type, elaborated below:

$$T_i = \sum_{j=1}^{F_i} \{ X(g_{ij}) - \bar{X}(g_i) \} \{ C_{ij} - \bar{C}_i \}$$

In this equation, $\bar{X}(g_i)$ represents the parental mating type-specific mean of the genotype coding for the affected children and $\bar{C}_i$ represents the parental mating type-specific mean for environmental exposure for the affected children (allowing for mean centering within strata). Statistical inference for FBAT-I is based on an algorithm that estimates the distribution of the test statistic under the null hypothesis. The algorithm independently permutes the residuals $X(g_{ij}) - \bar{X}(g_i)$ and $C_{ij} - \bar{C}_i$ within a given parental mating type. In the presence of g x e interactions, these residuals are correlated. The independent permutation breaks down this correlation so that computation of T on all of the possible

observed data permutations produces the distribution of the test statistic under the null hypothesis and permits calculation of exact p-values.

CASE-CONTROL ANALYSES

Our case-control approach was modeled after the methodology used by Caspi (Caspi et al., 2002; Caspi et al., 2003) to study g x e interactions in depression and conduct disorder, and utilized a modified regression framework (Aiken & West, 1990) to estimate an association between ADHD symptomatology (e.g., affection status, or age- and gender-regressed ADHD symptomatology) and (1) a specific psychosocial or bioenvironmental risk factor, (2) a particular ADHD risk allele, and (3) their interaction. The modified regression equation (with exemplar variables) is as follows:

$$ADHD = B_0 + B_1(Season\ of\ Birth) + B_2(DRD4*7R) + B_3(Season\ of\ Birth * DRD4*7R),$$

where:

B_0 is the intercept

B_1 is the regression coefficient associated with season of birth, coded in order of increasing photoperiod:

1 = Winter, 2 = Autumn, 3 = Spring, and 4 = Summer

B_2 is the regression coefficient associated with the effects of variations in the DRD4 gene, which here is coded so as to reflect the number of risk alleles, such that:

0 = No alleles, 1 = Heterozygous for 7R, 2 = Homozygous for 7R

B_3 is the coefficient associated with the interaction effect, and is the product of two variables (Season of Birth * DRD4*7R). As we primarily took a dimensional approach to analyses (so as to quell any concern about a valid "diagnosis" of ADHD), ordinary

least-squares regression (OLS) was used to evaluate the impact of genes and environment on age- and gender-regressed ADHD symptoms.

(4) SPECIFICITY OF GENE X ENVIRONMENT INTERACTIONS

The pattern of results that has emerged from previous studies examining interaction effects between an ADHD risk allele and an environmental risk factor has often demonstrated an increased risk for hyperactive-impulsive behaviors, while symptoms inattention were not affected. This suggests a possible dimensional specificity of g x e interactions in the manifestation of ADHD. As such, significant interactions were broken down into their constituent dimensions and replicated utilizing both within-family and case-control methodology.

EXPLORATORY ANALYSES

Many of the studies addressing g x e interactions conducted to date have focused on the dopaminergic system. However, the lack of convergent evidence across studies suggests that genes conferring moderate to large genetic risk may not exist. However, it is possible that several genes, acting in concert with one another, may confer substantially greater genetic risk and interact uniquely with specific environmental risk factors. As such, for each dopamine gene included in the present study (DAT1, DRD2, DRD4, DRD5, & DBH), a single risk allele was identified, taking into consideration both evidence from the literature, and (if significant) overtransmission to affected children within the present sample. The final composite score included the following risk alleles: DRD4*4R, DAT1*10R, the 5R dinucleotide repeat of DRD5, the A2 allele of DBH, and

the A2 allele of DRD2. A composite score was created by summing the number of identified risk alleles at a given locus (0, 1, or 2) for each of the 5 candidate genes impacting the dopaminergic system, yielding a final genetic risk score ranging from 0 – 10. Case-control analyses were then implemented, utilizing this score as an independent variable, in order to determine whether dopaminergic genes (as a group) interact with particular environmental risk factors.

Additionally, previous studies have also suggested a potential subtype specificity of ADHD symptomatology, such that children with particular risk alleles demonstrate increased risk for ADHD-Combined Type specifically. While dimensional analyses were pursued as part of our primary analytic approach due to their increased power to detect interactions, it is possible that crossover interactions exist such that risk alleles confer increased risk for severe ADHD (in this case, ADHD-C, which requires 6 or more symptoms of inattention and 6 or more of hyperactive-impulsive behavior) in one environment, while conferring decreased risk in another environment. Nearly all studies published to date have focused on the DRD4 and DAT1 risk alleles. Specifically, there is evidence from the literature that DAT1 may enter into interactions in the absence of main effects, and that it may exert effects on ADHD-C (or hyperactive-impulsive symptoms) specifically. Therefore, exploratory analyses were conducted targeting interactions between the identified the DAT1*9R and DAT1*10R alleles and environmental risk factors in order to determine whether there is evidence for dimensional (hyperactive-impulsive) or subtype (ADHD-C) specific interactions within the present sample.

Finally, it is important to note that, while extended family pedigrees (families including multiple sibs and/or extended family members) provide additional power to

detect omnibus effects and interactions, they allow for environmental variation which may introduce confounds into analyses. This sample, however, poses the advantage of being a twin sample, thus providing us a subset of families for which environment is (almost) perfectly controlled (as bioenvironmental and, to an extent, psychosocial risk factors are often "shared"). Were we to find significant g x e interactions in a sample of twins across whom symptomatology varied (represented by one affected and one unaffected twin) while environment was held constant, we would be more confident that our results were not driven by rG-Es of any kind. Thus, within-family analyses were replicated in a sample of discordant twin pairs and their parents in order to further refine our results.

SUPPLEMENTARY ANALYSES

Supplementary analyses were conducted as needed in order to clarify the nature of some of the results produced by our analyses. As these analyses arose on an as-needed basis, specific analytic details will be addressed in the RESULTS section.

POWER

In order to address issues of power to detect significant interaction effects, within-family and case-control analyses were evaluated for power independently. For within-family analyses, power calculations were conducted in PBAT, while for case-control analyses, power calculations specific to the interaction term had been previously published and were addressed in the context of the current sample.

RESULTS

The results of these analyses focus primarily on the four areas of interest previously described: 1) main effects of genotype on ADHD symptomatology, 2) main effects of specific bioenvironmental and psychosocial risk factors on the later manifestation of ADHD symptoms, 3) g x e interaction effects between those environmental risk factors and risk alleles substantiated by main effects, and 4) specificity of interactions to particular symptom dimensions of ADHD. Each of these areas will be addressed in turn, while exploratory and supplementary analyses, as well as power, will be discussed later in this section.

PRIMARY ANALYSES

(1) MAIN EFFECTS OF GENOTYPE ON ADHD SYMPTOMATOLOGY

WITHIN-FAMILY ANALYSES

Participants were 1,114 persons selected from the aforementioned sample (480 parents and 634 children), constituting 253 nuclear families. As FBAT automatically includes informative families (trios in which at least one parent contributes to the genotype variance in the offspring, meaning, generally, that at least one parent is heterozygous for the allele of interest) and excludes uninformative families (e.g., families missing parental or child data, trios lacking at least one heterozygote parent, etc.), further sample selection was unnecessary. Allele frequencies for all genes under consideration

were similar to those previously published (Waldman & Gizer, 2006). Multi-allelic FBAT analyses demonstrated an omnibus main effect of the DRD4 gene on overall age- and gender-regressed ADHD symptomatology (χ^2 = 11.168, p = 0.025, Table 6). This pattern of results did not change when FBAT analyses targeted affection status as the dependent variable. FBAT analyses focusing on individual alleles revealed a significant overtransmission of the DRD4 4-repeat allele (DRD4*4R) from parents to affected children (p = 0.009), and a simultaneous undertransmission of the DRD4*7R allele from parents to affected children (p = 0.020), suggesting that the DRD4*4R allele is preferentially *transmitted*, and the DRD4*7R allele is preferentially *non - transmitted* to children as ADHD symptoms increase (Table 7).

Table 6: FBAT Analyses – Main Effects of G on Overall ADHD Symptoms

Gene	χ^2	p-value (1-sided)
DAT1	1.826	0.410
DRD2	0.006	0.938
DRD4	11.168	0.025
ADRA2C	0.053	0.973
DBH	1.099	0.777
DRD5	5.496	0.240
5HTT	0.131	0.717

Table 7: FBAT Analyses – Main Effects of DRD4 Alleles on Overall ADHD Symptoms

DRD4 Allele	Allele Freq.	# Info Families	p-value (1-sided)	Direction of Transmission	Odds Ratio	95% CI
2	0.068	41	0.516	-	-	-
3	0.043	35	0.123	-	-	-
4	0.695	95	0.009	Overtransmitted	1.377	0.750 – 2.529
7	0.176	128	0.020	Undertransmittd	0.573	0.299 – 1.098

Note: DRD4 alleles with fewer than 10 informative families were automatically removed from analyses
Odds ratios are calculated for affection status, while p-values are calculated for ADHD symptoms

CASE-CONTROL ANALYSES

Participants were 656 children selected from the aforementioned sample. Following initial recruitment, the general sample was divided into "cases" and "controls." As the

blood and buccal samples were originally ascertained to accommodate within-family analyses (which focus primarily on affected children), children from a family in which at least one child was identified as having academic difficulties were preferentially genotyped. Thus, for the purposes of these analyses, "cases" were defined as children who met overall inclusion criteria, and were considered affected (i.e., children who met DSM-IV diagnostic criteria for ADHD, rated by either parent or teacher), and "controls" were defined as unaffected children (children who did not meet such criteria, but who nevertheless had been recruited because they themselves or a sibling had demonstrated academic difficulties). Within each candidate gene (including only children for whom genetic data on said candidate was available), one child from each family was randomly selected for inclusion in analyses. If a child randomly selected for inclusion in the "case" group was related biologically to a "control" subject, the "control" child was subsequently excluded (so as to remove a genetic confound from case-control analyses.) Case-control methodology was utilized to compare risk allele frequencies across ADHD (affected) and control (unaffected) groups (Table 8). In order to minimize number of comparisons, alleles were identified as "risk" if they were either 1) implicated through within-family methods, or 2) substantiated by multiple studies within the literature. Chi-square analyses demonstrated a trend towards association for the DRD4*4R allele (χ^2 = 3.278, p = 0.058, OR = 2.208) and the 5HTT Long allele (χ^2 = 2.709, p = 0.071, OR = 1.725), providing some support for a main effect of these risk alleles on a diagnosis of ADHD within this sample, and supporting within-family results for DRD4. Additionally, linear regression provided evidence that as the number of DRD4*4R alleles (0, 1 or 2) increased, age- and gender-regressed ADHD symptomatology increased as well (Adj. R

Square = 0.014, p = 0.046).

Table 8: Case-Control Analyses - Main Effects of Genotype

Allele	# Case	# Control	χ^2	p-value (1-sided)	Odds Ratio	95% CI	Adj. R Square	p-value
DRD4								
4	134	86	3.278	0.058	2.208	0.922 – 5.290	0.014	0.046
7	134	86	0.176	0.393	0.880	0.485 – 1.598	0.005	0.144
DRD2								
A1	91	43	0.006	0.554	1.033	0.461 – 2.315	-0.003	0.441
A2	91	43	0.686	0.371	0.410	0.046 – 0.362	-0.003	0.441
5HTT								
Long	132	83	2.709	0.071	1.725	0.878 – 3.315	-0.003	0.511
DAT1								
10	134	87	4.841	0.029	0.263	0.074 – 0.933	0.004	0.175
9	134	87	0.247	0.352	1.160	0.665 – 2.023	0.003	0.119
DRD5								
5	49	11	2.456	0.155	0.202	0.024 – 1.755	-0.011	0.560

In summary, within-family and case-control analyses support robust main effects of the DRD4*4R allele on ADHD symptomatology, while suggesting a potential association with the 5HTT*Long allele.

(2) MAIN EFFECTS OF ENVIRONMENT ON ADHD SYMPTOMATOLOGY

Participants were 1,473 children selected from the aforementioned sample. Random selection procedures were identical to those described in the previous section. However, as information on certain environmental variables was originally collected from all children recruited by the CLDRC (with no preference given to children demonstrating academic difficulties), we were able, for the purposes of case-control

analyses, to perform a more pure comparison of frequency of risk environments across affected and unaffected children. Therefore, for the purposes of these analyses, "cases" were defined as children who met overall inclusion criteria, and were considered affected (i.e., children who met DSM-IV diagnostic criteria for ADHD, rated by either parent or teacher), and "controls" were defined as unaffected children (children who did not meet such criteria) who were originally recruited into the control group by the CLDRC (i.e. presented with no academic difficulties), and who demonstrated no reading impairment as determined by a composite reading measure. Chi-square analyses examining the frequency of environmental risk across affected and unaffected groups (Table 9) found significant results for maternal smoking ($\chi^2 = 9.333$, $p = 0.002$, OR = 3.20), prenatal alcohol exposure ($\chi^2 = 6.087$, $p = 0.010$, OR = 2.03), spring/summer birth ($\chi^2 = 3.971$, $p = 0.029$, OR = 1.49), and parental education < 16 years ($\chi^2 = 9.587$, $p = 0.001$, OR = 2.08).

Table 9: Case-Control Analyses - Main Effects of Environment

Risk Factor	# Cases	# Controls	χ^2	p-value (1-sided)	OR	95% CI	Adj. R Square	p-value
Bioenvironmental Risk Factors								
Smoking	138	162	9.333	0.002	3.20	1.47 - 6.96	N/A	N/A
Drinking	136	161	6.087	0.010	2.03	1.15 – 3.59	N/A	N/A
Birth Weight	132	159	2.794	0.060	0.67	0.42 – 1.07	0.007	0.760
Season of Birth	176	236	3.971	0.029	1.49	1.01 – 2.21	0.026	0.001
Maternal Age	156	230	0.138	0.395	0.93	0.62 – 1.39	-0.001	0.395
Obstetric Optimality	81	117	N/A	N/A	N/A	N/A	-0.005	0.972
Psychosocial Risk Factors								
Parental Education	129	172	9.587	0.001	2.08	1.30 – 3.32	0.025	0.004
Family Size	158	232	N/A	N/A	N/A	N/A	-0.002	0.795
TV Viewing	176	234	N/A	N/A	N/A	N/A	0.007	0.045

Examined continuously, linear regression also provided support for an association between increasing photoperiod (Adj. R Square = 0.026, p = 0.001), mean years of parent education (Adj. R Square = 0.025, p = 0.004), and average hours of TV watched per week (Adj. R Square = 0.007, p = 0.045) with overall ADHD symptomatology when controlling for age and gender. It is important to note that environmental main effects yeilded higher ORs and smaller p-values than genetic main effects. However, environmental analyses were conducted in a larger sample, and posed the advantage of having a purer "control" group, and it is possible that the disparities in ORs from one analysis to the next are reflective of these sample differences.

Taken together, these data implicate maternal smoking, prenatal alcohol exposure, season of birth/increasing photoperiod, parental education, and television exposure as potential environmental risk factors in the manifestation of ADHD. However, prior to drawing any conclusions, it is important to first examine rG-Es.

GENE-ENVIRONMENT CORRELATIONS

Our rG-E analyses attempted to address 3 primary questions: a) Is maternal symptomatology confounded with environment?, b) Is maternal genotype confounded with environment?, and c) Is child genotype confounded with environment? The results of analyses targeting each question will be discussed in turn, as will the implications of these analyses for g x e interaction results.

To address question a) Is retrospective maternal ADHD symptomatology confounded with environment?, participants were those previously included in *MAIN EFFECTS OF ENVIRONMENT* analyses. The percentage of the sample for which the MSRADD (our measure of retrospective maternal symptomatology) was available varied

from 79% - 98% (Table 10). Independent t-tests indicated significantly higher MSRADD scores in children exposed in utero to smoking (t = -2.601, p = 0.014), and drinking (t = -2.041, p = 0.042). Regression analyses also demonstrated that increasing photoperiod (Adj. R Square = 0.015, p = 0.016) and hours of TV watched per week (Adj. R Square = 0.022, p = 0.004) were predictive of increasing MSRADD scores. Thus, it appears that

Table 10: Are Maternal ADHD Symptoms Confounded with Environment?
G-E Correlations Between MSRADD Scores and Environmental Risk

Risk Factor	% MSRADD	Risk Mean	Non-Risk Mean	t	p-value (2-sided)	Adj. R Square	p-value
Bioenvironmental Risk Factors							
Smoking	98	49.35	40.18	-2.601	0.014	-0.003	0.348
Drinking	98	44.12	40.39	-2.041	0.042	0.020	0.145
Season of Birth	80	41.86	39.38	-1.654	0.099	0.015	0.016
Psychosocial Risk Factors							
Parental Education	93	41.71	40.02	1.138	0.256	0.009	0.060
TV Viewing	79	N/A	N/A	N/A	N/A	0.022	0.004

for many of the environmental risk conditions for which analyses demonstrated main effects, results support rG-Es that may potentially influence g x e interaction analyses.

To address questions b) Is maternal genotype confounded with environment?, and c) Is child genotype confounded with environment?, additional rG-E analyses were conducted in the larger sample in order to maximize power to detect possible confounds (Ns are presented by analysis in Tables 11 & 12). No significant associations were found between levels of environmental risk and maternal genotype (Table 11), suggesting that the rG-Es presented in Table 10 are not driven by our identified risk factors. This disparity between maternal symptom – environment correlations and maternal genotype –

environment correlations may be reflective of a false negative, may suggest the presence of a systematic rater bias, or an alternate genetic risk factor that mothers are conferring to children, or may indicate that multiple alleles are acting synergistically to manifest a symptom – environment correlation in the absence of a genotype – environment

Table 11: Is Maternal Genotype Confounded with Environment?
G-E Correlations Between Maternal Genotype and Environmental Risk

Smoking	**N**	**χ^2**	**p-value (2-sided)**	**Drinking**	**N**	**χ^2**	**p-value (2-sided)**
DRD4*4R	168	0.076	0.782	**DRD4*4R**	167	0.365	0.546
DRD4*7R	168	0.082	0.774	**DRD4*7R**	167	0.956	0.328
5HTT Long	169	1.598	0.206	**5HTT Long**	168	0.702	0.402
DAT1*10R	171	0.403	0.525	**DAT1*10R**	170	0.690	0.406
DAT1*9R	171	0.148	0.700	**DAT1*9R**	170	0.030	0.863
Parental Education	**N**	**χ^2**	**p-value (2-sided)**	**Season of Birth**	**N**	**χ^2**	**p-value (2-sided)**
DRD4*4R	156	1.385	0.239	**DRD4*4R**	205	0.611	0.434
DRD4*7R	156	0.705	0.401	**DRD4*7R**	205	0.263	0.608
5HTT Long	160	0.101	0.750	**5HTT Long**	208	0.240	0.624
DAT1*10R	159	0.733	0.392	**DAT1*10R**	211	0.260	0.610
DAT1*9R	159	0.382	0.537	**DAT1*9R**	211	0.715	0.398
TV Viewing	**N**	**t**	**p-value (2-sided)**				
DRD4*4R	204	0.847	0.398				
DRD4*7R	204	-0.711	0.478				
5HTT Long	207	0.122	0.903				
DAT1*10R	210	0.731	0.465				
DAT1*9R	210	-1.919	0.056*				

** = Analyses reached trend or significance levels, but not in direction of risk/confound*

correlation. Interestingly, however, independent t-tests targeting whether the mean level of environmental risk differed across child genotype groups ("No risk alleles" vs. "Any risk alleles" at a particular locus) demonstrated significantly elevated levels of television watching in the DRD4*4R allele group (Table 12, t = -2.710, p = 0.007). Chi-square analyses focusing on whether level of environmental risk ("Risk group" vs. "No risk group") differed across levels of genotypic risk ("No risk alleles" vs. "Any risk alleles")

demonstrated trend-level elevated associations between the DAT1*9R allele and parental education (χ^2 = 2.740, p = 0.098). In summary, as we move into g x e interaction analyses, we must be aware that these data suggest (possible) alternate genetic risk factors that are correlated with environment, and furthermore, that any significant interactions involving DAT1*9R x Parental Education or DRD4*4R x TV Viewing Habits are confounded by rG-E.

Table 12: Is Child Genotype Confounded with Environment?
G-E Correlations Between Child Genotype and Environmental Risk

Smoking	**N**	**χ^2**	**p-value (2-sided)**	**Drinking**	**N**	**χ^2**	**p-value (2-sided)**
DRD4*4R	359	0.167	0.683	**DRD4*4R**	357	0.032	0.858
DRD4*7R	359	0.034	0.854	**DRD4*7R**	357	3.208	0.073*
5HTT Long	350	2.967	0.085*	**5HTT Long**	348	0.239	0.589
DAT1*10R	360	5.579	0.018*	**DAT1*10R**	358	4.392	0.036*
DAT1*9R	360	0.900	0.343	**DAT1*9R**	358	1.134	0.287
Parental Education	**N**	**χ^2**	**p-value (2-sided)**	**Season of Birth**	**N**	**χ^2**	**p-value (2-sided)**
DRD4*4R	348	0.171	0.679	**DRD4*4R**	446	2.325	0.127
DRD4*7R	348	0.882	0.348	**DRD4*7R**	446	1.138	0.277
5HTT Long	340	4.228	0.040*	**5HTT Long**	432	2.132	0.144
DAT1*10R	345	0.851	0.356	**DAT1*10R**	446	1.205	0.272
DAT1*9R	345	2.740	0.098	**DAT1*9R**	446	0.122	0.727
TV Viewing	**N**	**t**	**p-value (2-sided)**				
DRD4*4R	442	-2.710	0.007				
DRD4*7R	442	-0.338	0.736				
5HTT Long	428	0.007	0.995				
DAT1*10R	442	-1.248	0.213				
DAT1*9R	442	-0.726	0.468				

** = Analyses reached trend or significance levels, but not in direction of risk/confound*

(3) GENE-ENVIRONMENT INTERACTIONS

WITHIN-FAMILY ANALYSES

Participants included the 1,114 persons selected for initial within-family main effect analyses of G (comprised of 480 parents and 634 children). As analyses were

limited to trios for which genetic data on our particular risk alleles were available, the sample was ultimately constituted of 128 informative families for g x e analyses of DRD4*4R, and 149 informative families for 5HTT. G x e interaction analyses implemented in PBAT (Table 13) demonstrated significant FBAT-I values for DRD4*4R and season of birth ($p < 0.05$), as well as television viewing habits ($p = 0.046$). Additionally, FBAT-I values approached trend levels for DRD4*4R and parental education ($p = 0.102$). Heritability values (which indicate directionality of the interaction), showed a significant overtransmission of the allele + risk condition for all

Table 13: FBAT G x E Interactions Between Genetic and Environmental Risk Factors Impact on Overall ADHD Symptoms

DRD4*4R 128 Informative Families		**Categorical Analyses (e.g, High or Low Education)**		**Continuous Analyses (e.g, Years of Education)**	
		FBAT p-value	**FBAT-I p-value**	**FBAT p-value**	**FBAT-I p-value**
Environmental Risk	**Parental Education**	0.100	0.310	0.066	0.102
	Season of Birth	0.091	0.041	0.088	0.033
	Smoking	0.156	0.678	**N/A**	**N/A**
	Drinking	0.397	0.290	**N/A**	**N/A**
	TV Viewing	**N/A**	**N/A**	0.100	0.046
5HTT*Long 149 Informative Families		**Categorical Analyses (e.g, High or Low Education)**		**Continuous Analyses (e.g, Years of Education)**	
		FBAT p-value	**FBAT-I p-value**	**FBAT p-value**	**FBAT-I p-value**
Environmental Risk	**Parental Education**	0.658	0.377	0.714	0.450
	Season of Birth	0.971	0.812	0.615	0.726
	Smoking	0.826	0.674	**N/A**	**N/A**
	Drinking	0.805	0.655	**N/A**	**N/A**
	TV Viewing	**N/A**	**N/A**	0.419	0.768

significant interactions, (e.g., the combined presence of the DRD4*4R allele + spring/summer season of birth occurred more often in affected children, consistent with a diathesis-stress model). Analyses found no significant interaction effects for 5HTT.

CASE-CONTROL ANALYSES

Participants included the 656 children previously selected from the aforementioned sample for case-control main effect analyses of G. Designation of "cases" and "controls" as well as random selection procedures were identical to the aforementioned analyses, and differ only in that they were implemented by gene-environment group (e.g., random selection for DRD4*4R x parental education analyses were conducted in a subset of children for whom both the DRD4*4R allele status and parental education information were available). Results from case-control g x e interaction analyses are presented in Tables 14a & 14b. Regression analyses conducted with the identified DRD4*4R allele demonstrated a trend towards an interaction with years of parental education (Adj. R Square = 0.047, p = 0.090), and a significant interaction with maternal smoking (Adj. R Square = 0.057, p = 0.038), such that in the

Table 14a: Case-Control G x E Interactions Between Genetic and Environmental Risk Factors Impact on Overall ADHD Symptoms

DRD4*4R			Categorical Analyses (e.g, High or Low Education)			Continuous Analyses (e.g, Years of Education)		
		N	Adj. R Square	Omnibus p-value	Interaction p-value	Adj. R Square	Omnibus p-value	Interaction p-value
Environmental Risk	**Parental Education**	162	0.044	0.017	0.118	0.047	0.014	0.090
	Season of Birth	218	0.001	0.348	0.909	0.006	0.240	0.560
	Smoking	171	0.057	0.005	0.038	**N/A**	**N/A**	**N/A**
	Drinking	170	0.002	0.351	0.250	**N/A**	**N/A**	**N/A**
	TV Viewing	217	**N/A**	**N/A**	**N/A**	0.008	0.195	0.583

"risk" environment (e.g., presence of maternal smoking), ADHD symptoms increased as the number of DRD4*4R alleles increased (Figures 3 and 4), consistent with a diathesis-stress model. Analyses showed no significant interactions for 5HTT (Table 14b).

Figure 4: DRD4*4R X Maternal Smoking

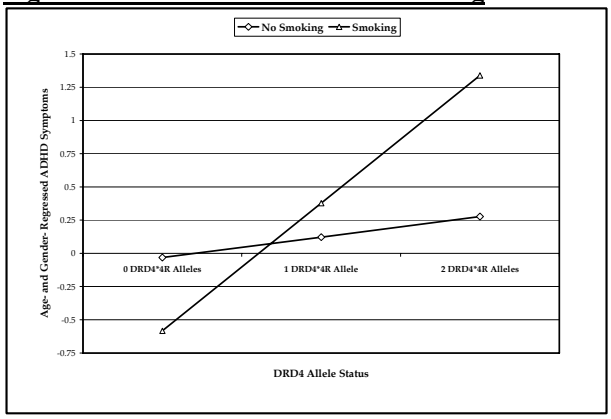
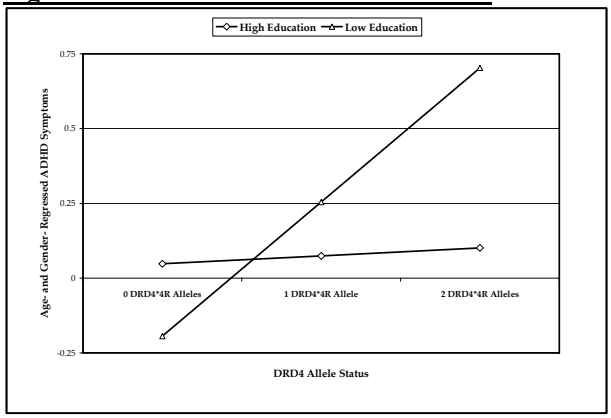


Figure 3: DRD4*4R X Parental Education



Taken together, within-family and case-control g x e interaction analyses suggest significant diathesis-stress g x e interactions between the DRD4*4R allele and season of birth, maternal smoking, and parental education (at the trend level). Although there is likewise some evidence for a potential interaction with television viewing

Table 14b: Case-Control G x E Interactions Between Genetic and Environmental Risk Factors Impact on Overall ADHD Symptoms

5HTT*Long			Categorical Analyses (e.g, High or Low Education)			Continuous Analyses (e.g, Years of Education)		
		N	Adj. R Square	Omnibus p-value	Interaction p-value	Adj. R Square	Omnibus p-value	Interaction p-value
Environmental Risk	Parental Education	163	-0.002	0.444	0.700	-0.003	0.467	0.490
	Season of Birth	216	0.001	0.352	0.912	0.032	0.019	0.083
	Smoking	171	0.001	0.377	0.144	**N/A**	**N/A**	**N/A**
	Drinking	170	0.008	0.228	0.055	**N/A**	**N/A**	**N/A**
	TV Viewing	215	**N/A**	**N/A**	**N/A**	0.005	0.248	0.366

habits, taken in the context of the previously illustrated rG-E between DRD4*4R and hours of television watched per week (as reported by parents), a meaningful test of g x e interaction is precluded by the presence of a significant rG-E in this case.

(4) DIMENSIONAL SPECIFICITY OF GENE X ENVIRONMENT INTERACTIONS

Participants were those previously included in within-family and case-control g x e analyses, respectively. Family-based g x e interaction analyses targeting symptoms of inattention demonstrated only a trend FBAT-I value for the DRD4*4R allele and season of birth ($p < 0.100$, Table 15). However, analyses targeting symptoms of hyperactivity-impulsivity demonstrated significant FBAT-I values for the DRD4*4R allele and parental education ($p = 0.050$), season of birth ($p < 0.050$), and television viewing habits ($p =$

Table 15: Dimensional Specificity of Significant FBAT G x E Interactions Impact on Inattention and Hyperactivity/Impulsivity

DRD4*4R 128 Informative Families		Categorical Analyses (e.g, High or Low Education)		Continuous Analyses (e.g, Years of Education)	
		FBAT p-value	FBAT-I p-value	FBAT p-value	FBAT-I p-value
Inattention	Parental Education	0.152	0.447	0.183	0.180
	Season of Birth	0.184	0.076	0.179	0.064
	Smoking	0.136	0.628	N/A	N/A
	Drinking	0.221	0.703	N/A	N/A
	TV Viewing	N/A	N/A	0.129	0.098
Hyperactivity-Impulsivity	Parental Education	0.097	0.145	0.046	0.050
	Season of Birth	0.059	0.041	0.055	0.027
	Smoking	0.241	0.943	N/A	N/A
	Drinking	0.168	0.059	N/A	N/A
	TV Viewing	N/A	N/A	0.089	0.028

0.028). Again, heritability values indicated an overtransmission of the allele + risk condition in the case of all significant interactions.

Case-control analyses of individual symptom dimensions showed similar patterns to within-family analyses. Regressions demonstrated no significant interactions between the DRD4*4R allele and any environmental risk factor or symptoms of inattention (Table 16). However, analyses did reveal significant interactions between DRD4*4R and parental education (p = 0.022) as well as maternal smoking, (p = 0.030) on symptoms of hyperactivity-impulsivity. In summary, these data support the notion that g x e interactions present in the manifestation of ADHD appear to preferentially exacerbate hyperactive-impulsive symptoms, although they suggest that some effects may be more diffuse across symptom dimensions (such as in the case of season of birth.)

Table 16: Dimensional Specificity of Significant Case-Control G x E Interactions Impact on Inattention and Hyperactivity/Impulsivity

DRD4*4R			Categorical Analyses (e.g, High or Low Education)			Continuous Analyses (e.g, Years of Education)		
		N	Adj. R Square	Omnibus p-value	Interaction p-value	Adj. R Square	Omnibus p-value	Interaction p-value
Inattention	Parental Educ.	162	0.025	0.071	0.229	0.025	0.073	0.326
	Season of Birth	218	0.000	0.401	0.905	0.008	0.199	0.843
	Smoking	171	0.023	0.079	0.115	N/A	N/A	N/A
	Drinking	170	-0.007	0.595	0.361	N/A	N/A	N/A
	TV Viewing	217	N/A	N/A	N/A	-0.006	0.623	0.632
Hyperactivity-Impulsivity	Parental Educ.	162	0.048	0.013	0.086	0.059	0.005	0.022
	Season of Birth	218	-0.003	0.522	0.938	0.000	0.391	0.337
	Smoking	171	0.070	0.002	0.030	N/A	N/A	N/A
	Drinking	170	0.004	0.300	0.244	N/A	N/A	N/A
	TV Viewing	215	N/A	N/A	N/A	0.023	0.049	0.633

EXPLORATORY ANALYSES

For exploratory g x e analyses of composite genetic risk, participants included 112 subjects for whom data was available on the five candidate genes under study that impact the dopaminergic system: DRD2, DRD4, DRD5, DBH, and DAT1. Subjects were screened for exclusion criteria, and one child was randomly selected from each family for inclusion in analyses. Final sample sizes, per analysis, ranged from 58 to 62 participants. Regression analyses found no significant interactions between composite genetic risk and environmental risk factors (Table 17). Analyses demonstrated a significant omnibus effect for the model including composite genetic risk and prenatal alcohol exposure, although the interaction value did not approach significance. However, it is worth noting that this study is considerably underpowered to detect such interactions (See POWER for further details), and would require the collection of additional genetic data in order to explore this important question.

Table 17: Case-Control G x E Interactions Between Composite Genetic and Environmental Risk Impact on Overall ADHD Symptoms

Composite Genetic Risk			Categorical Analyses (e.g, High or Low Education)			Continuous Analyses (e.g, Years of Education)		
		N	Adj. R Square	Omnibus p-value	Interaction p-value	Adj. R Square	Omnibus p-value	Interaction p-value
Environmental Risk	**Parental Educ.**	58	0.003	0.372	0.214	0.005	0.362	0.229
	Season of Birth	62	-0.001	0.405	0.511	-0.017	0.586	0.811
	Smoking	58	0.004	0.369	0.371	**N/A**	**N/A**	**N/A**
	Drinking	58	0.129	0.015	0.575	**N/A**	**N/A**	**N/A**
	TV Viewing	62	**N/A**	**N/A**	**N/A**	-0.021	0.632	0.491

For exploratory within-family g x e analyses of DAT1, participants included the 1,114 persons selected for initial within-family main effect analyses of G (comprised of

480 parents and 634 children). As analyses were limited to trios for which genetic data on our particular risk alleles were available, the sample was ultimately constituted of 117 informative families for g x e analyses of the DAT1*9R allele, and 119 informative families for the DAT1*10R allele. G x e interaction analyses implemented in PBAT (Table 18) demonstrated significant FBAT-I values for the DAT1*10R allele and season of birth (p = 0.044), as well as television viewing habits (p = 0.040), on hyperactive-impulsive symptomatology. Heritability values indicated that the DAT1*10R allele and

Table 18: Does DAT1 Enter Into FBAT G x E Interactions in the Absence of Main Effects? Impact on Hyperactivity/Impulsivity

DAT1*9R 117 Informative Families		**Categorical Analyses (e.g, High or Low Education)**		**Continuous Analyses (e.g, Years of Education)**	
		FBAT p-value	**FBAT-I p-value**	**FBAT p-value**	**FBAT-I p-value**
Hyperactivity-Impulsivity	**Parental Educ.**	0.500	0.267	0.650	0.353
	Season of Birth	0.181	0.065	0.177	0.095
	Smoking	0.413	0.732	**N/A**	**N/A**
	Drinking	0.446	0.455	**N/A**	**N/A**
	TV Viewing	**N/A**	**N/A**	0.145	0.059
DAT1*10R 119 Informative Families		**Categorical Analyses (e.g, High or Low Education Group)**		**Continuous Analyses (e.g, Years of Education)**	
		FBAT p-value	**FBAT-I p-value**	**FBAT p-value**	**FBAT-I p-value**
Hyperactivit-Impulsivity	**Parental Educ.**	0.380	0.212	0.584	0.328
	Season of Birth	0.130	0.044	0.144	0.069
	Smoking	0.371	0.836	**N/A**	**N/A**
	Drinking	0.354	0.431	**N/A**	**N/A**
	TV Viewing	**N/A**	**N/A**	0.068	0.040

Note: DAT1 10-repeat allele is overtransmitted for interaction effects, while the 9-repeat allele is undertransmitted.

the risk environment were preferentially overtransmitted together to children with higher hyperactive-impulsive symptom counts. Simultaneously, analyses demonstrated trends towards an undertransmission of the DAT1*9R allele + risk environment within the sample (which is to be expected, as the allele frequencies of the DAT1*9R and DAT1*10R alleles are so high that the polymorphism is nearly bi-allelic). Case-control analyses (conducted within the 656 previously selected) indicated a trend towards an interaction between the DAT1*10R allele and parental education in the manifestation of hyperactive-impulsive symptoms (Table 19, Adj. R Square = 0.003, p = 0.080). No significant interactions effects were found for the DAT1*9R allele.

Table 19: Does DAT1 Enter Into Case-Control G x E Interactions in the Absence of Main Effects? Impact on Hyperactive-Impulsive Symptoms

DAT1*9R			Categorical Analyses (e.g, High or Low Education)			Continuous Analyses (e.g, Years of Education)		
		N	Adj. R Square	Omnibus p-value	Interaction p-value	Adj. R Square	Omnibus p-value	Interaction p-value
Hyperactivity-Impulsivity	Parental Educ.	163	-0.001	0.409	0.133	-0.006	0.569	0.293
	Season of Birth	219	-0.008	0.761	0.712	-0.007	0.693	0.954
	Smoking	172	-0.012	0.802	0.926	N/A	N/A	N/A
	Drinking	171	-0.003	0.478	0.312	N/A	N/A	N/A
	TV Viewing	218	N/A	N/A	N/A	-0.012	0.950	0.942
DAT1*10R			**Categorical Analyses (e.g, High or Low Education)**			**Continuous Analyses (e.g, Years of Education)**		
		N	**Adj. R Square**	**Omnibus p-value**	**Interaction p-value**	**Adj. R Square**	**Omnibus p-value**	**Interaction p-value**
Hyperactivity-Impulsivity	Parental Educ.	163	0.003	0.320	0.081	0.001	0.372	0.123
	Season of Birth	219	-0.005	0.591	0.556	-0.004	0.562	0.860
	Smoking	172	-0.009	0.691	0.987	N/A	N/A	N/A
	Drinking	171	-0.001	0.410	0.336	N/A	N/A	N/A
	TV Viewing	218	N/A	N/A	N/A	-0.010	0.817	0.486

Additionally, interactions between the DAT1*10R allele and environmental risk factors were examined for their possible selective impact on the ADHD-C subtype. Analyses demonstrated significant FBAT-I values for the DAT1*10R allele and season of birth (p = 0.004), as well as parental education (p = 0.011) specific to the ADHD-C subtype (Table 20). Results also showed a trend towards a g x e interaction for the DAT1*10R allele and parental education (p = 0.083) for the ADHD-I subtype, indicating that interactions with that risk environment may have more diffuse effects on ADHD symptoms.

Table 20: Are DAT1 Interactions Subtype-Specific? Impact on ADHD-C Affection Status

DAT1*10R 119 Info Families		Categorical Analyses (e.g, High or Low Education)	
		FBAT p-value	FBAT-I p-value
ADHD-HI	Parental Educ.	0.670	0.443
	Season of Birth	0.914	0.772
	Smoking	0.513	0.406
	Drinking	0.208	0.142
	TV Viewing	N/A	N/A
ADHD-CO	Parental Educ.	0.027	0.011
	Season of Birth	0.001	0.004
	Smoking	0.024	0.235
	Drinking	0.009	0.109
	TV Viewing	N/A	N/A
ADHD-I	Parental Educ.	0.206	0.083
	Season of Birth	0.291	0.138
	Smoking	0.136	0.065
	Drinking	0.151	0.056
	TV Viewing	N/A	N/A

Given the findings that emerged from these analyses (that DAT1*10R appears to enter into interactions which selectively impact hyperactive-impulsive behavior and / or ADHD-C status), we decided to backtrack and examine main effects of DAT1*10R on symptom dimensions and diagnostic subtypes. Utilizing DRD4*4R as a basis for comparison (given its robust main effects),

FBAT analyses were conducted to determine omnibus and allele-specific effects of DAT1*10R on ADHD-C affection status and hyperactive-impulsive symptomatology. Analyses demonstrated a subtype-specific main effect of DAT1 (Table 21, $\chi^2 = 6.224$, p = 0.044), such that the DAT1*10R allele was preferentially overtransmitted to ADHD-C children ($p < 0.001$). Overtransmission of DAT1*10R for hyperactive-impulsive symptoms was at the trend level (p = 0.073).

Table 21: Omnibus Effects of DAT1 and DRD4 on ADHD-C and Hyperactivity-Impulsivity

	ADHD-C Affection Status		Hyperactivity-Impulsivity	
Gene	χ^2	**p-value (1-sided)**	χ^2	**p-value (1-sided)**
DAT1	6.224	0.044	2.853	0.240
DRD4	9.324	0.054	14.196	0.007

While examining the results of the g x e analyses targeting DAT1*10R, it was noted that the undertransmission of DAT1*10R + smoking or drinking to ADHD-I children (Table 20) may be suggestive of an overtransmission of DAT1*9R to children in those risk environments. When examined more closely, indeed this pattern did emerge (Smoking FBAT-I p-value = 0.094, Drinking FBAT-I value = 0.046). However, main effect analysis targeting impact of DAT1*9R on ADHD-I affection status and inattentive symptoms returned no significant results. Given the nearly bi-allelic nature of the DAT1 polymorphism, coupled with the significant undertransmission of DAT1*9R to ADHD children generally, this pattern may merely be a reflection of the undertransmission of DAT1*10R to ADHD-I children. Thus, these results must be interpreted with caution.

Finally, within-family analyses were run within a subset of discordant twin pairs and their parents in order to determine whether g x e interactions were upheld in an

environment in which there was genetic and symptomatic variation, while environment was (at least partially) controlled. Across all significant interactions, the pattern of transmission did not change. Analyses demonstrated a trend-level interaction for DRD4*4R allele and spring/summer season of birth ($p = 0.066$), as well as TV viewing habits ($p = 0.054$), in the manifestation of overall ADHD symptoms. Additionally, analyses demonstrated a significant interaction with length of photoperiod ($p = 0.039$). Interactions with parental education, and interactions that impacted the manifestation of hyperactivity-impulsivity or ADHD-C were not supported by these analyses. However, it is important to note that these analyses were substantially underpowered to detect omnibus and interaction effects (See *POWER*).

SUPPLEMENTARY ANALYSES

It is worth noting that, although the initial screening for environmental risk factors was conducted in the largest possible sample, main effects of environment were also performed by genotype for those subjects included in case-control g x e analyses to determine whether main effects were upheld in the smaller samples. Among those children included in DRD4 analyses, main effects for parental education (Adj. R Square = 0.021, $p = 0.038$), and smoking ($\chi^2 = 3.340$, $p = 0.061$) were supported, while main effects of season of birth approached trend levels ($\chi^2 = 1.599$, $p = 0.131$). Similarly, in the 5HTT sample, a main effect of length of photoperiod (Adj. R Square = 0.016, $p = 0.034$) was supported, while a main effect of parental education approached trend levels ($\chi^2 = 1.940$, $p = 0.109$). No main effects were supported within the DAT1 sample. Implications of these results will be addressed further in the DISCUSSION section.

Additionally, it is important to keep in mind that some environmental variables were obtained nearly a decade after original CLDRC data collection, and the method of ascertainment differed (self-report questionnaire as opposed to clinical interview). As such, it is possible that the frequency with which environmental risk factors are endorsed may differ across groups and may thereby impact g x e analyses. As such, levels of environmental risk were compared across participants whose environmental data was recently obtained, but whose genetic data was collected prior to 2000 (the *ENVIRONMENTAL DATA COLLECTION* sample) and those who provided environmental and genetic risk data at the time of original CLDRC data collection. Analyses demonstrated significantly elevated levels of reported maternal smoking ($\chi^2 = 8.807$, $p = 0.003$, OR = 2.108) in the environmental data collection sample (for which peri-natal environment data was collected by mail-in self-report questionnaire), as opposed to the original sample. These data suggest that either social desirability, or some cohort effect, may be at play in this sample. For example, mothers may be less inclined or report smoking behavior in the context of a clinical interview, or, since genetic and neuropsychological data on the environmental data collection sample was obtained before 2000 (when the PBIQ and MSRADD were first introduced into the battery), public health education on the dangers of maternal smoking may have increased. To address this disparity across samples, case-control g x e interactions were conducted in the environmental data collection sample independently for maternal smoking in order to determine whether the increased levels of reported maternal smoking would influence our results. Analyses returned no significant g x e interactions; however, sample sizes were very small (Ns

ranging from 36 to 37), and thus analyses were substantially underpowered to detect such interactions (see *POWER*).

POWER

Power analyses were addressed for case-control and within-family interactions separately. For within family analyses, power calculations implemented in PBAT for the continuous trait of age- and gender-regressed ADHD symptom counts demonstrated sufficient power to detect omnibus effects for all primary analyses (Table 22).

Table 22: PBAT Within-Family Power Calculations for Primary G x E Analyses

Risk Allele	Number of Informative Families	Allele Frequency	Power
DRD4*4R	128	0.695	0.938 – 0.939
DRD4*7R	95	0.176	0.806 – 0.836
5HTT Long	149	0.551	0.961 – 0.963
DAT1*10R	119	0.746	0.915 – 0.920
DAT1*9R	117	0.244	0.907 – 0.916

Although power cannot be calculated for the interaction term specifically (FBAT-I), FBAT analyses that include an interaction term produce omnibus values that incorporate both main effects and interactions. Therefore we have confidence that these analyses had sufficient power to detect within-family g x e interactions for our sample sizes and associated allele frequencies.

As all significant results were replicated in a subset of discordant twin pairs and their parents in order to investigate whether results were upheld under greater environmental control, power analyses were conducted within this sub-sample. The results are presented in Table 23. Power calculations indicated insufficient power to

detect omnibus and interaction effects for these exploratory analyses; thus, any failure of these analyses to support primary g x e analyses may be due to lack of power.

Table 23: PBAT Within-Family Power Calculations for Exploratory G x E Analyses

Risk Allele	Number of Informative Families	Allele Frequency	Power
DRD4*4R	37	0.702	0.453 – 0.478
DRD4*7R	31	0.188	0.349 – 0.389
5HTT Long	52	0.540	0.624 – 0.626
DAT1*10R	40	0.755	0.491 – 0.507
DAT1*9R	40	0.234	0.487 – 0.506

For case-control analyses, sample sizes required to test for the interaction term in the modified regression equation we utilized have been previously published: N = 143 for a small effect size (.06) at α= .05 (Aiken &West, 1991). As sample sizes for primary case-control g x e interaction analyses ranged from N = 162 to N = 216, the current sample had sufficient power to detect a significant interaction for all non-exploratory case-control analyses. However, it is important to note that one set of exploratory analyses addressing potential interactions between composite genetic risk and environmental risk factors (having sample sizes ranging from N = 58 to N = 62), and one set of supplementary analyses focusing on environmental data collection participants (having sample sizes ranging from N = 36 to N = 37) were significantly underpowered to detect such interactions. Thus the lack of positive results produced by these analyses may be due to lack of power.

DISCUSSION

The primary goal of this study was to test a diathesis-stress model of gene x environment (g x e) interactions in the etiology of Attention Deficit Hyperactivity Disorder (ADHD). To this end, this study sought to: (1) to explore the main effects of genotype on ADHD symptomatology by conducting an association study between ADHD and polymorphisms in the DRD2, DRD4, DRD5, DAT1, 5HTT, ADRA2C and DBH genes within a community sample, (2) to explore the main effects of specific bioenvironmental and psychosocial risk factors on the later manifestation of ADHD symptoms, (3) to test for g x e interaction effects between those environmental risk factors and risk alleles substantiated by main effects, and (4) to investigate whether these results were specific to particular DSM-IV symptom dimensions of ADHD.

OVERALL SUMMARY OF FINDINGS

In exploring main effects of genotype on the manifestation of ADHD symptoms, within-family and case-control analyses supported robust main effects of the DRD4*4R allele on ADHD symptomatology, while suggesting a potential association with the 5HTT*Long allele. Case-control analyses of environmental risk implicated maternal smoking, prenatal alcohol exposure, season of birth, increasing photoperiod, parental education, and television viewing habits as environmental risk factors at play in the manifestation of ADHD. However, rG-E analyses demonstrating significant associations

between MSRADD scores and maternal smoking, prenatal alcohol exposure, increasing photoperiod, and television viewing habits suggested possible confounds. Coupled with more fine-grained rG-E analyses demonstrating no evident correlations between maternal genotype and environmental risk factors, these data were suggestive of potential g x g interactions. Furthermore, rG-E analyses targeting child genotype and environment indicated that any meaningful test of g x e interactions involving DAT1*9R x parental education or DRD4*4R x television viewing would be precluded by the presence significant DAT1*9R-parental education and DRD4*4R-television viewing correlations. Taken in the context of rG-Es, therefore, within-family and case-control g x e interaction analyses supported significant interactions between the DRD4*4R allele and season of birth/increasing photoperiod, maternal smoking, and parental education (at the trend level). These g x e interactions appear to preferentially exacerbate hyperactive-impulsive symptoms, although they suggest that some effects may be more diffuse across symptom dimensions (such as in the case of season of birth.)

Exploratory analyses of g x e interactions of composite genetic risk and environmental risk factors revealed no significant findings; however, the sample was constrained by the scarcity of genotype information on some dopaminergic genes, and power analyses indicated that the sample was substantially underpowered to detect such interactions. Analyses of DAT1 revealed main effects of the DAT1*10R allele on the ADHD-C subtype (suggesting a possible subtype-specific etiology of ADHD-C) and, to a lesser extent, hyperactive-impulsive symptomatology. Furthermore, significant g x e interactions were demonstrated between DAT1*10R and season of birth, as well as television viewing habits, on the manifestation of hyperactive-impulsive symptoms.

Additional g x e interactions were revealed between DAT1*10R and parental education, as well as season of birth, on the manifestation of ADHD-C. Finally, g x e interaction analyses conducted within a sub-sample of discordant twin pairs and their parents supported trend-level g x e interactions between DRD4*4R and season of birth, length of photoperiod, and television viewing habits. These data support the validity of the DRD4*4R x season of birth interaction, and further suggest that television viewing habits (a nonshared environment) differ across affected and unaffected children via either an active or evocative correlation. For example, an affected child may evoke a response from his or her environment (e.g., an inattentive or hyperactive-impulsive child may prompt a parent to put on television programs that entertain and occupy them, or, conversely, an affected child may actively seek out visual stimulation (e.g., television) more frequently that an unaffected co-twin). This nonshared environment, coupled with a main effect of DRD4*4R in affected children, might lead to a spurious finding of a g x e interaction, even in a sub-sample of discordant twin pairs. While no other g x e interaction effects were supported within this sample, it is important to note that these analyses were found to be underpowered to detect such omnibus effects and interactions.

We believe that these results, taken together, are valid and provide numerous interesting insights into the etiology of ADHD. Furthermore, we believe that this study possesses a number of advantages over others of its kind, in that it rigorously pursued a methodological approach to g x e interactions, substantiating candidate risk factors with main effects in order to minimize number of comparisons while exploring a wide array of genes and environments, and investigated the specificity of g x e interactions to specific symptom dimensions (and, when appropriate, subtypes). However, given that these

finding are best interpreted in the context of more recent literature pertinent to DRD4, DAT1, and their interactions, for now we will turn to recent g x e publications, and further discussion of these results will occur in the *INTERPRETATION OF FINDINGS* section.

RECENT LITERATURE

Over the course of the past year, there have been a number of studies published focusing on g x e interactions in ADHD and associated disorders (e.g., Oppositional Defiant Disorder (ODD) and Conduct Disorder (CD)). Several of these studies have addressed novel genetic and/or environmental risk factors that were not ascertained for the purposes of this study, and are presented in Table 24. As these studies are outside of the scope of our investigation, they will not be a focus of discussion. However, several additional studies have targeted those genotypes and environmental risk factors included in this study, and will be addressed in more detail. The results of these investigations are presented in Table 25.

Table 24: G x E Interaction Studies Outside the Scope of the Present Study

		Genetic Risk				
	Risk	**CHRNA4*rs1044396**	**DAT1*10R**	**BDNF (3 SNPs)**	**DRD2*A2**	**5HTT*Short**
Environmental Risk	**Smoke Exposure**	Todd & Neuman (2007) OR = 3.0, (95% CI 1.2-13.1) ADHD-C				
	Psychosocial Adversity		Laucht et al., (2007) p = 0.013 – 0.017 HI Symptoms			Retz et al., (2008) P < 0.001 Dx of ADHD
	SES (Hollingshead)			Lasky-Su et al., (2007) P = 0.009 – 0.012 Inattentive Symptoms		
	Marital Status (Mother)				Waldman (2007) P = 0.009 Dx of ADHD	

Table 25: The Current State of G x E Interaction Literature Regarding ADHD

		Genetic Risk					
	Risk	**DRD4*7R**	**DAT1*10R**	**DAT1*9R**	**DAT1 Haplotype**	**DRD5*5R**	**5HTT*Long**
Environmental Risk	**Smoke Exposure**	Neuman et al., (2007) p = 0.0003 ADHD Symptoms Langley, et al. (2008) Dx of ADHD	Kahn et al., (2003) p = 0.01; HI p = 0.001; Opp Becker et al., (2006) P = 0.012; HI (males only) Langley, et al. (2008) Dx of ADHD P = 0.03, CD	Neuman et al., (2007) p = 0.001 ADHD Symptoms		Langley, et al. (2008) Dx of ADHD P = 0.002, ODD	Langley, et al. (2008) Dx of ADHD
	Alcohol Exposure	Langley, et al. (2008) Dx of ADHD	Langley, et al. (2008) Dx of ADHD		Brookes et al., (2006) p = 0.04 ADHD Symptoms	Langley, et al. (2008) Dx of ADHD	Langley, et al. (2008) Dx of ADHD
	Season of Birth	Seeger et al., (2004) p = 0.013 HD + CD Brookes et al., (2004) ADHD Symptoms					
	Birth Weight	Langley, et al. (2008) Dx of ADHD	Langley, et al. (2008) Dx of ADHD			Langley, et al. (2008) Dx of ADHD P = 0.004, ODD	Langley, et al. (2008) Dx of ADHD

Note: Studies highlighted in gray denote negative findings

A number of different patterns emerge when considering these g x e investigations concurrently. Firstly, as is evident from Table 25, g x e interaction studies targeting common risk alleles (most of which are involved in the dopaminergic system) have produced largely inconsistent findings, with negative results emerging as often as positive ones. Secondly, all of the g x e interaction studies to date that have included DRD4 as a candidate gene have focused on the DRD4*7R allele as opposed to the

DRD4*4R allele. Thirdly, although the literature has demonstrated some evidence that both the DAT1*10R and the DAT1*9R alleles enter into interactions, the DAT1*10R allele has been studied more frequently, and appears to selectively exacerbate hyperactive-impulsive symptoms. Each of these patterns will be discussed in turn.

Inconsistent findings across the literature may be indicative of a number of different phenomena. First, as there is often a publication bias towards positive results, it is possible that initial publication favors novel associations and interactions (which may be spurious), while replication studies (which are often published in reaction to novel results) often to reveal negative findings. This hypothesis is supported by the pattern of results demonstrated in Tables 24 and 25. Novel genetic associations and/or interactions (Table 24) are entirely positive in nature, while those studies published over the course of the past year targeting established associations have largely failed to replicate (non-significant findings highlighted in gray in Table 25). Secondly, the vast majority of these studies revealed interactions in the absence of main effects of gene and/or environment, a phenomenon which is very uncommon in the animal literature (e.g., Crabbe, Walston, & Dudek, 1999; Valdar, 2006). While it is possible that, in the manifestation of ADHD, some interactions are "crossover" in nature or not substantial enough to support main effects, this disparity from animal to human literature, coupled with the specificity of some results (e.g., g x e interactions targeting hyperactive-impulsive symptoms in males only) has led some to speculate that positive findings are the result of statistical "fishing expeditions". Furthermore, studies often do not report number of comparisons or seek to minimize comparisons in order to minimize Type I error. And finally, inconsistent g x e findings may be indicative of inaccurate genetic associations. As an example,

inconsistent findings across g x e studies for a particular allele may indicate that the targeted allele is not the true "risk" allele, rather is in linkage disequilibrium with a true "risk" allele nearby on the chromosome.

To date, all published g x e interaction studies that have included DRD4 as a candidate gene have focused on the DRD4*7R allele. DRD4 is a dopamine receptor gene which contains 2 (2R) to 11 (11R) variable number tandem repeats (VNTRs) of a 48bp sequence in exon 3. It has been reported that the 7R allele differs (albeit slightly) from the 2R and 4R variants of the gene, exhibiting blunted ability to reduce cAMP, which mediates gene expression and neurotransmitter biosynthesis. (Asghari et al., 1995). DRD4*7R also displays substantially more linkage disequilibrium to adjacent polymorphisms than DRD4*4R, suggesting it arose more recently. Initial studies suggested that the DRD4*7R allele was associated with novelty seeking (Ebstein et al., 1996) and ADHD (LaHoste et al., 1996). Since these initial reports, DRD4*7R has been extensively studied, with some investigations replicating an association, and others failing to do so. A meta-analysis conducted by Faraone and colleagues (2001) concluded that the association between DRD4*7R and ADHD was real, albeit small (ORs 1.4 – 1.9). Since the publication of this meta-analysis, two additional studies have been published examining DRD4*7R. The first reported an increased prevalence of the allele in a clinical sample compared to a control group (Roman et al., 2001), while the latter reported a significant preferential *non-transmission* of the DRD4*7R allele to affected children, and a significant preferential *transmission* of the "short" alleles (2R – 5R) to such children (Manor et al., 2002).

Concerning g x e interaction studies (Table 25), interaction effects have been largely inconsistent for DRD4*7R. Although two studies have suggested that DRD4*7R may enter into g x e interactions with maternal smoking and season of birth, Brookes and colleagues (2004) reported a preferential transmission of a "short" allele (DRD4*2R) to those born in spring and summer (this interaction did not surpass significance, although the difference in transmission between the two seasons was nominally significant). Taken together, these data suggest that DRD4*7R is more commonly associated with ADHD than other allelic variants of the gene (although positive findings have also been reported for "short" alleles). However, its role in interactions is less clear. These data pose somewhat of an intriguing paradox when considered in the context of our results, and will be addressed further in the *INTERPRETATION OF FINDINGS* section.

DAT1 is a dopamine transporter gene which expresses as a solute carrier protein responsible for reuptake of dopamine from the synaptic cleft back into the presynaptic neuron. Evidence from animal and human studies nominated DAT1 as a potential candidate for association with ADHD (e.g., "knockout" mice exhibiting more motor activity, methylphenidate inhibits the function of the dopamine transporter, etc.) and association studies (which have likewise been inconsistently replicated) have tenuously supported an association between DAT1*10R and ADHD (Waldman & Gizer, 2006). Although some evidence has suggested that both the 10R and 9R alleles of DAT1 enter into interactions with maternal smoking and prenatal alcohol exposure, the majority of g x e investigations have focused on DAT1*10R, for which findings have been largely inconsistent. However, it appears that positive findings have revealed interactions in the absence of main effects, and have demonstrated a selective impact of DAT1*10R x

maternal smoking on hyperactive-impulsive symptoms. These findings are largely consistent with those presented here, and will be discussed further in the next section.

Finally, given the nature of our results, it is important to supply some context as regards the season of birth association. Typical biological arguments regarding the potential association between season of birth and psychopathology have taken multiple forms. For example, season of birth may be a proxy for risk factors such as viral infections or amount of daylight exposure during gestation. Those born in spring and summer spend most of their gestation in fall and winter, seasons characterized by increased viral infections that may exert influence on the fetus. Maternal disorders, such as seasonal affective disorder, show seasonal variation and may confer prenatal risk. Additionally, it has been suggested that hours of daylight (i.e. length of photoperiod) could impact the dopaminergic system (Naber et al., 1981) through the increased synthesis of melatonin, which is known to inhibit dopamine release in numerous brain regions. Furthermore, dopamine is thought to inhibit the production of melatonin via DRD4 (e.g., Zisapel, 2001).

INTERPRETATION OF FINDINGS

This study represented a methodologically rigorous approach to g x e interaction analyses, and produced several primary findings that regard two candidate genes of interest: DRD4 and DAT1. Each will be discussed in turn.

DRD4

The literature has largely targeted the DRD4*7R allele as the "risk" allele in ADHD. This presents somewhat of an intriguing paradox when considered in the context

of our results, as our analyses revealed a significant association between the DRD4*4R allele and ADHD symptomatology, a finding that was robust across within-family and case-control analyses. Additionally, within our sample, the DRD4*4R allele appeared to enter into interactions with several substantiated environmental risk factors, namely season of birth, maternal smoking, and parental education (at the trend level), and selectively exacerbated hyperactive-impulsive symptomatology. Although these findings seem to contradict much of the literature, it is important to note that there has been some inconsistency as regards a genetic association between DRD4*7R and ADHD, and researchers have had even more difficulty replicating g x e interactions between DRD4*7R and proposed environmental risk factors. Taken together with studies demonstrating that, contrary to other findings, DRD4 "short" alleles confer risk for ADHD (Manor et al., 2002), and that these alleles may also enter into interactions with environmental risk factors previously shown to interact with DRD4*7R (Brookes et al., 2004; present study), the story behind the role of DRD4 in the manifestation of ADHD may be more complex than the literature has led us to believe.

Such contrary findings may suggest that it is neither the DRD4*7R nor the DRD4*4R allele that is the true "risk" allele in this case, rather that both of these alleles are independently in linkage disequilibrium with an alternate genetic risk factor (as an examplar hypothesis, a polymorphism in the regulatory region that influences DRD4 gene expression). As DRD4*7R also displays substantially more linkage disequilibrium to adjacent polymorphisms than DRD4*4R, it follows logically that DRD4*7R would find itself linked to such a polymorphism more frequently than DRD4*4R, and thus would demonstrate association with ADHD more commonly. Such an alternate risk

factor may also help to explain some of our rG-E results, which demonstrated strong associations between retrospective maternal ADHD symptomatology and environmental risk factors, while demonstrating no significant associations between specific maternal genotype and environmental risk. Given the high heritability of ADHD symptoms, such a disparity may be suggestive of an alternate source of genetic risk that is correlated with environment. In summary, our findings suggest that it is possible that the role of DRD4 in the manifestation of ADHD is a complex one, and it may be that an alternate polymorphism, to which DRD4*4R and DRD4*7R are "linked", is the true "risk" allele in the case of this gene.

DAT1

The literature has also demonstrated that, while association and g x e interaction analyses of DAT1 have produced inconsistent results, studies have suggested interactions in the absence of main effects, and have demonstrated a selective impact of DAT1*10R x maternal smoking on hyperactive-impulsive symptoms and/or increased risk for ADHD-C. Main effect analyses of DAT1*10R on ADHD-C and, to a lesser extent, hyperactive-impulsive symptoms, revealed a positive association. Exploratory analyses of DAT1*10R revealed significant interactions with season of birth and television viewing habits on hyperactive-impulsive symptoms, and further demonstrated significant interactions with season of birth and parental education on a subtype classification of ADHD-C. These data suggest that DAT1*10R may play into a subtype-specific etiology such that DAT1 interacts with environmental risk factors in order to manifest a severe form of ADHD (ADHD-C), while the effects of DRD4 may be more widespread across the hyperactive-impulsive and (to a small degree) inattentive symptom distributions. Given that

DAT1*10R appears to selectively exacerbate hyperactive-impulsive symptoms, as well as increase risk for ADHD-C (wherein children manifest 6 or more symptoms of hyperactive-impulsive behavior *and* 6 or more symptoms of inattention), but does *not* appear to confer risk for inattention, it is possible that the genetic underpinnings of inattention in the context of hyperactivity-impulsivity differs from inattention by itself. It is worth noting that a recent study by Lasky-Su and colleagues (2007, Table 24) utilizing within-family FBAT methods demonstrated a g x e interaction between genes influencing brain derived neurotrophic factor (BDNF) and socioeconomic status (SES) on the manifestation of inattention symptoms specifically. It is also notable that DAT1 entered into interactions with many of the same environmental risk factors as DRD4. While our analyses were underpowered to properly examine cumulative dopaminergic (genetic) risk, these data suggest that season of birth and parental education may specifically interact with polymorphisms in genes influencing the dopaminergic system in order to manifest hyperactive-impulsive symptoms and/or confer increased risk for ADHD-C.

CONCLUSIONS

Overall, this study supports a number of conclusions. Firstly, these analyses support the notion that, when g x e interactions occur in the manifestation of ADHD and hyperactive-impulsive symptoms, they tend to be diathesis-stress in nature. In the case of all significant interactions revealed by this study, the influence of a genetic risk factor on the manifestation of ADHD was enhanced in a risk environment. Secondly, these results suggest that the role of DRD4 in the etiology of ADHD is a complex one, and that studies focusing exclusively on the DRD4*7R allele may be doing so hastily. While it appears

that the DRD4 gene is interacting with specific environmental risk factors in order to increase hyperactive-impulsive symptomatology in this sample, these data, coupled with inconsistent g x e results for DRD4*7R in the literature, suggest that an alternate polymorphism of DRD4 in linkage disequilibrium with the 4R and 7R alleles may be conferring true genetic risk, in this case. Thirdly, these data support a possible subtype-specific role of the DAT1*10R allele in the etiology of ADHD-C, suggesting that either DAT1*10R confers risk for a severe form of ADHD, or that inattention in the context of hyperactive-impulsive symptomatology differs in etiology from standalone inattentive symptoms. Finally, these data suggest that dopaminergic genes may (on the whole) interact with season of birth, parental education and (in the case of DRD4*4R) maternal smoking to selectively exacerbate hyperactive-impulsive symptomatology.

As regards the final conclusion, selective exacerbation of hyperactive-impulsive symptomatology may seem to pose somewhat of a mystery when taken in the context of literature regarding the heritability of ADHD. While there is evidence in the literature that inattention is hightly heritable regardless of associated hyperactive-impulsive symptomatology, studies have suggested that the heritability of the hyperactive-impulsive symptom dimension disappears once the correlation between the two symptom dimensions of ADHD is accounted for (Willcutt, 2008; Willcutt, Pennington, & DeFries, 2000). If inattention is the dimension driving the heritability of ADHD, one would not necessarily expect g x e interactions to selectively exacerbate hyperactive-impulsive symptomatology. However, it seems plausible that inattention is heritable in the absence of environmental risk; that is, that the genetic underpinnings of inattention have yet to be fully elucidated, while the heritability of the hyperactive-impulsive symptom dimension

appears to involve the dopaminergic system, and appears to be enhanced by the presence of environmental risk.

Finally, it is worth noting that this study successfully demonstrated g x e interactions in the presence of genetic and environmental main effects, a pattern that is more in line with the animal literature. In the case of DRD4*4R, main effects of gene were robust across analytic strategies, and main effects of environment were revealed not only in the larger sample, but (in most cases) in those sub-samples included in DRD4*4R g x e interaction analyses. In the case of DAT1*10R, main effects of gene were found on ADHD-C (the subtype for which DAT1*10R appears to specifically confer risk) and, to a lesser extent, hyperactive-impulsive symptoms. As such, this study suggests that DRD4 and DAT1 are not "activated" by the presence of environmental risk, rather they confer risk independently for ADHD or associated symptom dimensions or subtypes, and that risk is enhanced by specific environmental risk factors.

LIMITATIONS AND FUTURE DIRECTIONS

Although this study possesses several advantages over others in the literature, addressing a wider array of genetic and environmental risk factors than have been examined previously, exploring dimensional specificity of interactions, methodically investigating potential rG-Es, and seeking to minimize Type I error, it nevertheless has a number of limitations.

Firstly, the scope of this study was limited to those genetic and environmental risk factors suggested by g x e interaction publications available at study inception. Therefore, we can draw no definitive conclusions about those risk factors presented in Table 24.

Therefore, future studies may seek to replicate the g x e interaction findings proposed by those studies, while also seeking to clarify the roles of more substantiated risk alleles and environments in the manifestation of ADHD.

Although this sample provided sufficient power in order to detect omnibus and interaction effects for all primary analyses, a number of exploratory analyses were underpowered to detect such effects. Given that DRD4*4R and DAT1*10R both entered into interactions with parental education and season of birth (suggesting that these environmental risk factors may interact specifically with polymorphisms that influence the dopaminergic system), it is particularly unfortunate that our investigations of cumulative genetic risk were underpowered to detect interaction effects. Future studies may choose to focus on the cumulative risk conferred by dopaminergic genes in the hopes of more thoroughly addressing this question.

The rG-E analyses employed in this study demonstrated significant correlations between levels of retrospective maternal ADHD symptomatology and environmental risk, and simultaneously failed to demonstrate a correlation between maternal genotype and such risk. These data, taken together, may be suggestive of a g x g interaction. That is, there may be an alternate genetic risk factor (not under consideration here) that is correlated with environment and interacts with our targeted risk allele in order to manifest ADHD symptoms. Alternatively, given that ADHD is a multifactorial disorder, it is possible that multiple genetic risk factors act synergistically to manifest a symptom – environment correlation, while failing to demonstrate a specific genotype – environment correlation. As such, once specific risk alleles are better characterized, future studies

may choose to further explore rG-Es for consistently identified risk alleles, or examine specific g x g interactions.

Additionally, although this study addressed multiple genetic and environmental risk factors, it did not target g x g, e x e, or three-way interactions (which it was underpowered to detect). However, given that the literature regarding g x e interactions is in such a state of flux, it may be wise for future studies to target replication of published associations and interactions in order to clarify and better characterize the roles of DRD4 and DAT1 in the etiology of ADHD before turning their focus to more complex interactions. Since the results of our analyses suggest a (possible) alternate polymorphism of DRD4 that is the true "risk" allele, any positive g x g interactions discovered between DRD4 and (as an example) DAT1 within this sample would likely not be replicated by other studies. Until these risk alleles are better (and more consistently) characterized, it is not feasible to accurately examine more sophisticated interplay among genes. Furthermore, given the breadth of environmental risk factors implicated in the manifestation of ADHD, and the extent to which those environments are correlated with maternal symptomatology (perhaps acting as a proxy for unknown genetic risk) within this sample, it would be impossible to determine to what extent positive e x e interactions were reflective of g x g or g x e interactions. As such, these lines of investigation were not pursued. It will be important for future studies to pursue these important questions once obstacles are lessened by consistent replication of genetic and environmental risk factors.

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