Maternal Smoking During Pregnancy: An environmental factor indexing a more homogenous subgroup of ADHD

Geeta Angeli Thakur

Integrated Program in Neuroscience
McGill University, Montreal

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"What the mind of man can conceive and believe, it can achieve"

Napoleon Hill
Author, 1883-1970

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To my dear parents
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ABSTRACT

Attention-Deficit/Hyperactivity Disorder (ADHD) is a prevalent neurobehavioral disorder with a complex etiology implicating both genetic and environmental factors. Although it is now established that multiple genes are involved in ADHD, no single risk gene has been identified. Furthermore, several environmental factors, such as maternal smoking, alcohol use, and stress during pregnancy, have been consistently associated with this disorder.

This thesis will describe how maternal smoking during pregnancy (MSDP) is indexing a more homogenous subgroup of ADHD children. By studying behavioral and neurocognitive characteristics in these children, we found that exposure to MSDP is associated with a form of ADHD characterized by more severe clinical manifestations and poorer neuropsychological performance.

Subsequently, we stratified children with ADHD by MSDP to investigate the implication of candidate genes in increasing the risk for ADHD. This strategy allowed the uncovering of differential associations between single nucleotide polymorphisms (SNPs) within the norepinephrine transporter gene (*SLC6A2*) and a number of endophenotypes in patients according to their exposure to MSDP.

Finally, we used comorbidity as a tool to investigate several SNPs identified through genome-wide association studies (GWAS) of smoking behavior, a phenotype comorbid with ADHD. These SNPs were investigated in relation to ADHD diagnosis, as well as behavioral and neurocognitive traits relevant to

ADHD, and we found that an allele of rs1329650 may be increasing risk for ADHD and smoking behavior through a common mechanism.

This work identifies a phenotypic signature associated with MSDP that may help to identify a more homogenous subgroup of children with ADHD and highlights significant associations between the *SLC6A2* gene and ADHD in children exposed to MSDP. Moreover, this is the first report of SNPs identified through GWAS of smoking behavior shown to be tentatively associated with ADHD.

RÉSUMÉ

Le trouble de déficit de l'attention avec hyperactivité (TDAH) est un désordre neurocomportemental répandu avec une étiologie complexe impliquant des facteurs génétiques et environnementaux. Bien qu'il soit maintenant établi que plusieurs gènes sont impliqués dans le TDAH, aucun seul gène de risque a été identifié. De plus, plusieurs facteurs environnementaux, tels que le tabagisme maternel, l'abus de l'alcool, et le stress maternel, ont été fortement associé à cette maladie.

Cette thèse décrira comment la cigarette durant la grossesse est un indexe pour un sous-groupe plus homogène d'enfants atteints d'un TDAH. En étudiant les caractéristiques comportementales et neurocognitives chez ces enfants, nous avons remarqué que l'exposition à la cigarette durant la grossesse est associée à une forme de TDAH, caractérisée par de graves manifestations cliniques et une plus basse performance neuropsychologique.

Par la suite, nous avons stratifié notre échantillon d'enfants atteints d'un TDAH par l'exposition à la cigarette pour enquêter sur l'implication des gènes candidats à augmenter le risque pour le TDAH. Cette stratégie nous a permis de découvrir des associations différentielles entre des polymorphismes nucléotidiques simples (SNP) du gène transporteur de la noradrénaline (*SLC6A2*) et un certain nombre d'endophénotypes chez les patients en fonction de leur exposition à la cigarette.

Enfin, nous avons utilisé la comorbidité comme un outil pour étudier plusieurs SNPs identifiés par des études d'association de l'ensemble du génome (GWAS) du

comportement des fumeurs, un phénotype de comorbidité avec le TDAH. Ces SNPs ont été étudiés en relation avec le diagnostic du TDAH, ainsi que des traits comportementaux et neurocognitifs pertinents pour le TDAH, et nous avons observé qu'un allèle du rs1329650 pourrait augmenter le risque pour le TDAH et le tabagisme par le biais d'un mécanisme commun.

Bref, ce travail identifie une signature phénotypique associée à la cigarette durant la grossesse qui pourrait aider à identifier un sous-groupe plus homogène d'enfants atteints d'un TDAH et met en évidence des associations significatives entre le gène *SLC6A2* et le TDAH chez les enfants exposés à la cigarette durant la grossesse. De plus, ceci est le premier rapport où des SNPs identifiés par des GWAS de tabagisme ont démontré une association avec le TDAH.

CONTRIBUTIONS OF AUTHORS

CHAPTER 1 - Introduction
Literature review and writing of text = G. Thakur

CHAPTER 2 – Study Design
Writing of text = G. Thakur

CHAPTER 3 – Research Manuscript
Design = R. Joober and N. Grizenko
Clinical assessments for subject recruitment = R. Joober and N. Grizenko
Data formatting = G. Thakur
Statistical analysis = G. Thakur, S. Sengupta, V. Pagé and N. Schmitz
Writing of text = G. Thakur
Supervision = R. Joober

CHAPTER 4 – Research Manuscript
Design = R. Joober and N. Grizenko
Clinical assessments for subject recruitment = R. Joober and N. Grizenko
Data formatting = G. Thakur
Statistical analysis = G. Thakur, S. Sengupta, Z. Choudhry
Writing of text = G. Thakur and S. Sengupta
Supervision = R. Joober

CHAPTER 5 – Research Manuscript
Design = R. Joober and N. Grizenko
Clinical assessments for subject recruitment = R. Joober and N. Grizenko
Data formatting = G. Thakur
Statistical analysis = G. Thakur, S. Sengupta, Z. Choudhry
Writing of text = G. Thakur
Supervision = R. Joober

CHAPTER 6 - Discussion
Writing of text = G. Thakur

ORIGINAL CONTRIBUTIONS

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- Chapter three deals with clinical and neurocognitive characterization of children with ADHD based on maternal smoking during pregnancy. The manuscript has been published as: Geeta A. Thakur, Sarojini M. Sengupta, Natalie Grizenko, Norbert Schmitz, Véronique Pagé and Ridha Joober. "Maternal Smoking During Pregnancy and ADHD: A Comprehensive Clinical and Neurocognitive Characterization" in *Nicotine and Tobacco Research.*

- Chapter four deals with investigating the relation between the norepinephrine transporter gene, maternal smoking during pregnancy, and ADHD. The manuscript has been accepted as: Geeta A. Thakur, Sarojini M. Sengupta, Natalie Grizenko, Zia Choudhry and Ridha Joober. "Comprehensive phenotype/genotype analyses of the norepinephrine transporter gene (*SLC6A2*) in ADHD: relation to maternal smoking during pregnancy" in *PLoS ONE* (In Press).

- Chapter five aimed to study comorbidity as a tool to understand ADHD. The manuscript has been published as: Geeta A. Thakur, Sarojini M. Sengupta, Natalie Grizenko, Zia Choudhry and Ridha Joober. "Family-Based Association Study of ADHD and Genes Increasing the Risk for Smoking Behaviours" in *Archives of Disease in Childhood*.

RELATED PUBLICATIONS

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LIST OF ABBREVIATIONS

ADHD	Attention-deficit/hyperactivity disorder
ANOVA	Analysis of variance
CBCL	Child Behavioral Checklist
CGI	Clinical Global Impression
CI	Confidence Interval
CNV	Copy number variant
Conners'-P	Conners' Global Index-Parents
Conners'-T	Conners' Global Index-Teachers
CPT	Continuous Performance Test
DA	Dopamine
DAT	Dopamine transporter
DISC-IV	Diagnostic Interview Schedule for Children-version IV
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, 4th Edition
EF	Executive Function
ES	Effect Size
FBAT	Family-Based Association Tests
G-E	Gene-environment interplay
GXE	Gene-environment interaction
rGE	Gene-environment correlation
GWAS	Genome-Wide Association Studies
5-HT	Serotonin
IQ	Intelligence Quotient
IRR	Incidence Rate Ratio
LD	Linkage disequilibrium
MPH	Methylphenidate
MRI	Magnetic Resonance Imaging
MSDP	Maternal smoking during pregnancy
nAChR	n-acetylcholine receptor
NE	Norepinephrine

NET	Norepinephrine transporter
OR	Odds Ratio
PBO	Placebo
PFC	Prefrontal cortex
RASS	Restricted Academic Situation Scale
SD	Standard Deviation
SE	Standard Error
SES	Socioeconomic Status
SNP	Single nucleotide polymorphism
SOPT	Self-Ordered Pointing Task
TOL	Tower of London
VNTR	Variable number of tandem repeats
WCST	Wisconsin Card Sorting Test
WISC	Wechsler Intelligence Scale for Children

CHAPTER 1

Introduction

Attention-Deficit/Hyperactivity Disorder (ADHD) is one of the most common neurobehavioral disorders among children (Faraone et al., 2003), affecting approximately 5% of school-age children worldwide (Polanczyk et al., 2007). Early descriptions of this disorder date back to the late 19th century under the denomination of 'hyperkinetic disorder of childhood', described mainly in boys and putting an emphasis on motor hyperactivity. The diagnosis of ADHD has since evolved to include inattention and impulsiveness, in addition to hyperactivity (Biederman, 2005). ADHD is a serious public health problem given the financial burden to society as a whole, stress to families of patients, as well as negative effects on self-esteem and adverse academic outcomes in patients (Barkley, 1998).

1.1 Clinical features

The clinical expression of ADHD is heterogeneous and characterized by developmentally inappropriate levels of attention, hyperactivity and impulsivity (Biederman and Faraone, 2005). Three behavioral subtypes of the disorder have been defined in the *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition (DSM-IV), namely primarily inattentive (20-30%), primarily hyperactive-impulsive (less than 15%), and combined subtype (50-75%). Symptoms must be present before the age of 7 in children and impair their functioning in at least two different environments, such as home and school (Klimkeit et al., 2010). Classification within each of these subtypes is based on DSM-IV diagnostic criteria as illustrated in Table 1.1.

Table 1.1: Diagnostic criteria for ADHD - Reproduced from the DSM-IV (American Psychiatric Association, 1994)

A. Either (1) or (2): (1) six (or more) of the following symptoms of inattention have persisted for at least 6 months to a degree that is maladaptive and inconsistent with developmental level: **Inattention** (a) often fails to give close attention to details or makes careless mistakes in schoolwork, work, or other activities (b) often has difficulty sustaining attention in tasks or play activities (c) often does not seem to listen when spoken to directly (d) often does not follow through on instructions and fails to finish schoolwork, chores, or duties in the workplace (not due to oppositional behavior or failure to understand instructions) (e) often has difficulty organizing tasks and activities (f) often avoids, dislikes, or is reluctant to engage in tasks that require sustained mental effort (such as schoolwork and homework) (g) often loses things necessary for task or activities (e.g., toys, school assignment, pencils, books, or tools) (h) is often easily distracted by extraneous stimuli (i) is often forgetful in daily activities (2) six (or more) of the following symptoms of hyperactivity-impulsivity have persisted for at least 6 months to a degree that is maladaptive and inconsistent with developmental level: **Hyperactivity** (a) often fidgets with hands or feet or squirms in seat (b) often leaves seat in classroom or in other situation in which remaining seated is expected (c) often runs about or climbs excessively in situations in which it is inappropriate (in adolescents or adults, may be limited to subjective feelings of restlessness) (d) often has difficulty playing or engaging in leisure activities quietly (e) is often "on the go" or often acts as if "driven by a motor" (f) often talks excessively	**Impulsivity** (g) often blurt out answers before questions have been completed (h) often has difficulty awaiting turn (i) often interrupts or intrudes on others (eg. butts into conversation or games) **B.** Some hyperactive-impulsive or inattentive symptoms that causes impairment were present before age 7 years. **C.** Some impairment from the symptoms is present in two or more settings (e.g., at school [or work] and at home). **D.** There must be clear evidence of clinically significant impairment in social, academic, or occupational functioning **E.** The symptoms do not occur exclusively during the course of a Pervasive Developmental Disorder, Schizophrenia, or Psychotic Disorder and are not better accounted for by another mental disorder (e.g., Mood Disorder, Anxiety Disorder, Dissociative Disorder, or a Personality Disorder) *Code* based on type: **314.01 Attention-Deficit/Hyperactivity Disorder, Combined Type:** If both Criteria A1 and A2 are met for the past 6 months **314.02 Attention-Deficit/Hyperactivity Disorder, Predominantly Inattentive Type:** If Criterion A1 is met but Criterion A2 is not met for the past 6 months **314.01 Attention-Deficit/Hyperactivity Disorder, Predominantly Hyperactive-Impulsive Type:** If Criterion A2 is met but Criterion A1 is not met for the past 6 months **Coding note:** For individuals (especially adolescents and adults) who currently have symptoms that no longer meet full criteria, "In partial remission" should be specified.

Furthermore, it is important to mention that the clinical definition of ADHD is currently being amended. Indeed, the DSM-V committee responsible for ADHD has proposed some changes. For example, one of the proposed revisions is to change the age of onset criterion from age 7 (onset of *impairing* symptoms) to age 12 (simply onset of symptoms). Since ADHD is a neurodevelopmental disorder, this new criterion is meant to convey that symptoms will begin in childhood, without necessarily causing impairment (Polanczyk et al., 2010). Another proposed change deals with changing the term 'subtype', indicating a more stable difference, to 'presentation', which is perhaps more current, and adding a fourth category called 'restrictive inattentive' (Willcutt et al., In press).

This illustrates that the definition of this behavioral syndrome remains to be established. Thus, identifying genetic and environmental factors contributing to this disorder may help to better delineate ADHD.

1.2 Comorbidity

In approximately 50-80% of cases, ADHD is associated with a number of comorbid disorders, namely externalizing disorders (oppositional defiant disorder and conduct disorder), internalizing disorders (mood and anxiety disorders), as well as learning disabilities (Klimkeit et al., 2010). Research has provided evidence that subgroups of children with ADHD and comorbid disorders exhibit a more severe clinical profile with other social, emotional, and psychological problems (Spencer, 2006). Although symptoms of childhood ADHD may decrease over time, they persist into adulthood in around 30-60% of cases

(Faraone et al., 2000) leading to a number of academic, occupational and social impairments. The comorbidity spectrum seen in ADHD subjects varies with age, from childhood to adolescence to adulthood, and is thus called 'developmental comorbidity' (Thome and Reddy, 2009). Most notably, ADHD is an important risk factor for antisocial personality and psychiatric disorders, such as depression, substance abuse, risk-taking behaviors, criminal offences and other addictive behaviors when left untreated, in adults (Biederman et al., 2006, Molina et al., 2009).

1.3 Executive Functions

Executive functions (EF) are top-down cognitive processes that facilitate the performance of a task. People use them to perform activities such as maintaining and updating information (working memory), integrating knowledge, planning, and organizing the optimal action (strategic planning and organization), paying attention to and remembering details, and regulating impulse and interference control.

Deficits in EF are believed to be at the very core of the ADHD syndrome, since affected children exhibit difficulties in many domains, namely problem solving, planning, cognitive flexibility, sustained attention, response inhibition, and working memory.

Barkley's neuropsychological theory of ADHD suggests that behavioral inhibition is a core deficit seen in ADHD (Barkley, 1997), with patients exhibiting difficulties in five main neuropsychological domains, namely working memory,

internalization of speech, self-regulation of affect/motivation/arousal, behavior analysis and synthesis and motor control/fluency/syntax (Figure 1.1).

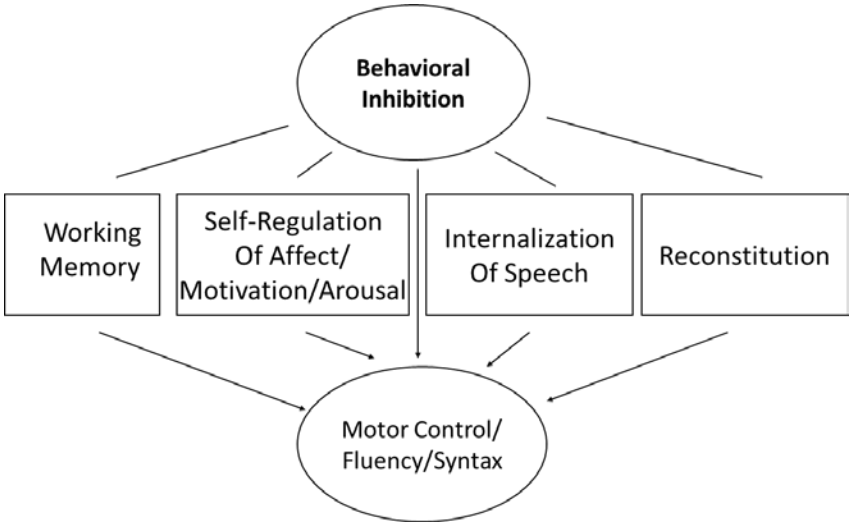

Figure 1.1: Barkley's Model of ADHD (Adapted from Barkley, 1997)

Furthermore, affective components, such as motivation and delay aversion, as well as sensory motor coordination are also affected in these children (Curatolo et al., 2010).

1.4 Endophenotypes

Endophenotypes can be understood as intermediate constructs that lie between genes and clinical symptoms (Castellanos and Tannock, 2002). These are often simple, quantifiable traits within a complex disorder that are rare in the general population, stable over time, specific to the disorder in question, and associated with genes that may be underlying the disorder (Waldman, 2005). They do not necessarily exist within each case, but can help to describe particular subtypes in a given disorder and are being used as quantitative traits in genetic studies.

Although understanding ADHD based on diagnosis has been useful from a clinical standpoint, it has not been very informative for genetic research. Thus, it has been suggested that it may be more fitting to investigate putative endophenotypes (Castellanos and Tannock, 2002, Del Campo et al., 2012, Doyle et al., 2005), which are purportedly less complex heritable traits that are more proximal to the biological etiology of a disorder than the clinical syndrome. Furthermore, studying endophenotypes, that may share one or more of the same genetic risk variants as the disorder at hand (Almasy and Blangero, 2001, Gottesman and Gould, 2003), may empower scientists to study underlying neurobiological mechanisms and detect genetic risks relative to ADHD.

A number of potential endophenotypes have been proposed for ADHD and may be grouped in three broad categories, namely neuropsychological, neuroimaging, and electrophysiological endophenotypes (Doyle et al., 2005).

Neuropsychological endophenotypes, related to deficits in executive function, have been proposed. One major example is deficient response inhibition, which is a measure of executive control allowing an individual to withhold a response in altered conditions (Aron and Poldrack, 2005). When a person exhibits a lack of inhibition, they become distractible and lose concentration, which can lead to errors and impulsiveness, as seen on certain neurocognitive tasks. These tasks are known to activate the PFC and basal ganglia, brain regions where the DA system is associated with executive functioning.

Neuroimaging, both structural and functional, studies in children with ADHD versus controls have reported abnormalities in frontal-subcortical networks, important for attention, inhibition, and motor behavior (Seidman et al., 2005). Data from structural imaging studies have yielded possible endophenotypes for ADHD, such as volumetric differences in the dorsolateral PFC, dorsal anterior cingulate cortex, the caudate nucleus, the putamen, and the globus pallidus (Castellanos and Tannock, 2002, Ernst et al., 1994, Faraone and Biederman, 1998, Giedd et al., 2001, Seidman and Valera, 2002, Zametkin et al., 1990). However, further research is needed in this new area, given the significant variability that has been reported across studies (Seidman et al., 2005).

Searching for ADHD endophenotypes derived from electroencephalographic (EEG) measures and event-related-potentials (ERPs) has also been suggested, given their reported association with a number of psychiatric disorders (de Geus, 2010). EEG measures record ongoing electrical activity in the brain, whereas ERPs measure changes in activity in response to specific stimuli (Doyle et al., 2005). An interesting finding from studies looking at differences between ADHD and controls across a number of ERP paradigms is that ADHD children have a reduced amplitude of the P3 wave, which reflects the activity of the locus-coeruleus-NE system (Nieuwenhuis et al., 2010) and is related to aspects of attention and working memory (Doyle et al., 2005). Together, these findings point to hypoarousal in certain brain areas including frontal regions, in children with ADHD.

By examining ADHD endophenotypes, trait markers for disease susceptibility, it may be possible for genetically homogeneous subgroups of patients to be identified. Also, small genetic effects that would otherwise be concealed may be detected and by defining precise phenotypes and then using them in quantitative trait analyses, it may be possible to highlight specific neurobiological mechanisms involved in the overall etiology of ADHD (Crosbie et al., 2008).

1.5 Pharmacological treatment of ADHD

Two major forms of pharmacotherapies exist, namely stimulant and non-stimulant medications, to treat ADHD symptoms (Curatolo et al., 2010). Both increase the levels of catecholamines, namely dopamine (DA) and norepinephrine (NE), in the brain. Given that ADHD involves dysregulation of both DA and NE neurotransmitter systems (Pliszka, 2005), drugs used to treat ADHD mainly act on these systems.

1.5.1 Stimulant medications

Psychostimulants are the most effective first-line treatment for ADHD and the most commonly used medications to treat ADHD symptoms. In Canada, it is estimated that approximately 6% of school-aged children use psychostimulants (Romano et al., 2005). Response to treatment has been shown to vary among children, where approximately 70% respond effectively. It has been suggested that genetic factors may underlie these differences in treatment response.

The two main types of psychostimulants that have been used over the last five decades in the treatment of ADHD are methylphenidate (MPH) and

dextroamphetamine (D-AMPH) (Wilens, 2008). Normally, DA is released into the synapse by dopaminergic neurons, and then signals through receptors (e.g. DRD4) to the post-synaptic neuron. The dopamine transporter (DAT) is then responsible for the re-uptake of DA into the presynaptic neuron (Figure 1.2).

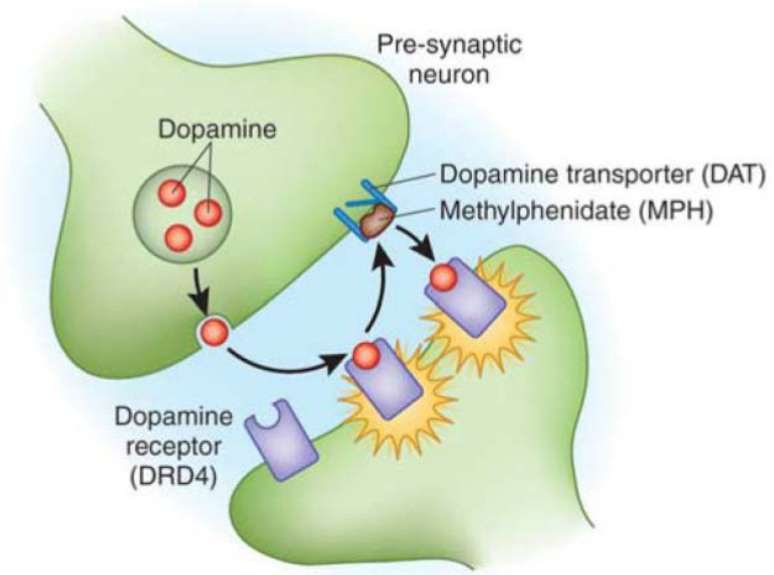

Figure 1.2: Normal action at a dopaminergic synapse (Bush, 2010)

Stimulants, such as MPH and D-AMPH, act in similar ways by blocking the DAT and norepinephrine transporter (NET), thereby blocking the re-uptake of DA leading to an increase in synaptic levels of both neurotransmitters (Zetterstrom et al., 1988). D-AMPH has an additional action since it also facilitates the release of these catecholamines into the extraneuronal space and inhibits the catabolic activity of monoamine oxidase (Kuczenski and Segal, 1975), an enzyme that breaks down catecholamines.

1.5.2 Non-stimulant medications

In addition to psychostimulants, non-stimulant medications, such as atomoxetine (ATX), which selectively inhibits the re-uptake of synaptic DA and increases extracellular levels of DA in the PFC, are used to treat ADHD (Del Campo et al., 2012). Another non-stimulant medication shown to be effective in reducing ADHD symptoms is guanfacine (Sallee et al., 2009). This drug acts on another neurotransmitter system, as it is a selective alpha2A adrenergic receptor agonist, which stimulates postsynaptic alpha2A adrenoceptors, highly concentrated in the PFC (Curatolo et al., 2010).

1.6 Pathophysiology of ADHD

Several neurotransmitter systems seem to be involved in ADHD. Three major neurochemical pathways are considered to be important in the pathophysiology of ADHD, namely dopamine (DA), norepinephrine (NE) and serotonin (5-HT) pathways (Aman et al., 1998, Durston, 2003, Faraone et al., 1995, Sagvolden and Sergeant, 1998) (Figure 1.3).

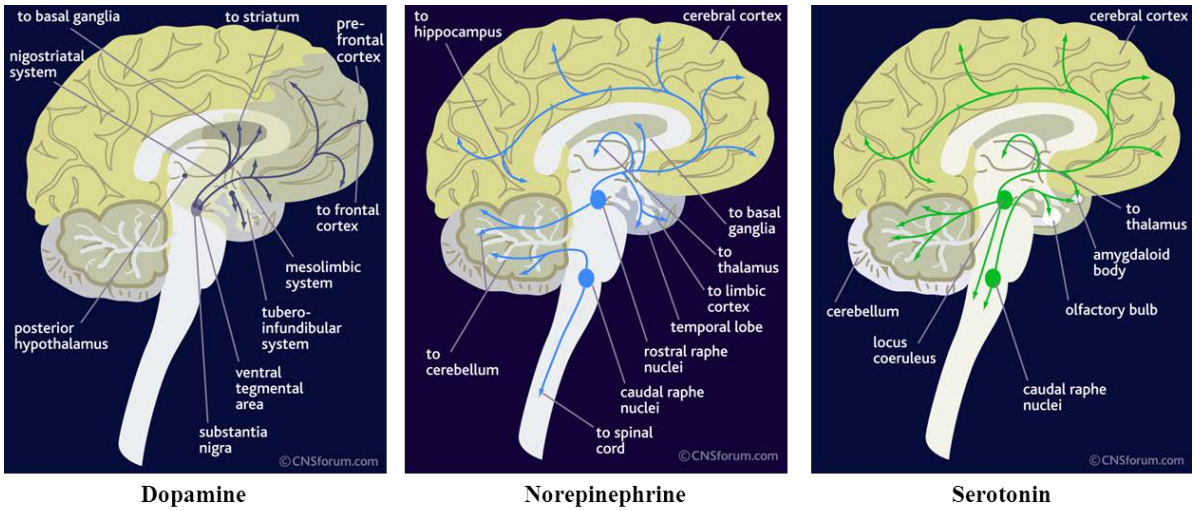

Figure 1.3: Three Major Neurochemical Brain Pathways involved in ADHD (Adapted from CNSforum, Lundbeck Institute website)

Since indirect evidence, derived mainly from animal studies, exists for the role of 5-HT in ADHD (Kostrzewa et al., 1994, Marx, 1999, Volkow et al., 2000), the next section will focus on DA and NE given their involvement in the mechanism of action of drugs used to treat ADHD, which increase brain levels of both these catecholamines.

1.6.1 Key neurotransmitters in ADHD

Dopamine and ADHD

Evidence from animal studies, pharmacology, neuroimaging as well as molecular genetic studies has recognized that dysregulation of the brain dopamine system underlies the pathogenesis of ADHD (Genro et al., 2010).

Several animal models have been proposed for ADHD, either where neurotoxins are used to create specific lesions, or rat strains are selected based on similar behavioral features seen in children with ADHD, or where specific genetic alterations are introduced (Genro et al., 2010). The commonality in these models is that when the DA system is manipulated, animals show hyperactivity and are calmed by stimulants, which is reminiscent of ADHD.

Psychostimulants used in the treatment of ADHD act within the DA system. However, since they also interact with the noradrenergic system, where non-stimulant medications, such as atomoxetine, are effective in treating symptoms, it will be important to consider both systems in ADHD.

Brain imaging studies have shown that impairment in the reward pathway may explain, in part, ADHD symptoms. Furthermore, differences have been seen in children with ADHD when compared to controls, with respect to overall brain reduction, especially in brain structures innervated by DA neurons, such as the caudate nucleus and globus pallidus (Castellanos and Tannock, 2002, Kieling et al., 2008). Functional neuroimaging studies are increasingly being conducted with initial reports showing decreased activation of the DA pathway (Durston, 2003).

Finally, given the significant role played by DA in ADHD etiology, candidate genes of the DA system, such as the dopamine transporter (*DAT1*) and DA receptor 4 (*DRD4*) genes, have been widely studied in relation to ADHD and associations with genetic variants in these genes have been reported.

Norepinephrine and ADHD

Norepinephrine (NE) is another brain catecholamine considered to be a major player in the pathophysiology of ADHD (Biederman and Spencer, 2002), given its involvement in visual attention, learning, and sustained attention (Ordway et al., 2007). Also, it has been shown that noradrenergic projections are quite abundant in the prefrontal cortex (PFC), a brain region critical for attentional control and high-level executive functions, such as working memory and behavioral inhibition, which are often impaired in children with ADHD (Robbins and Arnsten, 2009). Furthermore, low levels of NE in areas of the PFC have been linked to poor concentration and self-control, as well as greater motor activity (Klimkeit et al., 2010). Results from animal studies have shown that depletion of

NE increases distractibility and motor hyperactivity in rodents, whereas stimulation of the NE system decreases distractibility and improves cognitive function in non-human primates (Sengupta et al., 2012).

The NE pathway has also been implicated in the treatment of ADHD symptoms given that the selective NE reuptake inhibitor, atomoxetine, is effective in treating children with ADHD (Del Campo et al., 2011).

Given that NE-specific pharmacological agents have been clinically efficacious, the NE transporter gene (*SLC6A2*) has been considered an interesting candidate for genetic studies of ADHD. The *SLC6A2* gene is mapped to 16q12.2 with 14 exons spanning 45 kb (Figure 1.4a), and the resulting NET protein is a member of the sodium- and chloride dependent neurotransmitter transporter family containing 12 transmembrane domains (TMDs) (Figure 1.4b).

Therefore, dysregulation of the noradrenergic system, given the pivotal role of NET in the regulation of catecholamines and involvement in the re-uptake of both DA and NE into presynaptic terminals, in addition to DA, may also be an important player in the pathophysiology of ADHD (Arnsten, 2000).

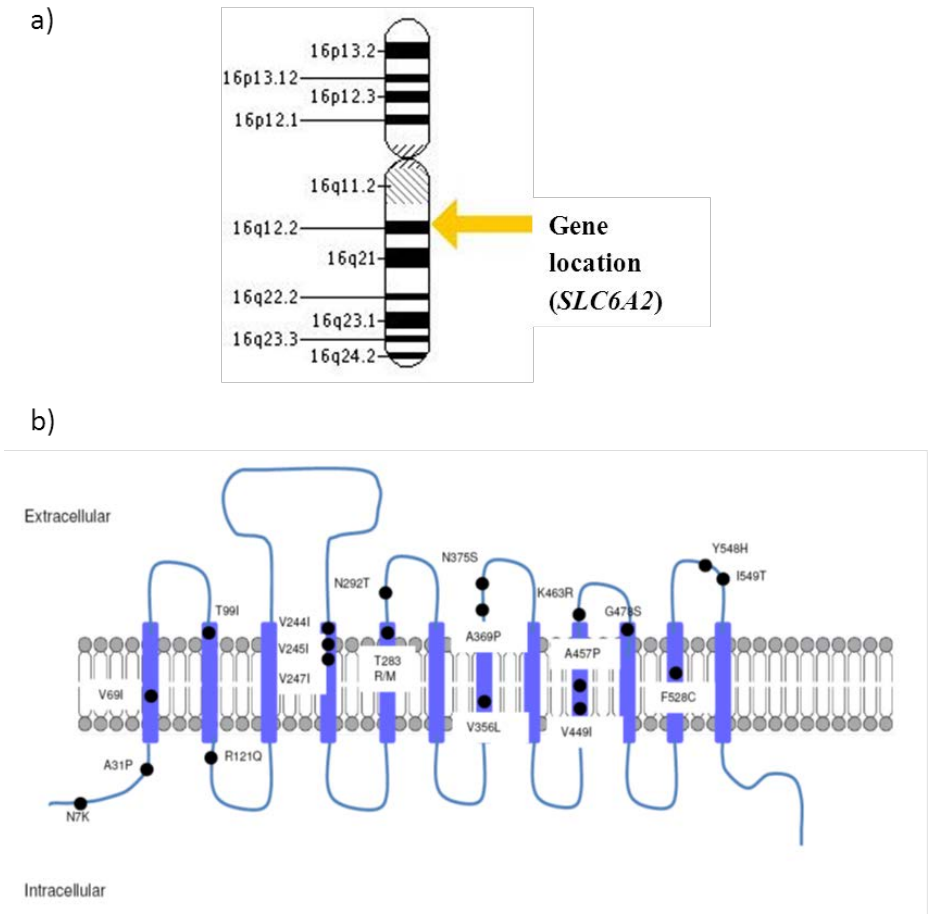

Figure 1.4: Illustration of the norepinephrine transporter (NET).
(a) Location of *SLC6A2* gene on chromosome 16 and (b) schematic representation of the NET protein, a transmembrane glycoprotein composed of 617 amino acids (Sengupta et al., 2012)

1.6.2 Brain imaging

Structural and functional imaging studies of ADHD are slowly surfacing and providing clues about subtle brain abnormalities in children with ADHD. Several brain regions have been studied, such as the caudate nucleus, cerebellum, corpus callosum, and prefrontal cortex (PFC), but findings have been inconsistent given the significant heterogeneity across regions and studies.

Two sets of brain networks are thought to be possible neural substrates for deficits seen in ADHD. Alterations in frontostriato-cerebellar circuits have been shown to underlie the deficits observed in prefrontal-dependent top-down control processes (Barkley, 1997), while disruptions in meso-cortico-limbic circuits are implicated in motivational abnormalities in ADHD (Castellanos et al., 2006, Sonuga-Barke, 2003).

Dysfunctions in the fronto-striatal network, which involves the lateral prefrontal cortex, the dorsal anterior cingulate cortex, and the caudate nucleus and putamen, have been implicated in ADHD. This may be related to the fact that many catecholamine systems, dopaminergic and noradrenergic, are present in this network, and are the main target sites of drug action (Curatolo et al., 2010). Reductions in total cerebral volume, the prefrontal cortex, the basal ganglia (striatum), the dorsal anterior cingulate cortex, the corpus callosum and the cerebellum have all been reported in children with ADHD (Emond et al., 2009).

An exciting new area of ADHD research with functional neuroimaging will help define endophenotypes which can then link them to specific gene variants to further our understanding of the underlying biological mechanisms (Thome and Reddy, 2009).

1.7 Etiology of ADHD

ADHD is a disorder with a rather complex etiology, with a number of genetic and environmental factors implicated. ADHD is also considered one of the most heritable psychiatric disorders, given that its mean heritability is estimated at 77%

(Figure 1.5), which means there is a significant genetic contribution to the disorder (Biederman, 2005).

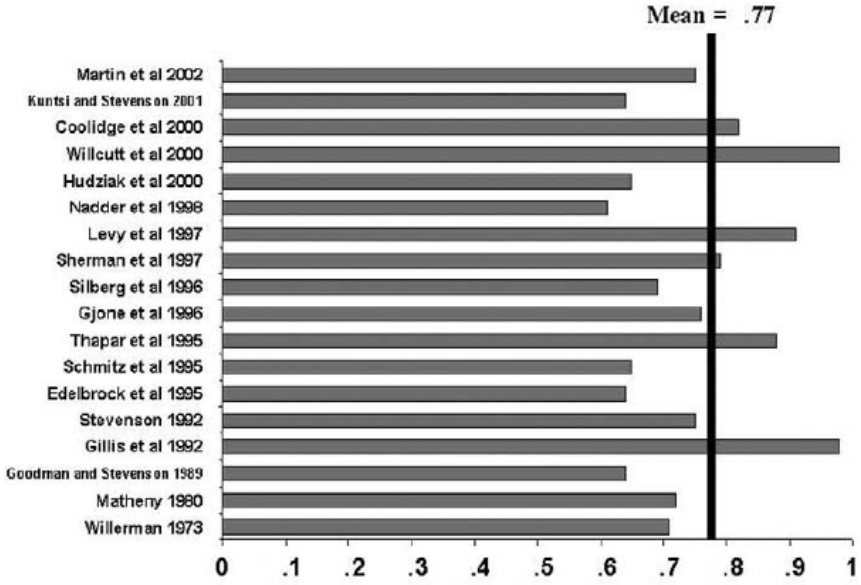

Figure 1.5: Heritability estimates of ADHD (Biederman, 2005)

However, the exact genes involved have yet to be discovered. Furthermore, environmental factors which account for the rest of the phenotypic variance of ADHD are very diverse and occur throughout development, namely at pre-natal, peri-natal, and post-natal stages.

1.7.1 Genetic Factors

It is now established that there is a significant contribution of genetic factors to the etiology of ADHD from family, twin, and adoption studies, which have shown that relatives of ADHD subjects are at a greater risk of developing the disorder, with higher rates in biological relatives (Faraone and Biederman, 1994, Faraone et al., 2005, Sprich et al., 2000). Furthermore, a large-scale twin study (n = 1,938) conducted in Australia by Levy and colleagues reported an 82% concordance rate

for ADHD in monozygotic twins as compared to 38% in dizygotic twins (Levy et al., 1997), highlighting the high heritability of ADHD.

In spite of over two decades of molecular genetic studies on ADHD, no single gene contributing significantly to the disorder has yet been identified with certainty (Franke et al., 2011, Franke et al., 2009). Many susceptibility genes (e.g. *DAT*, *DRD4*, *NET*) seem to contribute to the overall risk of ADHD, each having a small effect (Faraone et al., 2005). Therefore, identifying genes involved in ADHD has been quite a challenge.

Three main types of molecular genetic studies have been conducted, namely linkage studies, candidate gene association studies, and genome-wide association studies (GWAS) to obtain insight into which specific genes are involved.

Linkage studies

In a linkage study, the whole genome is screened with genetic markers in families with multiple affected individuals. When a marker co-segregates with the disorder, in this case ADHD, it indicates that the particular region (locus) is likely to contain risk genes for that disorder (Purper-Ouakil et al., 2011).

Specific chromosomal regions have been implicated in ADHD using affected sib-pairs and extended pedigrees. In a recent meta-analysis of seven linkage studies, ten chromosomal regions with linkage signals were identified. A genome wide significant finding was identified in the chromosome region from 16q23.1 to the q terminal (Zhou et al., 2008), however no previous candidate gene study had identified a gene in this region.

Since individual linkage studies are well-suited to capture strong genetic effects but lack power to detect linkage to genes of small effect (Faraone et al., 2005), it may be worthwhile to conduct these types of genome-wide linkage scans, to potentially discover novel genes associated with ADHD within these chromosomal regions (Zhou et al., 2008).

Candidate gene association studies

Candidate gene association studies (family-based and case-control) are based on *a priori* hypotheses, where genes are selected on the basis of their possible implication in the disorder. Familial studies examine whether there is an over-transmission from parents to offspring, while case-control studies compare frequencies of genetic variants in controls and affected probands (Purper-Ouakil et al., 2011).

These studies have primarily focused on dopaminergic, noradrenergic, and serotonergic systems given that psychostimulants used to treat ADHD symptoms act on these pathways. Two types of genetic markers are usually screened for in these association studies, namely single nucleotide polymorphisms (SNPs; one nucleotide position with a bi-allelic variation) and variable number tandem repeat (VNTR; a repeated sequence of nucleotides with multi-allelic variation).

Six genes, namely DA transporter (*SLC6A3/DAT1*), DA receptor D4 (*DRD4*), DA receptor D5 (*DRD5*), serotonin transporter (*SLC6A4/5HTT*), serotonin receptor 1B (*HTR1B*), and synaptosomal-associated protein 25 (*SNAP-25*) were implicated in ADHD in a recent meta-analysis (Gizer et al., 2009) as shown in Table 1.2.

Table 1.2: Summary of most significant meta-analytic results for candidate gene association studies in ADHD (Adapted from Gizer et al., 2009)

Gene	Location	Polymorphism	Risk allele	#Studies TDT/CC or HHRR	Meta-analysis (fixed/ random)	Results		Q statistic	
						OR (95% CI)	χ2 (P value)	χ^2 (P value)	I^2
DAT1	3 _**UTR**	**VNTR**	**10 repeat**	**34 (15/19)**	**Random**	**1.12 (1.00–1.27)**	**3.66 (0.028)**	**92.97 (<0.000001)**	**65**
	Intron 8	**VNTR**	**3 repeat**	**5 (4/1)**	**Random**	**1.25 (0.98–1.58)**	**3.35 (0.034)**	**11.22 (0.012)**	**64**
	Exon 8	rs6347	Unknown	6 (3/3)	Random	1.08 (0.94–1.22)	1.21 (0.272)*	5.81 (0.325)	14
	3 _**UTR**	**rs27072**	**'G' allele**	**7 (5/2)**	**Random**	**1.20 (1.04–1.38)**	**6.32 (0.006)**	8.29 (0.217)	28
	Intron 13	rs40184	'G' allele	4 (2/2)	Random	1.06 (0.90–1.24)	0.46 (0.249)	5.47 (0.141)	45
DRD4	**Exon 3**	**VNTR**	**7-repeat**	**26 (10/16)**	**Random**	**1.33 (1.15–1.54)**	**14.51 (0.00007)**	**54.32 (0.0006)**	**54**
	Promoter	In/Del	Unknown	8 (6/2)	Random	1.05 (0.86–1.31)	0.29 (0.590)*	**15.16 (0.033)**	**54**
	Promoter	**rs1800955**	**'T' allele**	**5 (3/2)**	**Fixed**	**1.21 (1.04–1.41)**	**6.01 (0.007)**	3.54 (0.472)	0
DRD5	**5 _ Flank**	**Dinucleotide repeat**	**148-bp allele**	**9 (6/3)**	**Random**	**1.23 (1.06–1.43)**	**7.73 (0.0027)**	***14.80 (0.063)***	***46***
SLC6A2	Exon 9	rs5569	Unknown	5 (3/2)	Fixed	1.06 (0.95–1.18)	1.17 (0.279)*	0.27 (0.992)	0
	Intron 13	rs2242447	Unknown	4 (2/2)	Random	1.04 (0.91–1.19)	0.29 (0.589)*	3.51 (0.319)	14
5HTT	**Promoter**	***5HTTLPR***	**Long allele**	**19 (10/9)**	**Random**	**1.17 (1.02–1.33)**	**5.40 (0.010)**	**52.80 (0.00003)**	**66**
	Intron 2	STin2	10-repeat	9 (2/7)	Fixed	1.01 (0.92–1.10)	0.03 (0.428)	4.08 (0.850)	0
	3 _ UTR	rs3813034	'T' allele	5 (2/3)	Random	1.05 (0.87–1.26)	0.26 (0.304)	5.71 (0.222)	30
HTR1B	**Exon 1**	**rs6296**	**'G' allele**	**9 (4/5)**	**Fixed**	**1.11 (1.02–1.20)**	**5.45 (0.010)**	7.92 (0.441)	0
SNAP25	Intron 4	rs362987	'A' allele	5 (4/1)	Random	1.00 (0.84–1.18)	0.00 (0.488)	***7.80 (0.099)***	***49***
	Intron 6	rs363006	'G' allele	7 (5/2)	Random	0.99 (0.86–1.15)	0.01 (0.547)	6.94 (0.326)	14
	3 _ **UTR**	**rs3746544**	**Unknown**	**7 (4/3)**	**Fixed**	**1.15 (1.01–1.31)**	**4.71 (0.030)***	2.69 (0.847)	0
	3 _ UTR	rs1051312	'T' allele	6 (4/2)	Random	1.06 (0.86–1.31)	0.30 (0.298)	***9.70 (0.084)***	***48***

Genome-Wide Association Studies (GWAS) of ADHD

Genome-Wide Association Studies (GWAS) are based on no prior hypothesis, and scan the entire genome by testing a large number of genetic variants (usually >100,000 SNPs), to identify genetic markers associated with a disorder. Thus far, five GWAS have been conducted in ADHD (Lasky-Su et al., 2008, Lesch et al., 2008, Mick et al., 2010, Neale et al., 2008, Neale et al., 2010) where 85 top-ranked ADHD candidate genes have been identified ($p<0.0001$). However, none of the findings passed the GWAS significance threshold (10^{-7}).

Although GWAS have been successful in other neurodevelopmental and neurodegenerative diseases, such as autism, schizophrenia, and Alzheimer's disease, results have been systematically pointed to genetic variants with small effect sizes. In the case of ADHD, no robust association through GWAS has been found until now, suggesting that further association studies with much larger sample sizes and more homogenous intermediate phenotypes or subgroups of the disorder will help in the identification of alleles associated with ADHD.

Copy Number Variants (CNVs)

Even though significant advances have been made in identifying risk variants with the genetic studies described thus far, they have only been able to explain a small portion of the overall variance (Eichler et al., 2010). Therefore, this has led researchers to alter their strategy by studying another type of variation, namely copy number variants (CNVs), which are large, rare duplications or deletions in the genome spanning an entire gene or multiple genes (Langley et al., 2011).

These studies determine whether the number of copies of a gene will increase or decrease in an individual (Ross, 2012).

Genome-wide analysis of CNVs has been conducted in ADHD (Elia et al., 2012, Lesch et al., 2011, Williams et al., 2012, Williams et al., 2010). Some key findings include the overrepresentation of CNVs affecting glutamatergic neurotransmission genes in many ADHD cohorts (Elia et al., 2012), while duplications at 15q13.3 have also been implicated as a novel risk factor for ADHD (Williams et al., 2012).

Results from these GWAS of CNVs suggest that rare structural variations may offer another alternative in detecting putative candidate genes which may be playing an important role in the etiology of ADHD.

1.7.2 Environmental Factors

Although ADHD is a highly heritable disorder, environmental risk factors play a significant role (approximately 30%) in disorder susceptibility. Epidemiological studies have strongly implicated a number of environmental factors in disruptive behavior disorders, such as ADHD, and categorized some of them based on the time period during which they occur, namely prenatal, perinatal, and postnatal factors (Latimer et al., 2012). Given that ADHD is a neurodevelopmental condition, it is now well established that environmental risk factors that occur during critical periods of development, such as fetal exposure, have a significantly detrimental effect on offspring (Banerjee et al., 2007). Furthermore, the earlier the

exposure occurs, the more widespread the negative consequences are likely to be (Tremblay, 2010).

Prenatal risk factors

Prenatal risk factors, such as maternal smoking and drinking, poor diet, as well as stress and anxiety during pregnancy (Latimer et al., 2012, Purper-Ouakil et al., 2011), have been highly associated with ADHD.

Several studies have linked maternal smoking during pregnancy to many adverse effects on pre- and postnatal growth, as well as poor cognitive and behavioral outcome in offspring (Banerjee et al., 2007). A number of large epidemiological studies have concluded that, even after controlling for factors such as socioeconomic status and parental psychopathology, there still exists a strong link between prenatal maternal smoking and increased rate of ADHD in offspring (Braun et al., 2006, Linnet et al., 2005).

Fetal alcohol syndrome (FAS) is the most severe consequence of maternal drinking during pregnancy, and is associated with mental retardation and other behaviors that strongly resemble those seen in children with ADHD, suggesting that alcohol may also play a causal role in ADHD. Exposure to alcohol prenatally has been shown to induce structural brain changes, notably in the cerebellum (Sowell et al., 1996), as well as cell loss. Children exposed to prenatal alcohol are known to be hyperactive, disruptive, at an increased risk to develop other psychiatric disorders, and have impaired cognitive abilities. Conflicting evidence

exists for the association between prenatal alcohol exposure and ADHD (Banerjee et al., 2007), indicating a need for further research in this area.

A number of studies from our group have shown that ADHD children whose mothers experienced moderate and severe stress during pregnancy tend to develop more severe symptoms of ADHD than those whose mothers experienced no or minimal prenatal stress (Grizenko et al., 2008) and that the association between maternal stress during pregnancy and ADHD symptomatology may be mediated by certain genetic factors (Choudhry et al., 2012, Grizenko et al., 2012).

Other prenatal risk factors include exposure to toxins, such as lead, mercury, manganese, and polychlorinated biphenyls (PCBs) (Froehlich et al., 2011). It has been shown that children exposed to lead contamination display a similar profile as children with ADHD, namely distractibility, hyperactivity, and lower intellectual functioning (Needleman, 1982). Exposure to mercury, a potent neurodevelopmental toxicant, has been shown to adversely affect IQ, language development, as well as memory and attention in offspring, while manganese hair levels have been associated with ADHD (Collipp et al., 1983). Although literature has shown that prenatal exposure to these neurotoxins has severe effects on offspring, further research is needed to draw stronger conclusions (Froehlich et al., 2011).

Obstetrical complications

An important category of risk factors for ADHD are pregnancy and delivery complications, which include preterm birth, eclampsia, fetal postmaturity and

distress, duration of labor, and antepartum hemorrhage (Ben Amor et al., 2005). These complications seem to predispose children to ADHD (Sprich-Buckminster et al., 1993), but studies till date have shown weak evidence and thus require further investigation. Brain structures, specifically the basal ganglia, should be studied in this context given that they are highly sensitive to hypoxic insults and often associated with ADHD (Banerjee et al., 2007).

Postnatal and infancy risk factors

Early postnatal influences, including neonatal anoxia, seizures, and brain injury, have also been reported in cases of ADHD.

Other very important factors are related to psychosocial adversity experienced by children in their early development (Banerjee et al., 2007). By studying prevalence of mental disorders in children living in two different geographical areas, classic studies conducted by Rutter and colleagues identified six risk factors, related to family environment, that were significantly associated with childhood mental disorders, namely severe marital discord, low social class, large family size, paternal criminality, maternal mental disorder, and foster placement (Rutter et al., 1975). They concluded that these adversity factors collectively rather than individually impaired offspring development.

Other psychosocial factors that have been highly correlated with childhood disorders include maltreatment and emotional trauma (Famularo et al., 1992, McLeer et al., 1994) where maltreated and traumatized children display concentration problems, avoidance of stimuli associated with trauma, social

withdrawal, and sleep disturbances (Krener, 1985) which mirror some of the symptom manifestations in children with ADHD (Weinstein et al., 2000).

Some important infancy factors are parenting style, maternal anxiety and depression, as well as early deprivation and separation (Latimer et al., 2012) which have been associated with increased risk of ADHD and associated comorbid disorders, such as oppositional defiant disorder and conduct disorder.

Taken together, all of the above-mentioned environmental factors that have been studied in ADHD till date embody a large proportion of the risk associated with ADHD for offspring. However, among all of them, maternal smoking during pregnancy (MSDP) is by far the strongest environmental factor associated with ADHD (estimated odds ratio = 2.39) (Langley et al., 2005).

1.8 In Utero exposure to nicotine – Effects on the fetus and brain

Nicotine is the major psychoactive compound in tobacco smoke (Oliff and Gallardo, 1999) and in utero exposure has both direct and indirect effects on the fetus and brain development.

When nicotine crosses the blood brain barrier (Luck et al., 1985), it is readily transferred to the fetus throughout pregnancy, where it binds to nicotinic acetylcholine receptors (nAChRs) in the fetal brain, and exerts a direct effect on its development (Hagino and Lee, 1985). Other indirect effects of MSDP are poor nutritional state of the mother (given the anorexigenic effect of nicotine) and carbon monoxide exposure (Abel, 1980, Perkins et al., 1994). Carbon monoxide increases the affinity of oxygen for hemoglobin, and disrupts oxygen uploading in

fetal tissue (Longo, 1972), thereby inducing hypoxia in the maternal-fetal unit which may result in indirect changes in brain growth and development (Slotkin, 1992).

Cigarette smoke also interferes with normal placental function and reduces uterine blood flow. It activates the adrenals and nerve cells, which trigger the release of catecholamines and cause vasoconstrictive effects. Further, when the cholinergic system is activated by nicotine, amino acid transport is depressed across the placenta, causing nutrient and oxygen deprivation to the fetus, which could lead to fetal intrauterine growth retardation (Naeye, 1978, Sastry, 1991).

Animal studies have demonstrated a significant dose-dependent effect of chronic exposure to nicotine during gestation on birth weight, locomotor activity, and cognitive performance, but results have been inconsistent (Banerjee et al., 2007). Furthermore, it has been shown that after prenatal exposure to nicotine, both dopaminergic and noradrenergic systems become hypoactive and hyporesponsive to exogenous stimulation. Thus, given that hypodopaminergic synapses were associated with ADHD (Banerjee et al., 2007) and drugs that increase synaptic levels of either dopamine or noradrenaline are therapeutic for ADHD, these disruptions in the development of catecholaminergic systems may explain the increased incidence of ADHD individuals prenatally exposed to nicotine.

Nicotine exerts its effects on various neurotransmitter systems, where binding of nicotine to nicotinic acetylcholine receptors enhances the release of DA, NE, 5-HT, γ-aminobutyric acid, and glutamate, in addition to acetylcholine, nicotine's

endogenous agonist, thereby affecting multiple neurotransmitter pathways with potential consequences on the programming of synaptic competence. Despite these informative reports from animal studies, it is not possible to directly extrapolate these findings to humans.

In addition to these effects, MSDP has also been associated with poor cognitive and behavioral outcomes in offspring, with increased incidence of disorders often comorbid with ADHD, such as conduct disorder, in children of mothers who smoked during pregnancy.

1.9 Maternal smoking during pregnancy (MSDP) and ADHD

MSDP is a highly prevalent and preventable behavior. In Canada, approximately 10-16% of women report smoking during pregnancy (Millar and Hill, 2004), and in the U.S, this number nearly doubles to 25% (Ernst et al., 2001). MSDP has important perinatal consequences including increased risk for fetal mortality (due to increased rates of spontaneous abortion) (Himmelberger et al., 1978, Kline et al., 1977) and morbidity (mainly low birth weight) (Eskenazi et al., 1995).

Compared to other maternal characteristics, such as alcohol consumption and psychosocial stress during pregnancy, MSDP has consistently been associated with ADHD (OR = 2.39) (Linnet et al., 2003). Milberger and colleagues reported that MSDP was associated with a 2.7-fold increased risk for ADHD in 140 cases and 120 controls (Milberger et al., 1996). Furthermore, a dose-response relationship between MSDP and hyperactivity has also been established (OR 1.30; 1.08–1.58) (Kotimaa et al., 2003). Although this association is now well

established, several studies have focused on ruling out potential confounders and reported that the association remained even after controlling for factors such as, including socioeconomic status (SES), family history of psychiatric disorders, and birth weight (Linnet et al., 2005, Obel et al., 2009).

Furthermore, children with ADHD exposed to MSDP have been shown to display lower scores on arithmetic and spelling tasks (Batstra et al., 2003), lower IQ scores (Milberger et al., 1998), as well as deficits in verbal learning, problem solving, and a slower response in eye-hand coordination compared to unexposed children (Cornelius et al., 2001).

Given the high comorbidity between smoking behavior and ADHD (McClernon and Kollins, 2008), other studies suggest that this association is predominantly due to shared genetic risks (Obel et al., 2011, Thapar et al., 2009) and that MSDP may be acting as a pointer for shared causal factors.

Thus, although MSDP is a core environmental factor in ADHD, with other factors such as obstetrical complications and maternal stress being related to smoking, further investigation is needed to dissect the pathways of these two co-occurring phenotypes.

1.10 Gene and Environment Interplay in ADHD

The etiology of ADHD is complex and it is now well understood that studying genes and the environment, as well as their interplay will be essential in accurately elucidating the pathogenesis of ADHD.

Gene-environment interplay is a term that encompasses both gene-environment interaction (GxE) and gene by environment correlation (rGE) (Knopik, 2009).

The following figure describes how genetic and environmental factors, as well as gene-environment interplay (G-E) have been implicated in the etiology of ADHD (Hyde et al., 2011).

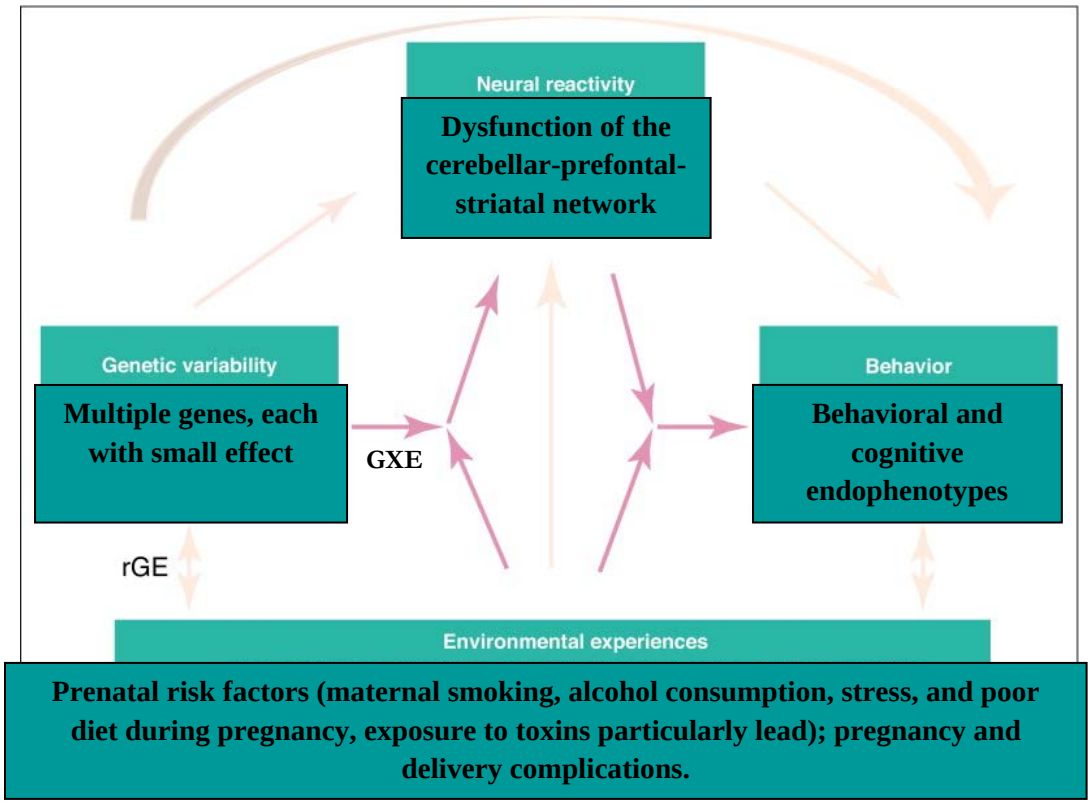

Figure 1.6: Depiction of the multifactorial etiology of ADHD
(Modified from Hyde et al., 2011)

GxE implies that an individual's genotype modulates the sensitivity or response to a specific environmental exposure (Moffitt et al., 2005). Whereas, rGE occurs when an individual's genotype affects the likelihood of his or her exposure to a particular environment, thereby suggesting that people somewhat shape and select their own environments through their behavior (Caspi and Moffitt, 2006).

Thus, in the case of ADHD symptomatology, it is clear that we must understand both the genetic predisposition, as well as environmental exposures to begin understanding the complex underlying mechanisms of the disorder.

Given strong pharmacological evidence, genes related to the dopaminergic neurotransmitter system, such as *DAT1*, *DRD4*, and *COMT*, have mostly been investigated in relation to environmental factors, such as MSDP, in children with ADHD. However, it may also be of interest to use another approach by investigating genetic variants of comorbid disorders, such as smoking behavior, in ADHD subjects, given that these two phenotypes (smoking and ADHD) are highly comorbid (McClernon and Kollins, 2008).

Despite a large body of research on genetic and environmental risk factors, the pathophysiology of ADHD is still poorly understood and GxE interactions have hardly been investigated.

Results from two studies looking specifically at children who had been exposed to prenatal maternal smoking found that (1) symptoms of hyperactivity and impulsivity were associated with the 480-bp *DAT1* risk allele, but only in exposed children (Kahn et al., 2003) and that (2) twins who had inherited the *DAT1* 440 allele and who had prenatal exposure to smoke were 2.9 times more likely to be diagnosed with the DSM-IV combined ADHD subtype, than their unexposed twins without the risk allele (Neuman et al., 2007).

In a study by Thapar and colleagues, it was reported that children who were carriers of the risk variant of the *COMT* gene and showed conduct disorder

symptoms in ADHD were more vulnerable to the harmful effects of lower birth weight (Thapar et al., 2005). Another GxE study conducted in children with ADHD from southeast England and Taipei, Taiwan reported a stronger association between a *DAT1* haplotype and ADHD, but only in cases where the mother had consumed alcohol during pregnancy (Brookes et al., 2006).

Taken together, these studies highlight the fact that GxE interactions may help to further understand the phenotypic complexity of ADHD (Banerjee et al., 2007). Although ADHD is a highly heritable disorder with a significant genetic contribution, its developmental course is certainly influenced by the way in which genes interact with and affect an individual's response to environment risk factors.

Therefore, it is important that future studies not only investigate genetic and environmental risk factors, but also their interactions. Furthermore, studying ADHD in a more thorough and comprehensive fashion, by not only looking at the diagnosis of ADHD itself, but also intermediate behavioral and neurocognitive endophenotypes, may help to understand the neurobiological mechanisms of this clinically and genetically heterogeneous disorder (Purper-Ouakil et al., 2011).

In this thesis, we hope to address some of these unanswered questions to further disentangle the etiology of ADHD by keeping the following hypotheses in mind:

Hypothesis

Our central hypothesis is that maternal smoking during pregnancy (MSDP) may be indexing a genetically more homogenous subgroup of children with ADHD, who display a more severe clinical and neurocognitive profile, and that examining specific genetic variants while stratifying children according to their exposure to MSDP may help to further clarify the interplay between genetic and environmental risk factors in ADHD.

Specific Objectives

- To determine whether children with ADHD exposed to maternal smoking during pregnancy show a distinctive clinical and neurocognitive profile when compared to unexposed children.

- To test the association between the *SLC6A2* gene and ADHD in two groups of children stratified based on maternal smoking during pregnancy.

- To investigate single nucleotide polymorphisms (SNPs) located in different genes and loci highly associated with different dimensions of smoking behavior, in relation to ADHD.

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CHAPTER 2

Study Design

Overview

The collection of phenotypic data presented throughout this thesis is based on a two-week double-blind, placebo-controlled, crossover randomized trial of methylphenidate (MPH) conducted at the Douglas Mental Health University Institute in children with ADHD between 6 and 12 years of age.

In this section, an overview of each of the methods used in this study will be presented. First, overall study context and inclusion and exclusion criteria for the patients will be presented. Then, evaluation of behavioral and treatment response within the two-week medication trial will be described, followed by a list of baseline evaluations and a detailed description of five neurocognitive tasks and the restricted academic situation scale (RASS). Finally, the principle behind family-based association tests will be explained.

Study Context:

Following baseline evaluations, children received either 1 week of placebo or 0.5mg/kg of MPH in a b.i.d dose and were crossed over during the second week. Response to treatment was then determined by examining the change scores on the different lab tests and improvement on the Conners' scales.

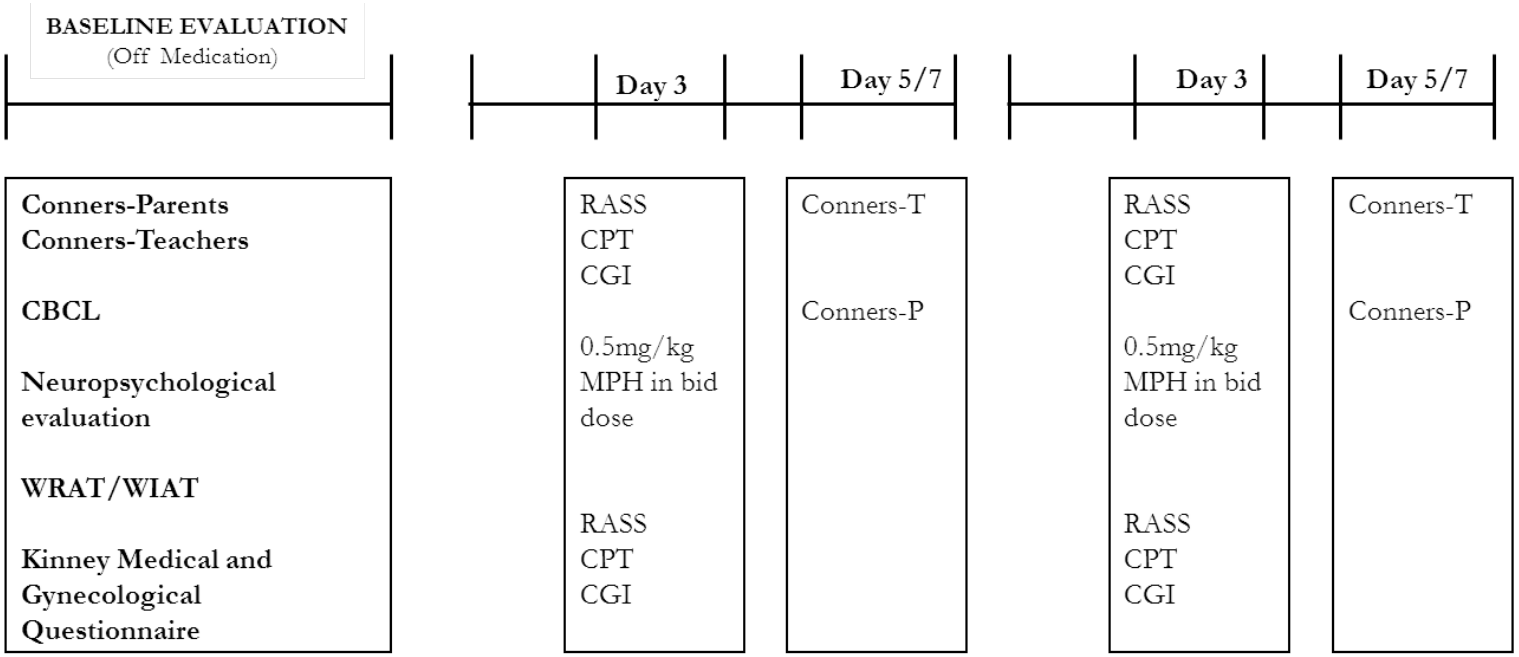


Figure 2.1: Timeline of the two-week double-blind, placebo-controlled crossover trial of methylphenidate

Recruitment of ADHD subjects
(With parents, and unaffected siblings)

Children with ADHD (between 6-12 years of age) were referred to the ADHD clinic at the Douglas Mental Health University Institute in Montréal, by schools, community social workers, family doctors, pediatricians, and child psychiatry outpatient clinics. The research protocol was approved by the Research Ethics Board of the Douglas Institute. All parents provided written informed consent and children gave their verbal assent to participate after details of the study were explained. Recruitment into the study was based on the following inclusion and exclusion criteria:

Inclusion Criteria:

1- Age: 6-12 years old

2- Best estimate diagnosis of ADHD (based on DSM-IV criteria), made by two experienced child psychiatrists.

3- Diagnosis was based on:

- Clinical interview of the child and at least one parent by a child psychiatrist
- Structured interview with parents using the Diagnostic Interview Schedule for Children-version IV (DISC-IV, parental report)
- Evaluation of behavior in school by teacher (including the Conners' Global Index-Teacher version), and at home by parents (CGI-Parents). At least one CGI-Parents or Teachers sub-score must be 65 or over.

Exclusion Criteria:

1- History of mental retardation with an IQ less than or equal to 70 as measured by the Wechsler Intelligence Scale for Children-III (WISC-III)

2- History of autism, Tourette's syndrome, pervasive developmental disorder or psychosis

3- Major medical condition or impairment that would interfere with the ability of the child to complete testing.

4- Concurrent treatment with any other medication except for methylphenidate (in particular, patients receiving anti-epilepsy drugs were excluded)

Figure 2.2: List of inclusion and exclusion criteria for study participants

Evaluation of behavioral and therapeutic response to methylphenidate

After baseline evaluations, the child received either MPH or placebo, each for a period of 7 days, in a randomized, double-blind sequence. Colored gelatine capsules were prepared by a clinical pharmacist not otherwise involved in the study. Capsules were sealed in individual, daily packets to help ensure accurate administration. MPH was prescribed in a divided b.i.d. dose (0.5 mg/kg/day; in the morning before school and at noon).

On day 3 of each treatment week, the child was evaluated in the clinic (RASS, CPT and SOPT), before taking the medication and then again 60 minutes after the medication. For each child, medication was administered daily at the same dose and time over the treatment period. The clinical staff completed the Clinical Global Impression for severity of illness and improvement based on their observation during the testing day and parental reports. On day 5, a research assistant collected information on therapeutic response from teachers (Conners'-T) and on day 7, information was collected from parents (Conners'-P).

The following figure is representative of a child receiving MPH in the first week followed by placebo in the following week:

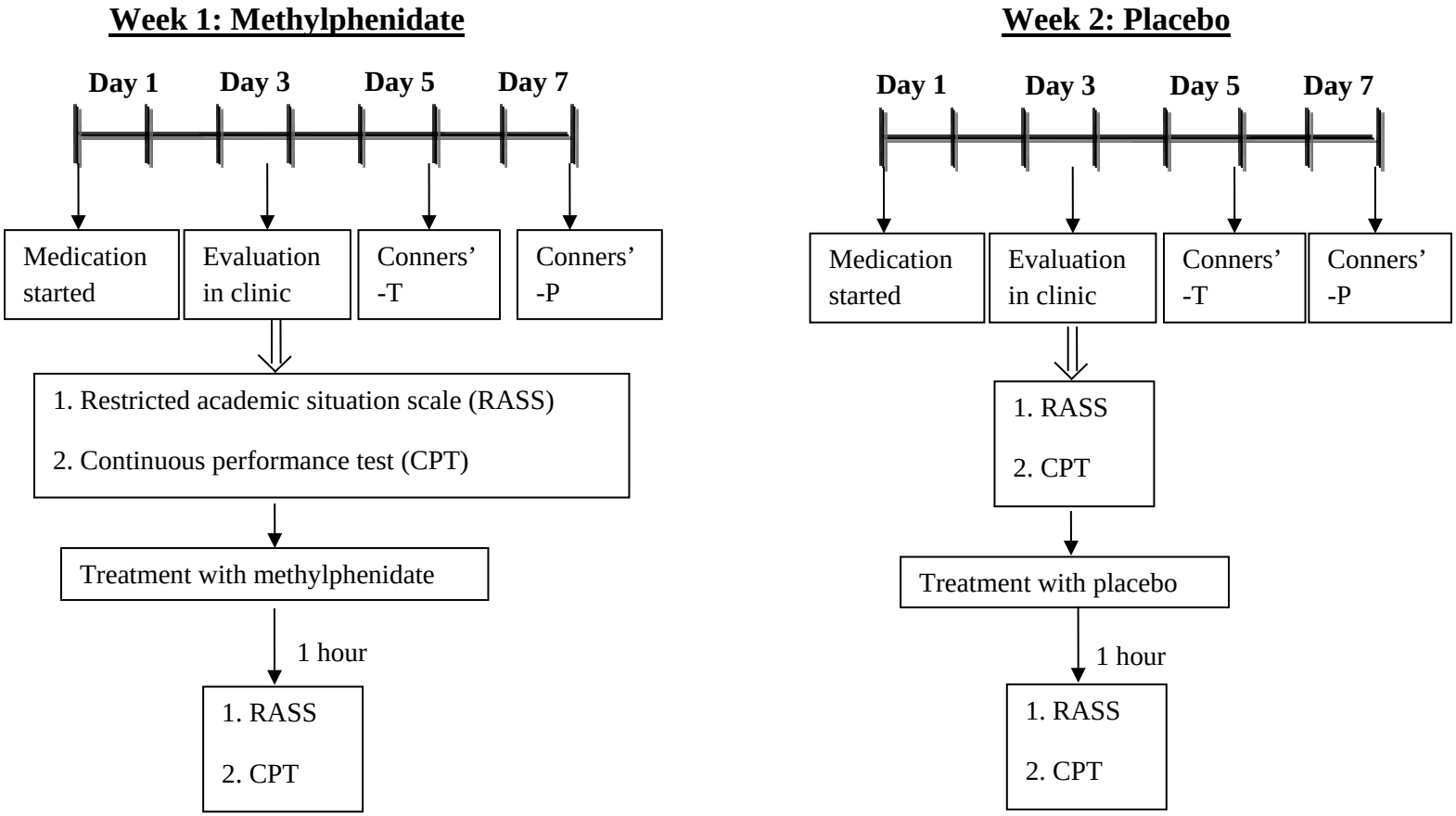


Figure 2.3: Description of behavioral measures and neurocognitive tasks administered during the two-week trial

Baseline Evaluations

During baseline evaluations: (1) diagnosis of ADHD, based on DSM-IV criteria, and comorbid disorders was established; (2) demographic data on the child and the family were collected; (3) Full scale, verbal and performance IQ were measured by the Wechsler Intelligence Scale for Children-III (WISC-III). Behavior was assessed by the psychiatrist and clinical research staff (Clinical Global Impression for severity, CGI-severity), parents (CBCL, Conners'-P), and teachers (Conners'-T); (4) pre-, peri- and postnatal environmental events were scored using the Kinney Medical and Gynaecological Questionnaire.

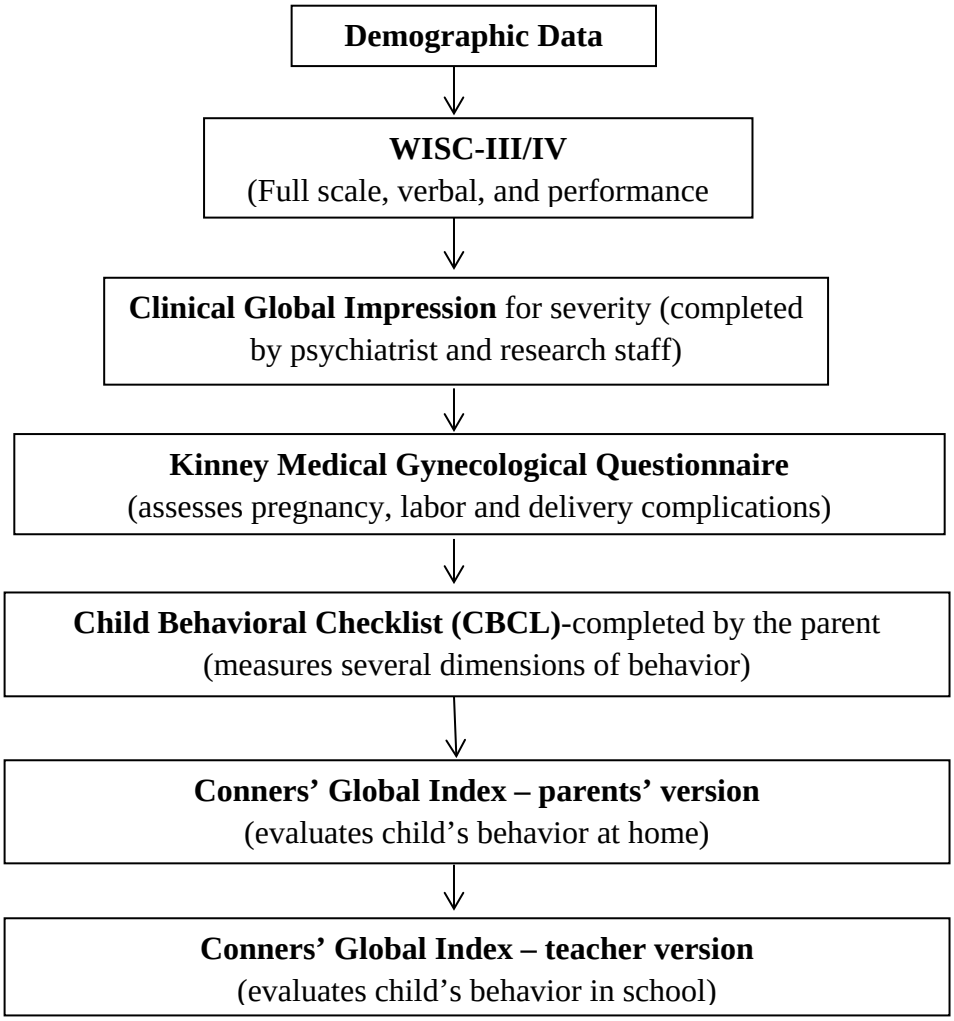


Figure 2.4 Outline of baseline evaluations conducted in study participants

Evaluation of cognitive function

To evaluate different domains of executive function in children with ADHD, a battery of five neurocognitive tasks was conducted (Table 2.1). A more detailed description of each task follows.

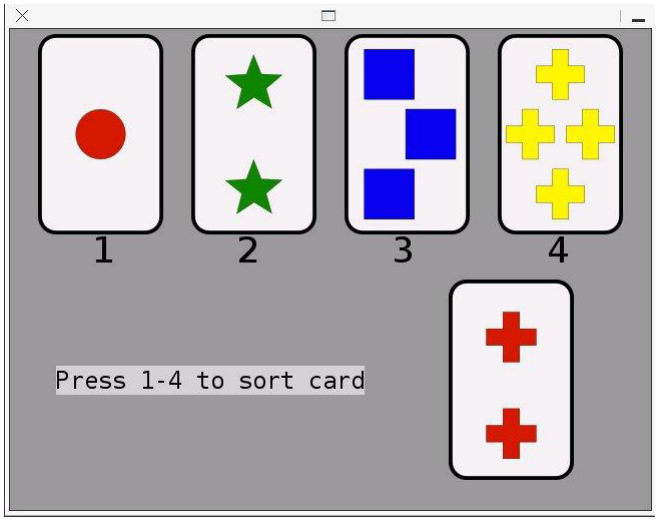
Name of Task:	**Measure of:**	**Assessment**
Wisconsin Card Sorting Test (WCST)	cognitive flexibility and set-shifting	Perseverative errors standard score Non-perseverative errors standard score Total errors standard score Number of categories completed
Finger Windows (FW)	spatial working memory	Standard score
Self-Ordered Pointing Task (SOPT)	spatial working memory, planning, and response inhibition	Total score
Tower of London test (TOL)	planning, organization, and problem-solving capacity	Standard score
Conners' Continuous Performance Test (CPT)	attention, response inhibition, and impulse control	Omissions (t-score) Commissions (t-score) Hit response time (t-score) Hit response time standard error Variability of standard errors Overall index

Table 2.1: List of five tasks conducted in order to evaluate cognitive function in children with ADHD

1. Wisconsin Card Sorting Test (Heaton et al., 1993)

The Wisconsin Card Sorting Test (WCST) measures the ability of a child to shift an established mental set ("set-shifting") and to manage distractions (interference control).

The child is presented with a set of "stimulus" cards (labeled 1-4 in diagram) and a "response" card (*pebl.sourceforge.net/cardsort.jpg*).



The child is asked to match the response card with one of the stimulus cards, according to 3 different criteria (number, color, or shape). However the child is not told of the sorting rule that has been set, and is only informed that the rule will change during the course of the test. As the child matches the response card to one of the stimulus cards, he/she is provided feedback about whether the choice was "right" or "wrong", leaving the child to decipher the sorting rule on their own.

After 10 consecutive correct matches, the sorting rule is changed, and the child must adapt his/her selection to the new rule.

"Perseverative errors" arise when the child continues to sort the cards according to the first rule long after that rule has been superseded.

The total number of errors is the sum of the perseverative and non-perseverative errors.

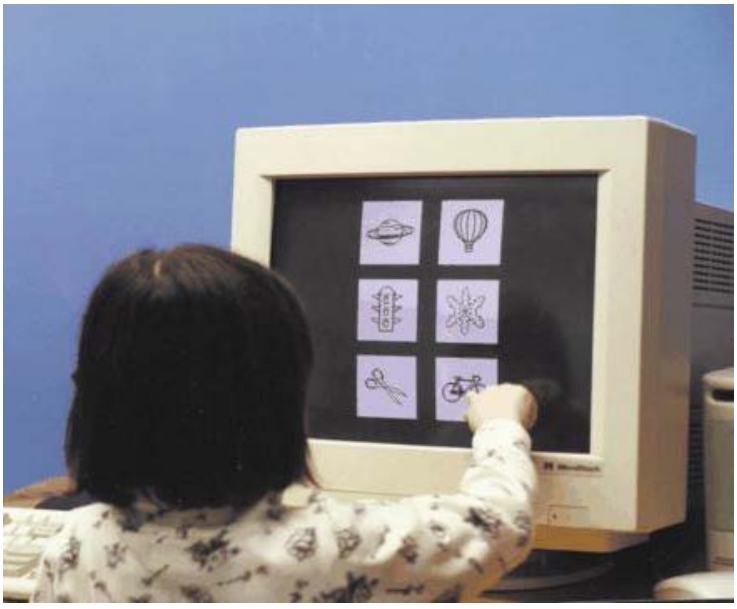
2. Finger Windows (Sheslow and Adams, 1990)

Finger Windows (FW) is a subtest of the Wide Range Assessment of Memory and Learning (WRAML).

In this test, the child is required to repeat the sequential placement of a pencil into a series of holes on a plastic card, as conducted by the examiner.

3. Self-Ordered Pointing Task (Petrides and Milner, 1982)

In addition to visual-spatial working memory, the Self-Ordered Pointing Task (SOPT) also measures planning and interference control. The task is depicted here, but in our study a manual version of the task is used (Diamond et al., 2004).



The child is presented with a rectangular grid containing 6 images and asked to point to an image and the page is turned. The next page contains the same grid of 6 images, but with the order shuffled and the child is asked to select a different image.

An error is recorded when the child points to an image previously selected on either of the preceding pages. The level of difficulty is increased by presenting matrices of 6, 8, 10 and 12 images. Each set is presented to the child 3 times.

4. Tower of London (Shallice, 1982)

The Tower of London (TOL) is designed to assess deficits in planning.

The testing device is composed of three colored beads placed on three rods.

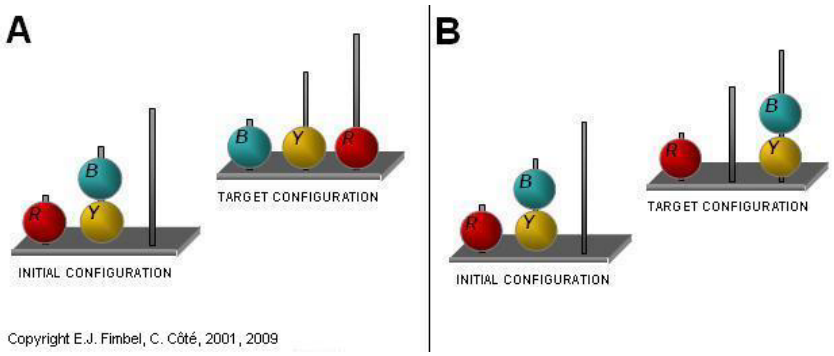
There are 36 X 36 pairs of possible configurations, schematically described below.

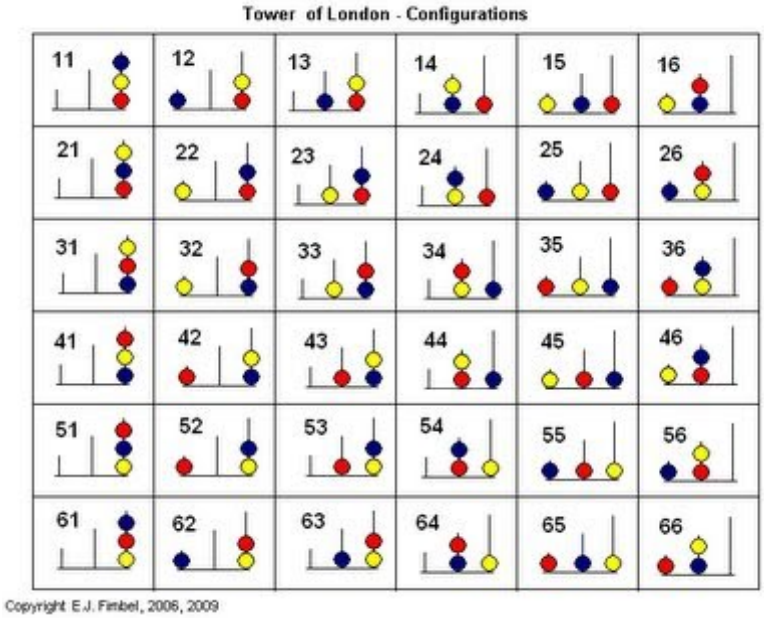
The goal is to reach a target configuration with a minimal number of moves.

Two examples are shown here:

Configuration 36 → 25 requires 2 moves;

Configuration 36 → 52 requires 8 moves.





5. Continuous Performance Test (Conners, 1995)

The Continuous Performance Test (CPT) is a computerized test where the child is instructed to press the space bar or mouse whenever they see any letter except for the letter "X".

Each letter is displayed for 250 milliseconds and the time intervals between presentations, called inter-stimulus intervals (ISIs), vary during the test (1, 2 and 4 seconds). The test structure consists of 6 blocks and 3 sub-blocks, each containing 20 trials (letter presentations), with varying presentation order of the different ISIs.

The picture below is a depiction of a child performing the CPT (*http://biof.com/images/take_iva.gif*):

Omission errors occur when the child fails to respond to the target sequence.

- They are a measure of vigilance/sustained attention.

Commission errors occur when the child responds to a sequence other than the target sequence (i.e. presses the spacebar when the letter “x” appears).

- They are a measure of “response inhibition” (the ability to withhold a pre-potent response).

Restricted Academic Situation Scale (RASS)

The RASS allows for a multi-dimensional evaluation of the child's behavior on a task that closely resembles features of everyday school life (Fischer and Newby, 1998). In a clinic playroom containing toys, a work table and chair and an intercom, the child is given a set of math problems below his/her current grade and is instructed to complete as many math problems as possible, not to leave the seat, and not to play with any of the toys in the room.

The child's behavior is assessed from behind a one-way mirror over a 15 minute time period.

Behavioral events are recorded at 30-second intervals according to five categories: "off-task", "fidgets, "out of seat", "vocalizes" and "plays with objects".

This scale has two factor structures: "task engagement" and "motor activation" (Karama et al., 2009).

Summary of Assessments

For each assessment or test carried out in this study, a score (T-score, total score, or standard score) was obtained. In some cases, a higher score indicates better behavior or performance, whereas in other cases, a lower score is indicative of improved behavior or performance. Below is a table with details on scoring for each assessment/test in the study.

Table 2.2: Explanation of scoring for each assessment/test in the study

Description of assessment/test	**Type of score (T/Standard score, average)**	**Is a higher score better or worse?**
IQ: WISC-III/IV	Standard score (average = 100)	Better
Clinical Global Impression (CGI)	Not standardized	Worse
Child Behavioral Checklist (CBCL)	T-score (average = 50; normal = 50-64; borderline = 65-69; problematic > 70)	Worse
Conners' Global Index-Parents (Conners'-P) and Teachers (Conners'-T)	T-score (average = 50; problematic > 65)	Worse
Restricted Academic Situation Scale (RASS)	Not standardized	Worse
Self-Ordered Pointing Task (SOPT)	Not standardized (Total score)	Worse
Finger Windows (FW)	Scale scores range from 1-19	Better
Wisconsin Card Sorting Test (WCST)	Standard score (average = 100)	Better
Tower of London (TOL)	Standard score (average = 100)	Better
Continuous Performance Test (CPT)	T-score (average = 50)	Worse

Family-Based Association Tests

Family-based association tests (FBAT) were used in Chapters 4 and 5 of this thesis (Laird et al., 2000). Single SNP tests of association were performed to investigate the association between selected markers with ADHD diagnosis and quantitative phenotypes relevant to ADHD. Analyses were conducted with the total sample and after stratification by maternal smoking during pregnancy (yes/no).

Offsets used in the FBAT analysis were based on average scores found in the population (e.g. 50 in the case of CBCL T-scores)

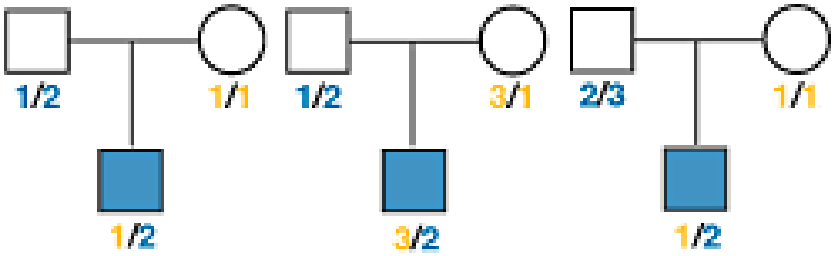


Figure 2.5: Depiction of allele transmission from parents to offspring

Principle: If a specific allele is associated with an abnormal level of a trait, it is expected to be transmitted more frequently than what is expected by chance, from parents to the child presenting an abnormal level of that trait. When this test is positive, it indicates the presence of both allelic association and linkage.

The over- or under-transmission from parent to affected offspring for each specific allele/haplotype is determined using the Transmission Disequilibrium Test (TDT). FBAT offer two major advantages over population-based (case/control) association studies, firstly they are not affected by population stratification, and secondly they may have increased statistical power (Haldar and Ghosh, 2011). Moreover, because the non-transmitted parental alleles are the control alleles themselves, this method controls for other possible sources of bias, such as socioeconomic status.

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CHAPTER 3

Maternal Smoking During Pregnancy and ADHD: A Comprehensive Clinical and Neurocognitive Characterization

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Preface

Genetic and environmental factors have been implicated in the etiology of ADHD. With a mean heritability of 77%, ADHD is a disorder with a strong genetic component. However, approximately 30% of the variance in the ADHD phenotype is attributed to the environment. Although numerous studies have examined a host of environmental risk factors, such as exposure to toxins, pregnancy and delivery complications, as well as fetal exposure to maternal smoking and alcohol, it has been a challenge to understand this variance given the myriad of exposures. With an odds ratio of 2.39, maternal smoking during pregnancy (MSDP) is a robust environmental factor that has been studied in ADHD for over 20 years. Although research has shown that MSDP is associated with ADHD, detailed information pertaining to profiles of exposed and unexposed children is still lacking.

In this chapter, a sample of children with ADHD was stratified based on MSDP, both with a qualitative and quantitative measure of smoking, and characterized with respect to several clinical and neurocognitive traits after adjusting for a number of socio-demographic confounders. With a wealth of phenotypic information, significant differences were observed among the two exposure groups. Children exposed to MSDP were characterized by more severe clinical manifestations and poorer neuropsychological performance, suggesting that MSDP may help in identifying a more homogenous subgroup of children with ADHD.

Abstract

Introduction: Evidence from epidemiological studies has consistently shown an association between maternal smoking during pregnancy (MSDP) and attention-deficit/hyperactivity disorder (ADHD). The objective of this study is to test the hypothesis that children with ADHD exposed to MSDP show a distinctive clinical and neurocognitive profile when compared with unexposed children. **Methods:** Four hundred and thirty-six children diagnosed with ADHD were stratified by exposure to MSDP and compared with regard to severity of illness, comorbidity, IQ, and executive function as assessed by a battery of neuropsychological tests. All comparisons were adjusted for socioeconomic status, ethnicity, mother's age at child's birth, and maternal alcohol consumption during pregnancy. **Results:** Exposed children had more severe behavioral problems with greater externalizing symptoms and more conduct and oppositional defiant disorder items, lower verbal IQ, and a sluggish cognitive profile on the Continuous Performance Test (CPT). Linear regression analyses revealed a dose-response relationship between the average number of cigarettes smoked per day during pregnancy and verbal IQ, CPT omission errors T score and several other clinical variables. **Conclusions:** These results suggest that MSDP is associated with a more severe form of ADHD, characterized by more severe clinical manifestations and poorer neuropsychological performance. This phenotypic signature associated with MSDP may help to identify a more homogenous subgroup of children with ADHD.

Introduction

Attention-Deficit/Hyperactivity Disorder (ADHD) is one of the most common early-onset behavioral disorders, affecting approximately 5% of school-age children worldwide (Polanczyk et al., 2007). It is a heterogeneous disorder characterized by inappropriate levels of attention, hyperactivity, and impulsivity. Family, adoption, and twin studies have established that there is a strong genetic component to the disorder (Shastry, 2004) with an estimated 80% heritability. Environmental factors, particularly nonshared ones account for the rest of the variance in the ADHD phenotype (Knopik et al., 2005).

Many maternal lifestyle factors have been investigated as risk factors for later psychopathology in offspring. One such environmental factor is maternal smoking during pregnancy (MSDP), which has been extensively investigated in relation to ADHD. In a systematic review of the literature assessing the relation between several maternal characteristics (smoking, alcohol and caffeine consumption, psychosocial stress during pregnancy) and behavioral problems related to ADHD, MSDP was consistently associated with ADHD (Linnet et al., 2003).

Although this association is now well established, most of the recent epidemiological studies have focused on ruling out potential confounders. Indeed, as smoking behavior and ADHD are highly comorbid (McClernon and Kollins, 2008), the association between MSDP and ADHD may reflect common genetic and/or environmental factors. Several studies found evidence for the association

between MSDP and ADHD after controlling for possible confounding factors. For example, in a longitudinal study based on Danish registers, it was reported that the adjusted relative risk (RR) for hyperkinetic disorder is increased (RR = 1.9; CI = 1.3–2.8) in children of mothers who smoked during their pregnancies after adjusting for a large number of potential confounders, including socioeconomic status (SES), family history of psychiatric disorders, birth weight, preterm delivery and Apgar score (Linnet et al., 2005). In another large prospective study, Obel et al. tested the hypothesis that genetic communality between ADHD and MSDP is the main link underlying the association between these two phenotypes. They made the assumption that genetic confounding between MSDP and ADHD would result in a stronger association between these two, in societies with low rates of smoking. The authors found that the strength of association between ADHD and MSDP is independent of the prevalence of smoking in the general population and concluded that the association between these two is not entirely due to genetic confounding (Obel et al., 2009).

In contrast, more recent studies suggest that the association between MSDP and ADHD could be mainly due to third genetic and/or environmental factors. In a large Swedish epidemiological study, Lindblad and Hjern found that MSDP was associated with an increased risk for being treated with psychostimulants (considered as a proxy for ADHD; OR = 2.86; CI = 2.66–3.07) but this association did not hold after controlling for SES and a large number of other confounding factors (OR = 1.89; CI = 0.95–1.58) (Lindblad and Hjern, 2010). Moreover, in a subsample of mothers with differential smoking during

pregnancies, the risk for ADHD in children born when the mother smoked during the pregnancy was comparable with the risk of their siblings where the mother did not (OR = 1.26; CI = 0.95–1.58). More recently, using a sibling design in a large Danish Cohort, Obel et al. (2011) concluded that shared family factors, including genes, could be the main factor underlying the association between maternal smoking and hyperkinetic disorders (Obel et al., 2011). This conclusion is in line with the study by Thapar et al. (2009) comparing the effect of MSDP in offspring conceived with Assisted Reproductive Technologies compared with biological offspring (Thapar et al., 2009). This study, although limited by its small sample size, did not find an effect of maternal smoking in the unrelated mother/offspring pairs, suggesting that the association between these two phenotypes is predominantly due to shared genetic risks.

Although a direct causal effect of cigarette smoking cannot be entirely ruled out, these studies collectively suggest that MSDP may be an indicator for genetic and environmental risk factors that are shared between ADHD and addiction to cigarette smoking. Consequently, it is possible that these shared causal factors will result in a specific “signature” on the ADHD phenotype, and that this signature may help to define a more homogenous subgroup of patients with ADHD.

In fact, many, but not all (Biederman et al., 2012), studies show differences in the clinical expression of ADHD in children born to mothers who smoked during pregnancy compared with those born to mothers who did not. For example, it was

reported that children with ADHD exposed to MSDP display lower scores on arithmetic and spelling tasks (Batstra et al., 2003), lower IQ scores (Milberger et al., 1998), as well as deficits in verbal learning, problem solving, and a slower response in eye-hand coordination (Cornelius et al., 2001) compared with those who were not exposed to MSDP. Other studies have demonstrated an association between heavy MSDP and slower reaction time and reaction time variability on the Continuous Performance Test (CPT) (Motlagh et al., 2011).

In this study, we compared the clinical and neurocognitive characteristics in ADHD children who had been exposed to MSDP with those who had not with the assumption that the former group has a set of genes and/or environmental factors (including nicotine exposure) that predisposes them to an ADHD/cigarette addiction phenotype with a distinctive clinical and neuropsychological profile compared with the latter group. The identification of such a phenotypic signature may in turn help to identify a more homogenous genetic subgroup of ADHD based on MSDP, thus further informing genetic studies.

Methods

Subjects

Four hundred and thirty-six nonrelated ADHD subjects (356 boys and 80 girls) between the age of 6 and 12 years were sequentially recruited from the Disruptive Behavior Disorders Program and the child psychiatry outpatient clinics at the Douglas Mental Health University Institute in Montreal. They were referred to these specialized care facilities by schools, community social workers, family doctors, and pediatricians.

Children were diagnosed with ADHD using DSM-IV criteria (Lahey et al., 1994), where diagnosis was based on clinical interviews of the child and at least one of the two parents by a child psychiatrist. A structured clinical interview of parents using the Diagnostic Interview Schedule for Children-IV (DISC-IV) (Shaffer et al., 2000) and school reports were used for the assessment. Mothers were primary informants in most cases. Details about diagnostic procedures have been described elsewhere (Grizenko et al., 2006)

Children with a history of Tourette's syndrome, pervasive developmental disorder, and psychosis were excluded. The research protocol was approved by the Research Ethics Board of the Douglas Institute. All parents provided written informed consent and children gave their verbal assent to participate.

Maternal Smoking during Pregnancy

The Kinney Medical Gynecological Questionnaire (McNeil et al., 1994) was used to systematically evaluate pregnancy, delivery, and perinatal complications. This questionnaire includes questions about smoking during the three trimesters of pregnancy. Mothers retrospectively reported maternal smoking (yes/no) during pregnancy. Children were coded as "unexposed" if mothers did not smoke at all during pregnancy, and "exposed" if mothers smoked during the three trimesters of their pregnancy. A small group of mothers (n = 28) who smoked intermittently during their pregnancy was excluded from further analyses since their smoking patterns were distributed across trimesters. Mothers also provided the average number of cigarettes smoked per day during the entire pregnancy.

Behavioral Evaluations

The Child Behavior Checklist (CBCL) (Achenbach, 1991), which assesses overall behavior of the child including behavioral and emotional problems (without a specific timeframe), was completed by the parents.

The Conners' Global Index for parents (CGI-P) and teachers (CGI-T) (Conners, 1999) were used to assess behaviors relevant to ADHD in home and school settings, respectively. The CGI-P and CGI-T are subsets of the original Conners' Rating Scales, which are widely used to assess ADHD symptoms and other psychopathology in children between 3 and 17 years of age. CGI-P and CGI-T are each comprised of two factors: "Emotional lability" and "Restless-impulsive

behavior". The raw total and factor scores are transformed into normalized T scores. All these assessments were completed while the children were not taking any medication.

Neurocognitive Assessment

A neuropsychological battery of tests was used to study executive function in these children. When children were medicated prior to their inclusion in the study, these assessments were carried out at the end of a 1-week washout period. The full scale, verbal, and performance IQ were evaluated for all children using the Wechsler Intelligence Scale for Children (WISC) (Weschler, 1991). Children with an IQ less than 70 were excluded from the study.

The Wisconsin Card Sorting Test (WCST) was administered to measure cognitive flexibility and set-shifting (Heaton et al., 1993), the Wide Range Assessment of Memory and Learning (WRAML) Finger Windows (FW) subtest was used to measure visual-spatial working memory (Sheslow and Adams, 1990), the Tower of London test (TOL) to assess planning, organization, and problem-solving capacity (Shallice, 1982), and the Self-Ordered Pointing Task (SOPT) to estimate visual working memory, planning, and response inhibition (Petrides and Milner, 1982).

Children were also evaluated with respect to their performance on the Conners CPT (Conners, 1995). The CPT measures attention, response inhibition, and

impulse control. Two types of errors (omission and commission) are recorded on the CPT. Omission errors result when the child does not respond to a target and commission errors occur when the child responds to a nontarget. Other relevant CPT outcome measures are response time, response time variability, and overall index, which is a weighted measure of different parameters reflecting attention problems.

Standard scores were obtained on the WCST, FW, and TOL, where a higher score is indicative of better performance. For the SOPT, the total score was corrected for age and a lower score indicates better performance. On the CPT, normalized T scores were computed.

Statistical Analysis

Demographic characteristics were compared between the groups of ADHD children stratified by exposure to MSDP (yes/no). Chi-square tests were used for categorical variables whereas analyses of variance (ANOVAs) were performed on continuous measures. Several variables were significantly associated with MSDP, namely mother's age at child's birth, mother's years of education, annual family income, and ethnicity. A measure of SES was computed based on annual family income and mother's years of education, where low SES was defined as having both an annual family income less than CAD $30,000 and the mother's education level less than high school (<11 years).

To compare clinical and neuropsychological characteristics of children with ADHD stratified according to their exposure to MSDP, we used univariate ANOVA tests for continuous variables, Poisson regression for count data, and logistic regression for categorical variables. We also performed linear regression analyses separately for each dependent variable to determine whether any of them were related to the average number of cigarettes smoked by the mother during pregnancy (dose-response analysis).

All these analyses were conducted while controlling for SES, ethnicity, mother's age at child's birth, and maternal alcohol consumption during pregnancy given their clinical significance. In order to appreciate the magnitude of observed effects, we also calculated effect sizes using Cohen's f2 method for ANOVAs and linear regressions, OR for logistic regression, and incidence rate ratios (IRR) for Poisson regression.

Results

Among the 436 children in the present study, 37.8% had been exposed to maternal cigarette smoking during the full gestational period. Demographic characteristics of these children stratified by exposure to MSDP are shown in Table 3.1. In the exposed group, mothers were younger when they had their children ($F_{1, 383} = 30.0$, $p < .001$), had completed less years of education ($F_{1, 399} = 58.6$, $p < .001$), came from a lower family income group ($\chi^2 = 50.4$, df = 1, $p < .001$), and were predominantly White ($\chi^2 = 12.2$, df = 1, $p < .001$).

As shown in Table 3.2, children with full gestational exposure to maternal smoking showed more severe presentation in almost all clinical and neuropsychological dimensions. These differences were particularly marked for the CBCL externalizing ($F_{1, 343} = 19.33$, $p < .001$) and total t scores ($F_{1, 343} = 11.15$, $p < .001$), the total baseline score ($F_{1, 312} = 6.16$, $p = .01$) as measured by the CGI-P, and WISC verbal ($F_{1, 316} = 15.1$, $p < .001$) and full-scale IQ ($F_{1, 316} = 9.7$, $p = .002$). With the exception of a few outcome measures (WISC performance IQ, WCST perseverative errors standard score and number of categories completed, as well as TOL standard score), children exposed to MSDP show poorer performance in most of the neurocognitive domains (Table 3.2).

On the CPT, children exposed to MSDP displayed a more sluggish cognitive profile as manifested by a slower reaction time ($F_{1, 334} = 6.38$, $p = 0.01$), more omission errors ($F_{1, 294} = 10.92$, $p = .001$), higher SE of the response time ($F_{1, 334} =$

8.29, p = .004), and higher variability of the response time SE ($F_{1, 334}$ = 6.44, p = .01). By contrast, children who were not exposed to MSDP were faster to respond to stimuli and committed more commission errors.

Other significant differences were observed with respect to number of hyperactivity (Wald statistic = 4.89, p = .03) and impulsivity symptoms (Wald statistic = 4.16, p = .04) on the DISC, as well as conduct disorder (CD; Wald statistic = 8.57, p = .003) and oppositional defiant disorder (ODD) items (Wald statistic = 13.49, p < .001) on the DISC (Table 3.4).

The largest effect sizes were seen with respect to CBCL externalizing behaviors (f=0.24) and WISC verbal IQ (f=0.22), which are in the medium effect size range, as well as CD items (IRR=1.54) and ODD items (IRR=1.33), where the incident rate for exposed children to have more CD items is 54% higher as compared with unexposed children.

Finally, we conducted linear regression analysis between the average number of cigarettes smoked per day during pregnancy and the severity of behavioral and neurocognitive dimensions while controlling for the same four covariates (Table 3.3). Notably, CBCL externalizing and total T scores, as well as the Conners baseline emotional lability score as rated by parents and teachers were significantly related to the average number of cigarettes smoked per day during pregnancy. In terms of neuropsychological features, verbal and full-scale IQ, CPT omission errors, hit response time SE, and overall index were all associated with

the number of cigarettes smoked during pregnancy. All effect sizes related to the linear regression analyses were in the low (f=0.11) to medium (f=0.21) range.

Furthermore, the number of hyperactivity (Wald statistic = 7.44, p = .006), impulsivity (Wald statistic = 15.18, p < .001), CD (Wald statistic = 14.19, p < .001) and ODD (Wald statistic = 25.43, p < .001) items, were all very significantly associated with the average number of cigarettes smoked per day by the mother during pregnancy (Table 3.4). For the quantitative exposure, the largest effect size was seen with CD items (IRR=1.03), which can be explained by the fact that if mothers increase their smoking consumption by 1 cigarette/day, the incidence rate of CD items in children will increase by 3%.

Tables

Table 3.1

Demographic Characteristics of Attention-Deficit/Hyperactivity Disorder Children With and Without Full Gestational Exposure to Maternal Smoking During Pregnancy

	Exposed (*n* = 165)	**Unexposed (*n* = 271)**	**Test statistic and *p* value**
Gender (% males)	80.6	82.3	$\chi^2 = 0.19$, $df = 1$, p = .66
Age	9.0 ± 1.7	8.9 ± 1.8	$F_{1,435} = 0.37$, p = .54
Mother's age at child's birth	26.0 ± 5.5	29.3 ± 5.6	$F_{1,383} = 30.0$, ***p* < .001**
Mother's years of education	11.6 ± 2.5	14.0 ± 3.3	$F_{1,399} = 58.6$, ***p* < .001**
Annual family income (% less than $30,000)	66.9	31.0	$\chi^2 = 50.4$, $df = 1$, ***p* < .001**
Ethnicity (% White)	92.7	80.4	$\chi^2 = 12.2$, $df = 1$, ***p* < .001**
Maternal alcohol during pregnancy (% yes)	23.9	18.1	$\chi^2 = 2.2$, $df = 1$, p = .14

Note. Values are M ± SD unless otherwise specified.

Table 3.2
Clinical and Neurocognitive Features of Attention-Deficit/Hyperactivity Disorder Children With and Without Full Gestational Exposure to Maternal Smoking During Pregnancy

	Exposed (*n* = 165)	**Unexposed (*n* = 271)**	**Test statistic and *p* value[a]**	**Effect Size[b]**
QUALITATIVE EXPOSURE				
Child behavior checklist				
Internalizing *t* score	65.5 ± 9.3	62.8 ± 10.0	$F_{1,343} = 1.88$, p = 0.17	0.07
Externalizing *t* score	71.5 ± 9.0	65.6 ± 10.0	$F_{1,343} = 19.33$, ***p < 0.001***	0.24
Total *t* score	71.2 ± 7.6	66.8 ± 8.8	$F_{1,343} = 11.15$, ***p = 0.001***	0.18
Conners Baseline scores				
Parent				
Emotional lability	68.2 ± 13.1	63.3 ± 13.1	$F_{1,312} = 5.73$, ***p = 0.02***	0.14
Restless-impulsive	76.0 ± 10.6	72.1 ± 10.7	$F_{1,312} = 4.56$, ***p = 0.03***	0.12
Total	75.5 ± 10.8	71.0 ± 11.1	$F_{1,312} = 6.16$, ***p = 0.01***	0.14
Teacher				
Emotional lability	68.6 ± 16.2	64.5 ± 16.2	$F_{1,315} = 2.91$, p = 0.09	0.10
Restless-impulsive	68.2 ± 9.9	68.4 ± 11.0	$F_{1,315} = 0.36$, p = 0.55	0.03
Total	70.6 ± 11.4	69.2 ± 12.6	$F_{1,315} = 0.25$, p = 0.62	0.03
WISC-III				
Verbal IQ	90.5 ± 13.9	97.0 ± 12.5	$F_{1,316} = 15.1$, ***p < 0.001***	0.22
Performance IQ	99.9 ± 14.8	102.9 ± 14.5	$F_{1,316} = 2.63$, p = 0.11	0.09
Full scale IQ	93.0 ± 13.3	98.2 ± 13.2	$F_{1,316} = 9.7$, ***p = 0.002***	0.18
Wisconsin Card Sorting Test				
Perseverative errors standard score	97.6 ± 11.8	100.2 ± 13.4	$F_{1,307} = 2.84$, p = 0.09	0.10
Non-perseverative errors standard score	91.9 ± 15.5	95.7 ± 15.2	$F_{1,307} = 4.63$, ***p = 0.03***	0.12

Total errors standard score	94.2 ± 13.9	97.8 ± 14.2	$F_{1,307} = 5.04$, ***p = 0.03***	0.13
Number of categories completed	4.2 ± 1.8	4.5 ± 1.7	$F_{1,307} = 2.94$, p = 0.09	0.10
WRAML Finger Windows				
Standard score	8.6 ± 2.9	9.8 ± 3.0	$F_{1,241} = 7.2$, ***p = 0.008***	0.17
Tower of London				
Standard Score	108.3 ± 13.9	108.7 ± 15.1	$F_{1,294} = 0.06$, p= 0.81	0.00
Self-Ordered Pointing Test				
Total score	16.8 ± 7.7	15.1 ± 7.5	$F_{1,340} = 4.37$, ***p = 0.04***	0.11
Continuous Performance Test				
Omissions (t-score)	64.8 ± 22.1	56.8 ± 15.0	$F_{1,294} = 10.92$, ***p = 0.001***	0.19
Commissions (t-score)	51.8 ± 8.4	53.6 ± 7.7	$F_{1,334} = 2.86$, p = 0.09	0.09
Hit response time (t-score)	56.5 ± 13.3	51.9 ± 11.2	$F_{1,334} = 6.38$, ***p = 0.01***	0.14
Hit response time standard error	63.6 ± 10.9	59.0 ± 11.5	$F_{1,334} = 8.29$, ***p = 0.004***	0.16
Variability of standard errors	61.3 ± 8.8	57.8 ± 10.2	$F_{1,334} = 6.44$, ***p = 0.01***	0.14
Overall index	10.4 ± 10.4	6.5 ± 8.9	$F_{1,334} = 11.4$, ***p = 0.001***	0.18

Note. Values are M ± SD unless otherwise specified.

[a]Corrected for socioeconomic status, ethnicity, mother's age at child's birth, and maternal alcohol consumption during pregnancy.

[b]Cohen's *f* effect size used in analysis of variance.

Table 3.3
Linear Regression Analysis of Attention-Deficit/Hyperactivity Disorder Children by Exposure to Maternal Smoking During Pregnancy (Average Number of Cigarettes Smoked/Day) With Respect to Clinical and Neurocognitive Features

	Unstandardized Coefficients		T score	*p* value[a]	Effect size[b]
	Beta	***SE***			
QUANTITATIVE EXPOSURE					
CBCL					
Internalizing t-score	0.13	0.07	2.03	***0.04***	0.11
Externalizing t-score	0.27	0.07	3.96	***< 0.001***	0.21
Total t-score	0.20	0.06	3.45	***0.001***	0.18
Conners Baseline scores					
Parent					
Emotional lability	0.27	0.10	2.89	***0.004***	0.16
Restless-impulsive	0.03	0.08	0.45	0.66	0.03
Total	0.11	0.08	1.38	0.17	0.08
Teacher					
Emotional lability	0.26	0.12	2.18	***0.03***	0.12
Restless-impulsive	-0.07	0.08	-0.92	0.36	0.05
Total	0.05	0.09	0.51	0.61	0.03
WISC-III					
Verbal IQ	-0.29	0.09	-3.18	***0.002***	0.17
Performance IQ	-0.13	0.10	-1.32	0.19	0.07
Full scale IQ	-0.24	0.09	-2.52	***0.01***	0.14
Wisconsin Card Sorting Test					
Perseverative errors standard score	-0.15	0.09	-1.66	0.10	0.09
Non-perseverative errors standard score	-0.20	0.11	-1.88	0.06	0.10

Total errors standard score	-0.19	0.10	-1.96	***0.05***	0.11
Number of categories completed	-0.02	0.01	-1.27	0.20	0.07
WRAML Finger Windows					
Standard score	-0.03	0.03	-1.04	0.30	0.07
Tower of London					
Standard Score	-0.15	0.10	-1.41	0.16	0.08
Self-Ordered Pointing Test					
Total score	0.02	0.05	0.43	0.67	0.02
Continuous Performance Test					
Omissions (t-score)	0.31	0.15	2.03	***0.04***	0.11
Commissions (t-score)	-0.10	0.06	-1.84	0.07	0.10
Hit response time (t-score)	0.08	0.08	1.01	0.31	0.05
Hit response time standard error	0.21	0.08	2.62	***0.009***	0.14
Variability of standard errors	0.12	0.07	1.71	0.09	0.09
Overall index	0.18	0.07	2.74	***0.007***	0.15

Note. [a]Corrected for SES, ethnicity, mother's age at child's birth, and maternal alcohol consumption during pregnancy.

[b]Cohen's *f* effect size used in regression analysis.

Table 3.4
Regression Analysis of Attention-Deficit/Hyperactivity Disorder (ADHD) Children by Exposure (Qualitative and Quantitative) to Maternal Smoking during Pregnancy With Respect to ADHD Symptom Severity, Comorbidity, and Subtype

	Wald	*df*	*p* value[a]	Effect size[b]	95% *CI* Lower bound	95% *CI* Upper bound
QUALITATIVE SMOKE EXPOSURE						
DISC ADHD						
# inattention items	0.10	1	0.76	1.01	0.95	1.08
# hyperactivity items	4.89	1	***0.03***	1.13	1.01	1.25
# impulsivity items	4.16	1	***0.04***	1.11	1.00	1.24
Total # ADHD items	3.27	1	0.07	1.06	1.00	1.13
DISC comorbidity						
# CD items	8.57	1	***0.003***	1.54	1.15	2.04
# ODD items	13.49	1	***<0.001***	1.33	1.14	1.54
ADHD Subtype						
Combined/Hyperactive vs. Inattentive	2.30	1	0.13	1.47	0.89	2.42
QUANTITATIVE SMOKE EXPOSURE						
DISC ADHD						
# inattention items	0.07	1	0.79	1.00	1.00	1.00
# hyperactivity items	7.44	1	***0.006***	1.01	1.00	1.01
# impulsivity items	15.18	1	***<0.001***	1.01	1.00	1.01
Total # ADHD items	5.11	1	***0.02***	1.00	1.00	1.01
DISC comorbidity						
# CD items	14.19	1	***<0.001***	1.03	1.01	1.04
# ODD items	25.43	1	***<0.001***	1.02	1.01	1.02
ADHD Subtype						
Combined/Hyperactive vs. Inattentive	0.51	1	0.47	1.01	0.98	1.04

Note. DISC = Diagnostic Interview Schedule for Children; ADHD = attention-deficit/hyperactivity disorder; ODD = oppositional defiant disorder; CD = conduct disorder.

[a]Corrected for SES, ethnicity, mother's age at child's birth, and maternal alcohol consumption during pregnancy

[b]For DISC items, Poisson regression was used and incidence rate ratios (IRR) are presented. For ADHD subtype, logistic regression was used and *ORs* are presented

Discussion

MSDP is an environmental risk factor that has been examined in ADHD and a number of other psychiatric disorders. Because MSDP is highly frequent in many populations (Rogers, 2009) and is a preventable behavior, understanding its link to ADHD may lead to beneficial public health actions. There is strong evidence supporting the association between prenatal tobacco exposure and later developmental consequences in children (Cornelius and Day, 2009). However, the causal relation between MSDP and neurodevelopmental disorders, such as ADHD, is not clear.

Recent human epidemiological studies suggest that MSDP may be a proxy for several genetic and environmental factors increasing the risk for ADHD (Obel et al., 2011). Also, neuropharmacological studies have shown that exposure of the developing brain to nicotine results in many neurochemical alterations that affect the major neuromodulatory pathways, including catecholamines (Dwyer et al., 2009, Oliff and Gallardo, 1999).

Although some animal studies have shown that exposure to nicotine during gestation results in cognitive impairments that are reminiscent of cognitive deficits in children with ADHD, these effects seem to be rather minor and require further investigation (Winzer-Serhan, 2008).

There is a large body of literature showing that ADHD and smoking are highly comorbid (McClernon and Kollins, 2008). This comorbidity could be, at least in part, the consequence of shared genetic factors between these two phenotypes. Under this assumption, it is likely that mothers who smoked during their pregnancy will transmit this genetic predisposition to their children, which will in turn express itself as behavioral problems in the ADHD spectrum and higher risk for smoking later in life.

We hypothesized that ADHD children exposed to MSDP may represent a more homogenous subgroup with a particular phenotypic signature compared with unexposed children.

To this end, we investigated the effect of full gestational smoke exposure, as a categorical variable, on many clinical and neuropsychological outcome measures in ADHD children to provide a comprehensive and comparative profile of exposed versus unexposed children with respect to severity of illness, comorbidity, and neurocognitive profile. In these comparisons, we controlled for potential socioeconomic confounders, ethnicity, and maternal alcohol consumption during pregnancy.

Overall, exposed children had a more severe clinical presentation with more ADHD symptoms and a higher level of comorbidity. They also showed deficits in a wide range of neurocognitive domains, including attention and executive function. Notably, exposed children had a sluggish attention profile as manifested

by a slower response time (longer hit reaction time), significantly more omission errors, and higher variability of response, as measured by the CPT, which is in line with other studies.

For example, Motlagh et al. (2011) investigated the effect of heavy MSDP on attentional control in an upper middle class sample with no difference in SES, yet they also identified a difference on attentional indices as measured by the CPT, where children exposed to heavy MSDP showed slower reaction time and higher reaction time variability (Motlagh et al., 2011). However, in this study, they did not identify statistically significant clinical differences between the two groups of children although exposed children had higher inattentive and hyperactivity scores ($p = .08$). Given the small sample size of the exposed group ($n = 12$), the lack of statistical significance is likely due to a lack of statistical power.

Most recently, Biederman et al. (2012) compared ADHD children with regard to the frequency of each ADHD defining symptom in children exposed or not to prenatal tobacco smoke (Biederman et al., 2012). They found no significant differences between the two groups with respect to the rates of any of the fourteen ADHD symptoms, as defined in the DSM-III-R. However, they did not compare the children with regard to individual measures of symptom severity, although other measures reflecting severity such as age at onset of ADHD, ADHD impairment and persistence were not significantly different between groups after controlling for SES measures.

It is possible that the discrepancy in clinical profiles observed by Biederman et al. and the present study may be due, at least in part, to the fact that in the latter all subjects had been evaluated off medication while in the former there is no mention of the treatment regimen at the time of evaluation.

Further, our study shows a linear relationship between the number of cigarettes smoked during pregnancy and severity of attention and clinical problems. Although dose-response relations are among the criteria used to establish causal effects between risk factors and outcome variables, it is also possible that this linear association is due to confounding factors, such as the severity of psychopathology in mothers that could correlate with the severity of smoking during pregnancy and the severity of ADHD in children. This potential causal relation has also been suggested in animal studies.

Indeed, controlled exposure to nicotine during gestation in laboratory animals has repeatedly shown behavioral and cognitive developmental abnormalities in exposed animals. For example, Schneider et al. recently showed that prenatal nicotine exposure is associated with performance deficits on the 5-choice serial reaction time test (5-CSRTT) in adult rats compared with rats who were born to mothers not exposed to nicotine during gestation (Schneider et al., 2011). These deficits on the 5-CSRTT are considered similar to those observed on the CPT in children. However, it is also possible that this dose-response relation is due to genetic factors controlling the quantity of cigarettes smoked that could also control the severity of ADHD behavioral manifestations (Lips et al., 2010).

Compared to previous studies with similar designs, this study has a number of strengths. First, to our knowledge, this is the largest and most comprehensive study comparing symptom and neuropsychological profiles of children with ADHD stratified according to their exposure to MSDP. Second, assessments were conducted using different scales in three different environments (home, school, and laboratory) and by different observers (parents, teachers, and research staff). The profile of differences observed between these two groups was consistent across the different settings and raters. Third, in addition to the categorical classification of maternal smoking behavior, we have included a quantitative measure for each trimester of pregnancy with the average number of cigarettes smoked per day. This quantitative approach helped to establish a potential dose-response relationship. Finally, all clinical and neurocognitive assessments were carried out while the children were not taking any medication.

However, certain limitations should be kept in mind when interpreting these results. First, the measure of MSDP was based on retrospective mother reports. However, smoking behavior of mothers during pregnancy is usually corroborated by another family member and the medical notes on the pregnancy, which are systematically collected. Further, it is more likely that mothers underreport smoking behavior during pregnancy, although this may reduce our capacity to detect differences. Second, we do not have information on familial psychopathology, most notably history of ADHD in the mother, which would have been helpful in dissecting the possible association between MSDP and ADHD given that ADHD has a strong genetic component, and the same genes

may also be risk factors for smoking behavior. Thus, it is possible that mothers with ADHD may be more prone to smoking during pregnancy and may have children with more severe symptoms of ADHD.

In summary, these results suggest that cigarette smoking during pregnancy in mothers of children with ADHD is likely to index genetic and/or environmental factors that increase the risk for both ADHD and smoking. These factors are associated with a more severe form of ADHD as manifested both by clinical and neuropsychological profile. In addition, it is possible that exposure to MSDP further impairs the developing brain thus aggravating the clinical and neuropsychological outcome of children. It is therefore probable that the subgroup of children exposed to MSDP is etiologically a more homogenous subgroup that will help to identify the genetic factors implicated in ADHD and smoking behavior.

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CHAPTER 4

Comprehensive Phenotype/Genotype Analyses of the Norepinephrine Transporter Gene (*SLC6A2*) in ADHD: Relation to Maternal Smoking During Pregnancy

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Preface

In the previous chapter, we established that children exposed to maternal smoking during pregnancy (MSDP) displayed a more severe ADHD phenotype. Given their high comorbidity and the fact that monoamine dysregulation is implicated in both ADHD and cigarette smoking phenotypes; we thought to explore a candidate gene previously investigated in ADHD. Norepinephrine (NE), which is involved in visual attention, learning, and sustained attention, is a major player in the pathophysiology of ADHD, given that NE-specific pharmacological agents (eg. atomoxetine) have been clinically efficacious. The NE transporter (NET) protein is a pivotal player in the regulation of catecholamines given its involvement in the re-uptake of both dopamine and NE into presynaptic terminals. Thus, strong *a priori* evidence suggests that the NET gene (*SLC6A2*) is an interesting candidate for genetic studies of ADHD.

In this chapter, we tested the association between 30 single-nucleotide polymorphisms in the *SLC6A2* gene and ADHD in two groups of children stratified based on MSDP. By conducting a detailed genotype and phenotype characterization, we examined a number of endophenotypes related to clinical and neurocognitive traits, as well as response to medication. Stratifying our sample by MSDP helped to reveal a number of highly significant associations between tag SNPs within *SLC6A2* and ADHD diagnosis, behavioral and cognitive measures relevant to ADHD and response to methylphenidate. Thus, results indicate that genetic variation in *SLC6A2* may be an important factor in a more severe subtype of ADHD.

Abstract

Objective: Despite strong pharmacological evidence implicating the norepinephrine transporter in ADHD, genetic studies have yielded largely insignificant results. We tested the association between 30 tag SNPs within the SLC6A2 gene and ADHD, with stratification based on maternal smoking during pregnancy, an environmental factor strongly associated with ADHD. **Methods:** Children (6-12 years old) diagnosed with ADHD according to DSM-IV criteria were comprehensively evaluated with regard to several behavioral and cognitive dimensions of ADHD as well as response to a fixed dose of methylphenidate (MPH) using a double-blind placebo controlled crossover trial. Family-based association tests (FBAT), including categorical and quantitative trait analyses, were conducted in 377 nuclear families. **Results:** A highly significant association was observed with rs36021 (and linked SNPs) in the group where mothers smoked during pregnancy. Association was noted with categorical DSM-IV ADHD diagnosis (Z=3.74, P=0.0002), behavioral assessments by parents (CBCL, P=0.00008), as well as restless-impulsive subscale scores on Conners'-teachers (P=0.006) and parents (P=0.006). In this subgroup, significant association was also observed with cognitive deficits, more specifically sustained attention, spatial working memory, planning, and response inhibition. The risk allele was associated with significant improvement of behavior as measured by research staff (Z=3.28, P=0.001), parents (Z=2.62, P=0.009), as well as evaluation in the simulated academic environment (Z=3.58, P=0.0003). **Conclusions:** By using maternal smoking during pregnancy to index a putatively more homogeneous

group of ADHD, highly significant associations were observed between tag SNPs within *SLC6A2* and ADHD diagnosis, behavioral and cognitive measures relevant to ADHD and response to MPH. This comprehensive phenotype/genotype analysis may help to further understand this complex disorder and improve its treatment.

Clinical trial registration information: Clinical and Pharmacogenetic Study of Attention Deficit with Hyperactivity Disorder (ADHD); www.clinicaltrials.gov; NCT00483106.

Keywords: ADHD, norepinephrine transporter, family-based association tests, maternal smoking during pregnancy.

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a highly prevalent psychiatric disorder, with rates ranging from 5.9-7.1% in children and adolescents (Willcutt, 2012). It is heterogeneous in its clinical expression, with core symptoms of poor sustained attention, impulsivity, and hyperactivity. It is often associated with cognitive deficits, particularly in executive function and sustained attention. ADHD has an important genetic component, with a mean heritability estimate of 76% (Biederman and Faraone, 2005), and it has been suggested that multiple genes are involved, each having a small effect (Faraone et al., 2005).

Psychostimulants, mostly methylphenidate (MPH) (Greenhill et al., 2002) are the first-line of treatment for ADHD. These medications are known to block the dopamine (DA) and norepinephrine (NE) transporters, resulting in increased synaptic concentration of both neurotransmitters (Krause et al., 2000, Madras et al., 2005, Volkow et al., 2001). Short-term trials have concluded that MPH is efficacious in reducing ADHD symptoms in approximately 70% of affected children (Greenhill et al., 2002) and adults (Faraone et al., 2004). NE-specific pharmacological agents (including clonidine, guanfacine, desipramine, and atomoxetine) are effective in treating ADHD, thereby implicating this catecholamine as a major player in the pathophysiology of the disorder (Biederman and Spencer, 2002). These studies reinforced the early evidence from neurochemical research that NE is involved in ADHD (Hanna et al., 1996, Shekim et al., 1983). Neuroimaging (Del Campo et al., 2011) and animal studies

(Arnsten and Pliszka, 2011) have provided further evidence for the role of NE in ADHD.

The NE transporter protein is a pivotal player in the regulation of catecholamines, involved in the re-uptake of both NE and DA into presynaptic terminals. Thus, it plays a key role in controlling the intensity and duration of signal transduction. The NE transporter is a member of the sodium- and chloride-dependent neurotransmitter transporter family, a transmembrane glycoprotein (Uhl and Johnson, 1994). It is encoded by *SLC6A2* which has been mapped to 16q12.2 (Bruss et al., 1993). The gene includes 14 exons spanning 45 kb (Porzgen et al., 1995), predicting a protein of 617 amino acids (Pacholczyk et al., 1991). Given the clinical efficacy of agents that block the NE transporter (including psychostimulants, MPH and amphetamine, and the NE-specific agent, atomoxetine), there has been considerable interest in *SLC6A2* as a candidate in genetic and pharmacogenetic studies of ADHD. Importance of the NE transporter has been further emphasized since it is responsible for the reuptake of both NE and DA in the prefrontal cortex (PFC), a brain region critical for attention regulation and where there is a scarcity of the dopamine transporter (Chen et al., 2004, Lachman et al., 1996), thus pointing to a potentially greater role of the norepinephrine transporter.

Several family-based (Barr et al., 2002, Biederman et al., 2008, Bobb et al., 2005, Brookes et al., 2006, Cho et al., 2008, De Luca et al., 2004, Kim et al., 2008b, McEvoy et al., 2002, Renner et al., 2011, Xu et al., 2008, Xu et al., 2005) and

case-control (Bobb et al., 2005, Cho et al., 2008, Joung et al., 2010, Kim et al., 2006, Kim et al., 2008a, Xu et al., 2008, Xu et al., 2005) studies have investigated the association between specific polymorphisms within *SLC6A2* and ADHD. While initial studies were conducted with a limited number of single nucleotide polymorphisms (SNPs) (Barr et al., 2002, Bobb et al., 2005, De Luca et al., 2004, McEvoy et al., 2002), recent association studies have used arrays of SNPs covering the entire gene (Biederman et al., 2008, Brookes et al., 2006, Kim et al., 2008b, Xu et al., 2008, Xu et al., 2005). A number of studies have examined the association between a functional SNP in the promoter region of the gene [-3081(A/T), rs28386840] and ADHD (Cho et al., 2008, Joung et al., 2010, Kim et al., 2006, Kim et al., 2008a, Renner et al., 2011). Furthermore, association between *SLC6A2* and ADHD endophenotypes, including neurocognitive measures (Kollins et al., 2008, Song et al., 2011a) or quantitative symptom scores (Retz et al., 2008), has also been studied.

Although many studies have been conducted thus far, findings have been limited and difficult to replicate. An earlier study reported an association between rs3785157 and rs998424 and ADHD (Bobb et al., 2005). Later, an independent group reported a trend for association with both these SNPs, however opposite alleles were conferring risk for the disorder in this study (Xu et al., 2005). Although these results were not confirmed in the International Multi-Centre ADHD Gene (IMAGE) project, associations were reported with two other SNPs (rs3785143, rs11568324), (Brookes et al., 2006) and these were confirmed in two independent samples (Kim et al., 2008b, Xu et al., 2008). Several related groups

have reported an association between ADHD and a functional promoter SNP rs28386840 [-3081(A/T)], using a case-control study design (Joung et al., 2010, Kim et al., 2006, Kim et al., 2008a). However, two large family-based studies (one with more than 99% power), conducted by independent groups, failed to replicate this association (Cho et al., 2008, Renner et al., 2011).

Although several pharmacogenetic studies, including a genome-wide association study (Mick et al., 2008), have examined the association between *SLC6A2* SNPs and response to MPH (Kim et al., 2010, Song et al., 2011b, Yang et al., 2004), or OROS-MPH (Cho et al., 2011, Lee et al., 2011) treatment, only limited association was observed with a few polymorphisms (rs5569, rs28386840, rs17841329, and rs192303) with little replication between studies.

We have conducted a family-based study to test the association between a panel of 30 SNPs within *SLC6A2* and ADHD. In addition to the DSM-IV diagnosis of ADHD, quantitative behavioral and cognitive phenotypes, as well as response of these measures with MPH treatment, were tested for association. The panel of SNPs included those analyzed in the IMAGE project (excluding SNPs having a minor allele frequency ≤ 0.02) (Brookes et al., 2006), two SNPs selected to extend the 3' flanking region examined (rs15534, rs7188230), and the functional promoter SNP, rs28386840.

Given that high comorbidity between ADHD and cigarette smoking (35%-45%) is well documented (Pomerleau et al., 1995), and that children with ADHD are

consistently reported to have higher exposure to cigarette smoking during pregnancy compared to the general population (OR=2.39), (Langley et al., 2005) analyses were conducted based on stratification by maternal smoking during pregnancy (MSDP). Also, it has been suggested that shared pathways to the two pathologies may exist, at least in some groups of individuals (McClernon and Kollins, 2008), and more precisely with respect to monoamine dysregulation. The aim of the current study was to examine the differential association (if any) of genetic polymorphisms within *SLC6A2* after MSDP stratification.

Methods

Ethics Statement

The study was approved by the Douglas Mental Health University Institute (DMHUI) Research and Ethics Board. All participating children agreed to take part in the study, and parents provided written consent.

Subjects

Four hundred and seventy-five children with ADHD between 6 and 12 years of age [mean=9; SD=1.8] were included in this study. They were referred by schools, social workers, family doctors and pediatricians, and were recruited from the Disruptive Behavior Disorders Program and the children's outpatient clinics of the DMHUI, a psychiatric teaching hospital in Montreal, Canada.

Each child was diagnosed with ADHD according to DSM-IV criteria. Further details pertaining to diagnostic procedures have previously been described (Grizenko et al., 2006, Sengupta et al., 2012). Of the total number of affected children, 77.9% were male and 82.5% were of Caucasian ethnicity. 54.1% met DSM-IV criteria for the combined subtype, while 35.6% and 10.3% were diagnosed with the inattentive and hyperactive subtypes, respectively. Among comorbid disorders, 40.6% had oppositional defiant disorder, 22.9% had conduct disorder, 46.2% had anxiety disorder (including specific phobias), and 8.8% had a

mood disorder (either/or major depressive episode, dysthymic disorder, manic episode, hypomanic episode).

Evaluations

The Conners' Global Index for Parents (Conners'-P) and for Teachers (Conners'-T) (Conners et al., 1998b, Conners et al., 1998a) were used to evaluate the behavior of the child at home and in the classroom, respectively. The Conners' Global Index scale has been validated from a genetic point of view, with research showing that genetic factors account for up to 78% of its variance (Hudziak et al., 2005).

Parents were also asked to complete the Child Behavior Checklist (CBCL), a comprehensive rating scale (113-item questionnaire) with well-established metric characteristics and representative norms (Achenbach, 1991). The raw scores of these scales were transformed into standardized *T* scores with an average of 50; where a score higher than 65 is considered to be clinical. The mean (standard deviation) for the total CBCL, Conners'-P, and Conners'-T scores were: 68.6 (8.9), 73.1 (11.4), and 69.5 (12.7), respectively, in this sample of children. Since it has been shown that a low to moderate correlation exists between parent and teacher reports of ADHD symptoms, and that each may assess a different dimension of the child's behavior (Mitsis et al., 2000, Thapar et al., 2006, Touliatos and Lindholm, 1981), by collecting information from both parents and teachers, a comprehensive assessment of the child's behavior was obtained.

In addition to clinical dimensions of ADHD, neuropsychological measures, mainly of executive function (EF), were included as quantitative traits in the genetic association analyses. EF encapsulates the range of cognitive abilities that are important for self-regulation and goal-directed behaviors, including response inhibition, sustained attention, working memory, set-shifting, planning, and organization. Deficits in EF have been implicated in the underlying pathophysiology of ADHD (Willcutt et al., 2005).

The following tests were included in the neuropsychological battery: Wisconsin Card Sorting Test (WCST; measure of cognitive flexibility and set-shifting) (Heaton et al., 1993), Tower of London test (TOL; planning, organization, and problem-solving capacity) (Shallice, 1982), Self-Ordered Pointing Task (SOPT; visual working memory, planning and response inhibition) (Petrides and Milner, 1982), Conners' Continuous Performance Test (CPT; attention, response inhibition, and impulse control) (Conners, 1995), and Finger Windows (FW; visual-spatial working memory) (Sheslow and Adams, 1990). The WCST, TOL, SOPT, and CPT were performed as described elsewhere (Gruber et al., 2007, Taerk et al., 2004). FW is a subtest of the Wide Range Assessment of Memory and Learning (WRAML). In this test, the child is required to repeat a sequential placement of a pencil into a series of holes on a plastic card, as conducted by the examiner. When children were medicated prior to their inclusion in the study, clinical and neuropsychological assessments were carried out at the end of a one-week washout period to limit variability due to medication effects (Kebir et al., 2009).

In addition to these EF measures, IQ (full scale, verbal, and performance) was evaluated using the Wechsler Intelligence Scale (WISC-III/IV) (Weschler, 1991).

Response to treatment with methylphenidate (MPH) was assessed in a double-blind, placebo-controlled, within-subject (crossover) randomized control trial conducted over a two-week period, as described (trial registration number: NCT00483106) (Grizenko et al., 2006). Following a one-week wash-out period, subjects received either one week of treatment with placebo (PBO) or one week of treatment with 0.5 mg/kg of MPH in a divided b.i.d. dose (0.25 mg/kg, morning and noon), and were then crossed over. At the end of each treatment week, parents and teacher were asked to evaluate the child's behavior using the Conners'-P and Conners'-T, respectively. Assessments were performed before and after the administration of PBO and MPH.

In addition, the clinical staff completed the Clinical Global Impression (CGI)-overall improvement scale based on their half day of behavioral observation while the child was completing various tasks in the clinic. In this study, MPH was used as a pharmacological probe to dynamically study the genetics of ADHD, rather than a classical trial of response to medication.

The Restricted Academic Situation Scale (RASS) was used to assess task-oriented behavior. During a simulated independent academic situation within a clinic setting (Barkley, 1990), the child is assigned a set of math problems and the RASS (coding system) is used to record the child's behavior as well as his or her

ability for sustained attention to routine, repetitive academic work in the presence of potential distractions, with no adult supervision (Fischer and Newby, 1998). The task has previously been described in detail (Sengupta et al., 2008). Over a 15 minute period, behavioral events are recorded at 30 second intervals, according to five categories: *off-task* (looking away from the task sheet), *playing with objects* (touching any object not directly used in the task), *out of seat* (lifting buttocks off chair or moving chair), *vocalizing* (any vocal noise, whether task related or not), and *fidgeting* (repetitive, purposeless movements). The RASS score is the total number of recorded behavioral events, and the difference score is obtained by subtracting the score after MPH administration from the score obtained after PBO. We have previously reported results from principal component analysis of the RASS (Karama et al., 2009) showing that off-task, out-of-seat, and playing with objects consist of one factor, while vocalizing and fidgeting appear to be independent factors.

Genotyping

Families were invited to participate in the genetic component of the study, where DNA was extracted, for each parent and child, from a blood sample, buccal swab, or saliva sample, if the subject was only amenable to the latter. Of the 377 nuclear families with one or more children diagnosed with ADHD, 184 were complete trios with information from both parents, 11 were trios with two affected children, 67 were trios with information from one parent and one or more

unaffected sibling, 103 were duos including the proband and one parent, while 12 were families with two affected siblings and one parent.

Tag SNPs within *SLC6A2*, previously examined in the IMAGE project, were genotyped (Brookes et al., 2006). Those with a very low minor allele frequency (MAF $\leq$ 0.02) were excluded, with one exception: rs11568324 (MAF= 0.01), since this SNP was shown to be associated with ADHD in the original IMAGE study (Brookes et al., 2006) and in a subsequent replication study (Kim et al., 2008b). Another SNP (rs28386840) which encodes a functional polymorphism in the upstream promoter region of *SLC6A2*, was also included in the panel, since it has been associated with ADHD (Kim et al., 2006). In order to extend the flanking region examined in *SLC6A2*, two SNPs (rs15534, present in exon 14; rs7188230, present in the 3' intergenic region) not genotyped in the IMAGE study, were also included in this study.

Sequenom iPlex Gold Technology (Ehrich et al., 2005) was used to genotype the panel of SNPs, where each plate included duplicates of two reference samples to estimate genotyping error. Genotypes for these samples were read with 100% accuracy on each of the plates. Five SNPs in the original panel in the IMAGE study (rs7201099, rs3760019, rs1362620, rs1861647, rs1566652) could not be genotyped on the Sequenom platform. Since these SNPs were in strong linkage disequilibrium (LD) with other SNPs in the panel, and were not shown to be specifically associated in any previous studies, they were excluded from subsequent analyses. The genotype distribution at each of the markers analyzed in

this study did not depart from Hardy-Weinberg equilibrium (Sengupta et al., 2012). By using genotype information from the current study (Stephens et al., 2001) and the default definition in Haploview (Gabriel et al., 2002), an LD plot was generated in Haploview v4.0. In this method, 95% confidence bounds on D' are generated for each pairwise comparison. A SNP block is formed if 95% of the informative comparisons are in strong LD with each other. As indicated by the color coded cells seen in Tables 1, 2 and 4, three major haplotype blocks exist in *SLC6A2*.

Statistical Analyses

Family-based tests of association (which examine the transmission disequilibrium of a specific allele/haplotype from parent to affected offspring) were conducted using the FBAT statistical package (version 2.0.3).(Horvath et al., 2001) All analyses were performed under the assumption of an additive model, with a null hypothesis of no linkage and no association. Tests were first conducted with the total sample, and then by maternal smoking during pregnancy (MSDP) stratification.

Of the total number of nuclear families in the study (n=377), we had information related to MSDP for 366 families, where 206 were coded as 'non-smoking' and 160 as 'smoking'.

Results

As noted in Tables 4.1.1, 4.1.2, and 4.1.3, marginal association was observed with several behavioral and cognitive dimensions of ADHD in the total sample. However, the most significant result was noted when FBAT analysis was conducted in the stratified group where mothers smoked during pregnancy (Table 4.2.1 and Supplementary Table). Whereas a marginal association was observed with rs36021 in the total sample (Z=2.54, P=0.01), a highly significant association was observed on every measure tested, as well as treatment response in the stratified sample. The *T* allele of this SNP appears to be the risk allele for ADHD, showing an association with the categorical DSM-IV diagnosis (Z=3.74, P=0.0002). In the quantitative FBAT analysis, the *T* allele was over-transmitted to the higher number of inattention (Z=3.91, P=0.00009), hyperactivity (Z=3.33, P=0.0009), and impulsivity (Z=2.93, P=0.003) items on the DISC-IV, higher CBCL total scores (Z=3.95, P=0.00008) (as well as each of the dimensional scores), higher restless-impulsive subscale scores of Conners'-T (Z=2.72, P=0.006) and Conners'-P (Z=2.75, P=0.006). Taken together, this suggests that the *T* allele is associated with more severe psychopathology, as assessed in the home, school, and clinic.

In terms of cognitive function, the risk allele was associated with worse performance on the SOPT (Z=3.69, P=0.0002), CPT and WCST (Table 4.2.2). Since the SOPT score is not a standardized score, higher scores imply worse performance, i.e. poor spatial working memory, planning, and response inhibition.

A highly significant association was observed with the CPT overall index (a weighted sum of all measures within the CPT) (Z=3.49, P=0.0005). The risk allele was over-transmitted to the higher scores, with higher *T*-scores implying worse performance. In particular, an association was noted with several dimensions evaluated in this test – hit reaction time (RT) standard error (SE) (Z=3.5, P=0.0005) and variability of SE (Z=3.0, P=0.003). High *T*-scores on these measures indicate highly variable reactions to the "target" and "non-target", often related to inattentiveness (Conners, 2000). Highly significant association was also observed with hit RT block change (Z=3.74, P=0.0002) and hit SE block change (Z=2.86, P=0.004). Here, the higher *T*-scores indicate a slowing in reaction time, as well as a loss of consistency, which together suggest a loss of vigilance, as the test progresses. The risk allele was also associated with poor performance on the WCST, which measures cognitive flexibility and set-shifting. The *T* allele showed an under-transmission (negative *Z* score) to the higher scores, specifically with non-perseverative errors (the higher standard scores imply a better performance on the test) (Z=-3.44, P=0.0006). No association was observed with perseverative errors or responses. On the WCST, perseverative errors occur due to an inability to shift set, despite negative feedback (Heaton et al., 1993). Non-perseverative errors are incorrect categorizations not related to perseveration, and usually arise from distractibility as well as deficits in updating and monitoring working memory. Therefore, it appears that in the group where mothers smoked during pregnancy, children with the *T* allele at rs36021 exhibit EF deficits, specifically sustained attention (characterized by distractibility during the task and loss of

vigilance as the test progresses), spatial working memory, planning, and response inhibition.

The *T* allele was also associated with response to MPH treatment (Table 4.2.3). The risk allele was associated with greater improvement as indexed by a higher change score (score after PBO – score after MPH) on the CGI (Z=3.275, P=0.001), Conners'-P (Z=2.62, P=0.009), as well as evaluation in the simulated academic environment, (Z=3.58, P=0.0003). Based on the factor structure of the RASS (Karama et al., 2009), change scores were examined for fidgeting, vocalizing and task disengagement. Association was observed with the task disengagement factor (Z=3.44, P=0.0006), but not with the other factors.

FBAT analysis in the group where mothers smoked during pregnancy also showed significant association between other SNPs towards the 5'end of *SLC6A2* and one or more behavioral/cognitive measures. These included: rs41154, rs187714, and to a lesser extent, rs4783899, rs2397771, and rs192303. Based on calculation of D' and r^2 in Haploview, it was noted that these markers are in strong LD with rs36021 (Table 4.3), explaining the parallel association observed on several of the measures. Conversely, markers that are not in strong LD with rs36021 (such as rs36023 and rs36024) do not show an association with ADHD or any of the relevant dimensions in this sub-group.

In the sample where mothers did not smoke during pregnancy, marginal association with rs3785152 was observed on several behavioral and cognitive

dimensions (Tables 4.4.1, 4.4.2, 4.4.3). In contrast, this SNP showed a highly significant association with treatment response. As with rs36021, the *C* allele was associated with significant improvement on behavioral evaluations; $CGI_{[PBO-MPH]}$ (Z=3.5, P=0.0005), RASS task-disengagement$_{(PBO-MPH)}$ (Z=3.58, P=0.0003). No association was observed with rs36021 in this group.

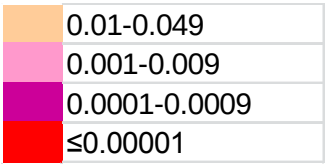
It is interesting that two adjacent SNPs (rs36021 and rs3785152) show highly divergent association in the two groups. In fact, LD between these two SNPs is low (Table 4.3). Therefore, it is likely that a recombination event at or close to these two SNPs resulted in at least two distinct variants of *SLC6A2*. Association was also observed with rs1814269, rs5569, rs998424, and rs36009 in this group, though the significance was marginal.

Tables

Table 4.1.1: Association between *SLC6A2* SNPs and ADHD behavioral dimensions in the total sample

Tag SNPs/Haplotypes	rs28386840	rs4783899	rs1362621	rs2397771	rs168924	rs2242446	rs3785143	rs192303	rs41154	rs187715	rs36024	rs187714	rs36023	rs36021	rs3785152	rs1814269	rs36017	rs10521329	rs3785155	rs5564	rs11568324	rs2279805	rs8047672	rs5569	rs998424	rs36009	rs1800887	rs2242447	rs15534	rs7188230
Alleles	A	T	A	G	T	T	C	G	G	A	C	C	C	T	C	A	G	C	G	T	C	C	G	C	C	G	T	T	C	A
Behavioral dimensions																														
ADHD																														
• Total number of DISC ADHD items																														
• Number of DISC inattentive items																														
• Number of DISC hyperactivity items																														
• Number of DISC impulsivity items																														
• Number of DISC oppositional defiant disorder items																														
• Number of DISC conduct disorder items																														
CBCL Total score																														
• CBCL Internalizing behavior																														
• CBCL Externalizing behavior																														
- CBCL Withdrawn																														
- CBCL Somatic complaints																														
- CBCL Anxious/depressed																														
- CBCL Social problems																														
- CBCL Thought problems																														
- CBCL Attention problems																														
- CBCL Delinquent behavior																														
- CBCL Aggressive behavior																														
Conners' P restless-impulsive baseline																														
Conners' T restless-impulsive baseline																														
Conners' P emotional lability baseline																														
Conners' T emotional lability baseline																														
Conners' P baseline																														
Conners' T baseline																														

Significance (*P*) values are provided according to this color scale:



Legend: Solid color indicates over-transmission of the risk allele and striped color indicates under-transmission. Three major haplotype blocks in *SLC6A2* are depicted above: block 1 in red, block 2 in blue, and block 3 in green. *Standard scores were used for all WCST and TOL measures, and T-scores were used for CPT measures (excl. overall index). DISC = Diagnostic Interview Schedule for Children, CBCL = Child Behavior Checklist, Conners' P = Conners' Parents, Conners' T = Conners' Teachers, WISC = Wechsler Intelligence Scale, SOPT = Self-Ordered Pointing Task, FW = Finger Windows, CPT = Continuous Performance Test, SE = standard error, RT = reaction time, ISI = inter-stimulus interval, WCST = Wisconsin Card Sorting Test, TOL = Tower of London, CGI = Clinical Global Impression, PBO = placebo, RASS = Restricted Academic Situation Scale.

Table 4.1.2: Association between *SLC6A2* SNPs and ADHD cognitive dimensions in the total sample

Tag SNPs/Haplotypes	rs28386840	rs4783899	rs1362621	rs2397771	rs168924	rs2242446	rs3785143	rs192303	rs41154	rs187715	rs36024	rs187714	rs36023	rs36021	rs3785152	rs1814269	rs36017	rs10521329	rs3785155	rs5564	rs11568324	rs2279805	rs8047672	rs5569	rs998424	rs36009	rs1800887	rs2242447	rs15534	rs7188230
Alleles	A	T	A	G	T	T	C	G	G	A	C	C	C	T	C	A	G	C	G	T	C	C	G	C	C	G	T	T	C	A
Cognitive dimensions																														
WISC IQ																														
• WISC Verbal IQ																														
• WISC Performance IQ																														
SOPT Total score																														
FW Score																														
CPT measures																														
• Overall index																														
• Omission errors																														
• Commission errors																														
• Hit Reaction Time																														
• Hit Reaction Time standard error																														
• Variability of standard error																														
• Detectability																														
• Response Style																														
• Perseveration																														
• Hit reaction time block change																														
• Hit SE block change																														
• Hit RT ISI change																														
• Hit SE ISI change																														
WCST Total errors standard score																														
WCST Perseverative responses																														
• WCST Perseverative errors																														
• WCST Non-perseverative errors																														
TOL																														

Table 4.1.3: Association between *SLC6A2* SNPs and treatment response in the total sample

Tag SNPs/Haplotypes	rs28386840	rs4783899	rs1362621	rs2397771	rs168924	rs2242446	rs3785143	rs192303	rs41154	rs187715	rs36024	rs187714	rs36023	rs36021	rs3785152	rs1814269	rs36017	rs10521329	rs3785155	rs5564	rs11568324	rs2279805	rs8047672	rs5569	rs998424	rs36009	rs1800887	rs2242447	rs15534	rs7188230
Alleles	A	T	A	G	T	T	C	G	G	A	C	C	C	T	C	A	G	C	G	T	C	C	G	C	C	G	T	T	C	A
Treatment response																														
CGI - improvement (PBO-active)																														
Conners' P restless-impulsive (PBO-active)																														
Conners' T restless-impulsive (PBO-active)																														
Conners' P emotional lability (PBO-active)																														
Conners' T emotional lability (PBO-active)																														
Conners' P (PBO-active)																														
Conners' T (PBO-active)																														
RASS total difference score (PBO time2-Active time2)																														
RASS fidgeting difference score																														
RASS vocalization difference score																														
RASS task disengagement difference score																														

Table 4.2.1: Association between *SLC6A2* SNPs and ADHD behavioral dimensions in the group where mothers smoked during pregnancy (MSDP)

Tag SNPs/Haplotypes	rs2386840	rs4783899	rs1362621	rs2397771	rs168924	rs2242446	rs3785143	rs192303	rs41154	rs187715	rs36024	rs187714	rs36023	rs36021	rs3785152	rs1814269	rs36017	rs10521329	rs3785155	rs5564	rs2279805	rs8047672	rs5569	rs998424	rs36009	rs1800887	rs2242447	rs15534	rs7188230
Alleles	A	T	A	G	T	T	C	G	G	A	C	C	C	T	T	A	G	C	G	T	C	G	A	T	G	T	T	C	A
Behavioral dimensions																													
ADHD																													
• Total number of DISC ADHD items																													
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• Number of DISC impulsivity items																													
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CBCL Total score																													
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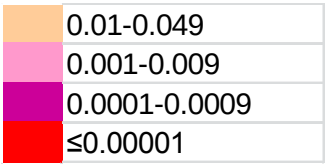
Significance (*P*) values are provided according to this color scale:



Legend: Solid color indicates over-transmission of the risk allele and striped color indicates under-transmission. Three major haplotype blocks in *SLC6A2* are depicted above: block 1 in red, block 2 in blue, and block 3 in green. *Standard scores were used for all WCST and TOL measures, and T-scores were used for CPT measures (excl. overall index). DISC = Diagnostic Interview Schedule for Children, CBCL = Child Behavior Checklist, Conners' P = Conners' Parents, Conners' T = Conners' Teachers, WISC = Wechsler Intelligence Scale, SOPT = Self-Ordered Pointing Task, FW = Finger Windows, CPT = Continuous Performance Test, SE = standard error, RT = reaction time, ISI = inter-stimulus interval, WCST = Wisconsin Card Sorting Test, TOL = Tower of London, CGI = Clinical Global Impression, PBO = placebo, RASS = Restricted Academic Situation Scale.

Table 4.2.2: Association between *SLC6A2* SNPs and ADHD cognitive dimensions in the MSDP group

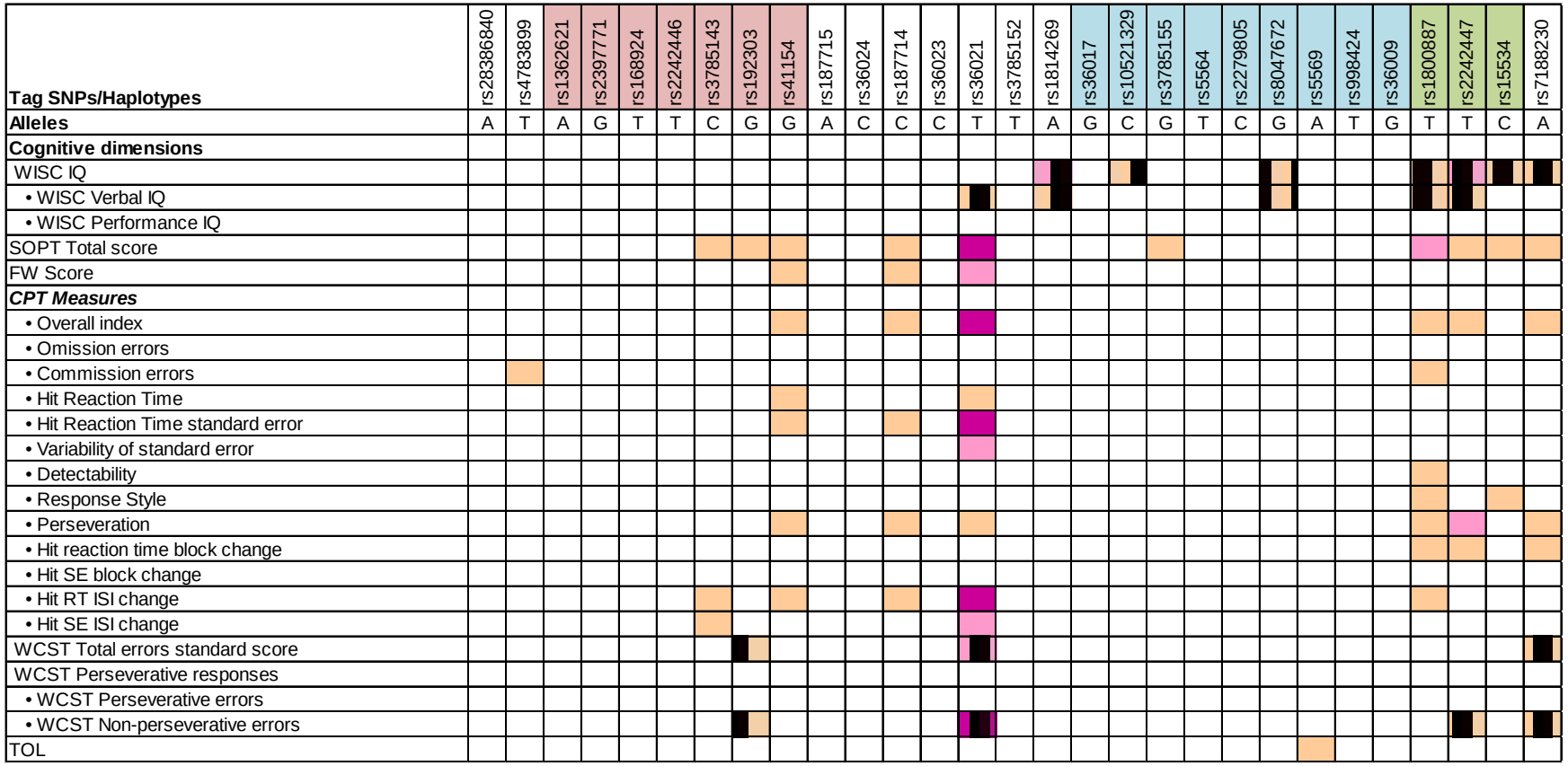


Table 4.2.3: Association between *SLC6A2* SNPs and treatment response in the MSDP group

Tag SNPs/Haplotypes	rs28386840	rs4783899	rs1362621	rs2397771	rs168924	rs2242446	rs3785143	rs192303	rs41154	rs187715	rs36024	rs187714	rs36023	rs36021	rs3785152	rs1814269	rs36017	rs10521329	rs3785155	rs5564	rs2279805	rs8047672	rs5569	rs998424	rs36009	rs1800887	rs2242447	rs15534	rs7188230
Alleles	A	T	A	G	T	T	C	G	G	A	C	C	C	T	T	A	G	C	G	T	C	G	A	T	G	T	T	C	A
Treatment response																													
CGI - improvement (PBO-active)																													
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Conners' P (PBO-active)																													
Conners' T (PBO-active)																													
RASS total difference score (PBO time2-Active time2)																													
RASS fidgeting difference score																													
RASS vocalization difference score																													
RASS task disengagement difference score																													

Table 4.3: Linkage disequilibrium between *SLC6A2* markers

LD with rs36021	D'	r-square		LD with rs3785152	D'	r-square
rs28386840	0,234	0,031		rs28386840	0,221	0,003
rs4783899	0,596	0,266		rs4783899	0,181	0,005
rs1362621	0,209	0,023		rs1362621	0,043	0
rs2397771	0,35	0,101		rs2397771	0,154	0,002
rs168924	0,659	0,096		rs168924	0,438	0,004
rs2242446	0,219	0,025		rs2242446	0,046	0
rs3785143	1	0,136		rs3785143	0,889	0,01
rs192303	0,78	0,333		rs192303	0,542	0,016
rs41154	0,919	0,431		rs41154	0,241	0,011
rs187715	0,958	0,05		rs187715	0,183	0
rs36024	0,333	0,109		rs36024	0,08	0,001
rs187714	0,917	0,445		rs187714	0,338	0,022
rs36023	0,202	0,03		rs36023	0,274	0,006
rs36021				rs36021	0,418	0,018
rs3785152	0,418	0,018		rs3785152		
rs1814269	0,091	0,004		rs1814269	0,483	0,021
rs36017	0,124	0,01		rs36017	0,152	0,003
rs10521329	0,296	0,023		rs10521329	0,033	0
rs3785155	0,386	0,026		rs3785155	0,511	0,005
rs5564	0,572	0,014		rs5564	0,535	0,123
rs11568324	1	0,009		rs11568324	1	0,002
rs2279805	0,143	0,014		rs2279805	0,009	0
rs8047672	0,29	0,022		rs8047672	0,071	0
rs5569	0,002	0		rs5569	0,133	0,001
rs998424	0,002	0		rs998424	0.1330	0,001
rs36009	0,444	0,011		rs36009	0,272	0,04
rs1800887	0,239	0,021		rs1800887	0,035	0,001
rs2242447	0,083	0,005		rs2242447	0,392	0,039
rs15534	0,319	0,025		rs15534	0,029	0,001
rs7188230	0,235	0,02		rs7188230	0,022	0

Legend: Five SNPs towards the 5'end of *SLC6A2* (rs41154, rs187714, rs4783899, rs2397771, rs192303) are in strong linkage disequilibrium (LD) with rs36021. LD = linkage disequilibrium

Table 4.4.1: Association between *SLC6A2* SNPs and ADHD behavioral dimensions in the sample where mothers did not smoke during pregnancy

Tag SNPs/Haplotypes	rs28386840	rs4783899	rs1362621	rs2397771	rs168924	rs2242446	rs3785143	rs192303	rs41154	rs187715	rs36024	rs187714	rs36023	rs36021	rs3785152	rs1814269	rs36017	rs10521329	rs3785155	rs5564	rs2279805	rs8047672	rs5569	rs998424	rs36009	rs1800887	rs2242447	rs15534	rs7188230
Allele	A	T	A	G	C	T	C	C	G	A	C	C	C	T	C	A	G	A	A	T	C	A	G	C	G	T	T	T	A
Behavioral dimensions																													
ADHD																													
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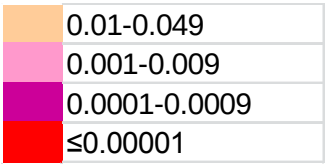
Significance (*P*) values are provided according to this color scale:



Legend: Solid color indicates over-transmission of the risk allele and striped color indicates under-transmission. Three major haplotype blocks in *SLC6A2* are depicted above: block 1 in red, block 2 in blue, and block 3 in green. *Standard scores were used for all WCST and TOL measures, and T-scores were used for CPT measures (excl. overall index). DISC = Diagnostic Interview Schedule for Children, CBCL = Child Behavior Checklist, Conners' P = Conners' Parents, Conners' T = Conners' Teachers, WISC = Wechsler Intelligence Scale, SOPT = Self-Ordered Pointing Task, FW = Finger Windows, CPT = Continuous Performance Test, SE = standard error, RT = reaction time, ISI = inter-stimulus interval, WCST = Wisconsin Card Sorting Test, TOL = Tower of London, CGI = Clinical Global Impression, PBO = placebo, RASS = Restricted Academic Situation Scale.

Table 4.4.2: Association between *SLC6A2* SNPs and ADHD cognitive dimensions in the sample where mothers did not smoke during pregnancy

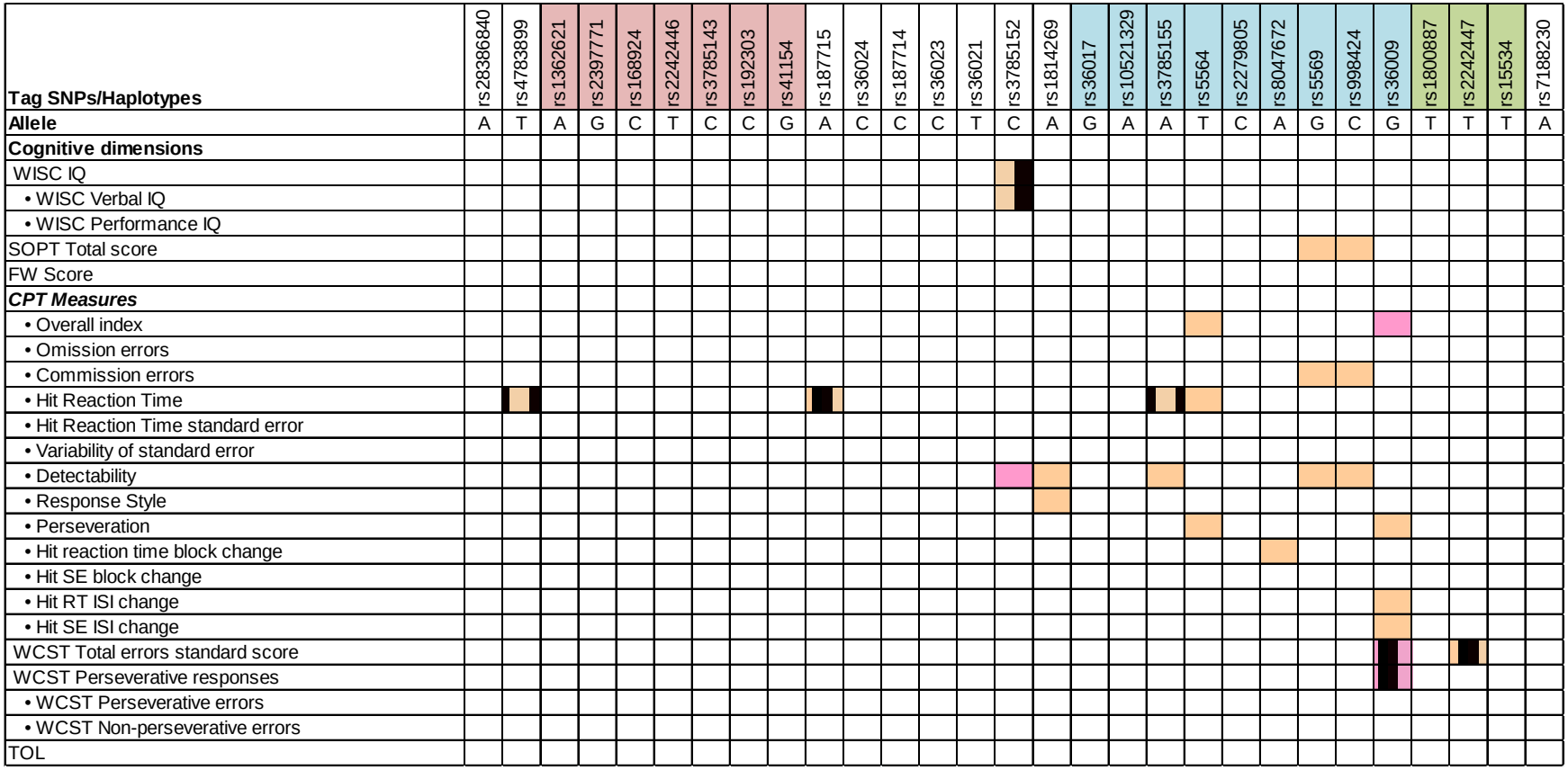


Table 4.4.3: Association between *SLC6A2* SNPs and treatment response in the sample where mothers did not smoke during pregnancy

Tag SNPs/Haplotypes	rs28386840	rs4783899	rs1362621	rs2397771	rs168924	rs2242446	rs3785143	rs192303	rs41154	rs187715	rs36024	rs187714	rs36023	rs36021	rs3785152	rs1814269	rs36017	rs10521329	rs3785155	rs5564	rs2279805	rs8047672	rs5569	rs998424	rs36009	rs1800887	rs2242447	rs15534	rs7188230
Allele	A	T	A	G	C	T	C	C	G	A	C	C	C	T	C	A	G	A	A	T	C	A	G	C	G	T	T	T	A
Treatment response																													
CGI - improvement (PBO-active)																													
Conners' P restless-impulsive (PBO-active)																													
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Conners' T (PBO-active)																													
RASS total difference score (PBO time2-Active time2)																													
RASS fidgeting difference score																													
RASS vocalization difference score																													
RASS task disengagement difference score																													

Supplementary Table:

Effect sizes for the association between *SLC6A2* tag SNPs and ADHD in the maternal smoking during pregnancy (MSDP) group

Marker	Allele	afreq	No of informative families	*Z*	*P* value	Effect size
rs28386840	A	0.71	52	1.74	0.08193	0.24
rs4783899	T	0.50	72	2.22	0.02622	0.26
rs1362621	A	0.71	55	1.11	0.26763	0.15
rs2397771	G	0.62	65	2.01	0.04495	0.25
rs168924	T	0.86	51	1.21	0.22650	0.17
rs2242446	T	0.71	55	1.22	0.22295	0.16
rs3785143	C	0.91	30	1.78	0.07454	0.32
rs192303	G	0.71	66	2.15	0.03191	0.26
rs41154	G	0.41	67	2.97	0.00298	0.36
rs187715	A	0.95	20	0.65	0.51925	0.15
rs36024	C	0.57	62	1.58	0.11452	0.20
rs187714	C	0.42	65	2.76	0.00582	0.34
rs36023	C	0.62	68	0.94	0.34523	0.11
rs36021	T	0.58	63	3.75	0.00018	0.47
rs3785152	T	0.12	26	0.18	0.85644	0.04
rs1814269	A	0.43	62	0.43	0.66501	0.05
rs36017	G	0.44	63	0.44	0.65694	0.06
rs10521329	C	0.81	50	1.01	0.31393	0.14
rs3785155	G	0.88	42	1.5	0.13265	0.23
rs5564	T	0.96	14	0.51	0.60952	0.14
rs2279805	C	0.46	66	0.64	0.52504	0.08
rs8047672	G	0.82	50	1.01	0.31393	0.14
rs5569	A	0.35	56	0.36	0.71798	0.05
rs998424	T	0.35	55	0.12	0.90277	0.02
rs36009	G	0.94	18	0.46	0.64921	0.11
rs1800887	T	0.78	61	1.95	0.05134	0.25
rs2242447	T	0.67	66	1.87	0.06195	0.23
rs15534	C	0.83	48	1.62	0.10516	0.23
rs7188230	A	0.78	55	1.62	0.10427	0.22

***Note*:** The most significant result is seen for SNP rs36021 with a large effect size of 0.47.

Discussion

Conducting stratified analyses based on MSDP provides great insight into the complex association between *SLC6A2* and ADHD. Although pharmacological, imaging, and neuropsychological studies have extensively implicated the norepinephrine transporter in ADHD, genetic studies have shown a minimal association. Although associations have been reported, non-replication between studies has resulted in a lack of overall significance when a meta-analysis was conducted (Forero et al., 2009). Results presented here, and in an earlier report (Choudhry et al., 2012), support the view that the lack of replication between studies may be explained, at least in part, by the inherent clinical and etiological complexity of the disorders.

The association between MSDP and ADHD is one of the most investigated in the field of environmental psychiatric epidemiology. Although consistently replicated (Linnet et al., 2003, Linnet et al., 2005, Obel et al., 2009) and high in magnitude, there is now relative consensus that this association has little causal significance (Lindblad and Hjern, 2010, Obel et al., 2011) and may instead be driven by other variables that are shared by the behavior of smoking during pregnancy in mothers and ADHD in their children. While environmental factors may play a role in this association, it is believed that genetic factors shared by mother and child play an important role in smoking during pregnancy in the former and ADHD in the latter.

In this study, MSDP was used to index a subtype of ADHD with putatively more homogeneous genetic determinants shared within families of children with ADHD where mothers smoked during pregnancy.

Consistent with this hypothesis, we have reported (Thakur et al., 2012) that children in this subgroup present a more severe clinical picture with greater behavioral problems and lower cognitive function, when compared to children whose mothers did not smoke during pregnancy, and that this difference in clinical phenotype is significant even when important environmental factors are controlled for. The results of the current study emphasize the genetic differences in these two subtypes. Polymorphisms (rs36021 and linked SNPs) are important genetic determinants of behavior, cognition, and treatment response in ADHD children whose mothers smoked during pregnancy, and who may represent a more homogeneous group of ADHD patients, as previously reported (Thakur et al., 2012). In the subtype where mothers did not smoke during pregnancy, an association with a different region of the gene (towards the 3' end of *SLC6A2)* is observed.

Given that the association between ADHD and rs36021 (and linked SNPs) is highly significant only in those children whose mothers smoked during pregnancy may suggest a true interaction between exposure to maternal smoking and carrying the risk allele(s) in the SLC6A2 gene. Indeed, the adverse consequences of *in utero* exposure to the toxic effects of nicotine are well documented, from animal and human studies (Ernst et al., 2001). MSDP is associated with pre- and

peri-natal complications, deficits in cognitive development as well as long-term behavioral problems. Alternatively, but not exclusively, the etiology of smoking behavior and ADHD may involve closely related, but distinct pathways. Indeed, it is possible that the complex genetic background underlying smoking behaviors in mothers (which is transmitted in part to their children), interacts with risk alleles in *SLC6A2* to increase the risk for ADHD in children. Under the latter scenario, MSDP may be considered as a phenotypic index used to select a subgroup of children with relatively more homogeneous genetic etiology.

Irrespective of the precise links between these pathways, this study strongly suggests that genetic variation in the *SLC6A2* is an important factor in a more severe subtype of ADHD. If replicated in independent studies, this may represent an important step towards *personalized medicine* in treating children with ADHD (Wallis, 2010).

Results of the present study are perfectly congruent with reports by Song *et al* (Song et al., 2011a) and Yang *et al* (Yang et al., 2004), but only in the group where mothers did not smoke during pregnancy. In this group, a significant over-transmission of the *G* allele to the higher difference scores was observed in the quantitative FBAT analysis on the Conners'-T (Table 4.4.2). Most of this effect appears to arise from the restless-impulsive factor scores, observed only in the group of non-smoking mothers. It is noted that when treatment response was assessed using the CGI-Improvement scale, two previous studies (Kim et al., 2010,

Lee et al., 2011), as well as the current study, did not find an association with 1287(G/A) (rs5569) (Table 4.4.3).

Several other previously-reported associations were replicated in the present study. Three studies had reported an association with rs3785143 and rs11568324 (Brookes et al., 2006, Kim et al., 2008b, Xu et al., 2008). These markers are in complete LD with rs36021 (*D'*=1; albeit with a low correlation coefficient, r^2; Table 4.3), indicating that the 3 SNPs are in one haplotype block not separated by a recombination event. In the total sample, rs3785143 showed marginal association with ADHD, but a significant association with all CBCL dimensional scores (Table 4.1.1). No association was observed when stratified analyses were carried out. Similarly, no association was observed with rs11568324 despite the fact that it is in complete LD with rs36021. This is most likely a result of the low heterozygosity of these markers, which make them non-informative in the FBAT analysis (as indicated by the number of informative families in Table 4.1.1). Two other previously-implicated SNPs, rs998424 and rs36017, showed marginal association with dimensions of ADHD in the sample where mothers did not smoke during pregnancy and the total sample, respectively.

Kim and colleagues (Joung et al., 2010, Kim et al., 2006, Kim et al., 2008a) reported an association between ADHD and a functional promoter SNP rs28386840 [-3081(A/T)] in several independent case-control studies. This association was not replicated in the current study, neither in the total sample, nor in the samples stratified by MSDP (Tables 4.1.1, 4.2.1, 4.4.1). The lack of

association with ADHD was also reported in two other family-based studies (Cho et al., 2008, Renner et al., 2011). A study examining the association between this polymorphism and treatment response reported an association with CGI-improvement scores (Kim et al., 2010), where *T*-allele carriers showed a better response to MPH treatment. In the current study, only a marginal association was observed with difference scores on the restless-impulsive subscale of the Conners'-T in the group where mothers smoked during pregnancy (Table 4.2.3).

In a previous report (Sengupta et al., 2012), we investigated the association between ADHD and the panel of 30 SNPs examined in the present study, and noted that a complex pattern of association emerged between *SLC6A2* SNPs/haplotypes, ADHD subtypes and gender. Gender and subtype are considered two dimensions that might help in reducing genetic heterogeneity in the ADHD syndrome. Although these results helped explain some of the discrepancies noted among previous studies, stratification according to these dimensions did not yield as strong an association with *SLC6A2* as the stratification based on MSDP, which may suggest that the latter is more pertinent for future efforts to map genes implicated in ADHD.

SNPs that showed the most significant association in this study (rs36021 and rs3785152, in particular) are within introns, opening up two possibilities. The first possibility is that these intronic variants are involved in gene regulation. The second is that these polymorphisms are not the causal mutation, but are in LD with a functional variant. Fine-mapping of the region is required to identify the

causal mutation(s) followed by molecular analysis to determine if the mutation affects transcriptional regulation of the gene or structure and function of the protein.

While we conducted a large number of comparisons and some correction for multiple testing is warranted, it is important to note that when we correct for multiple testing in relation to our primary hypothesis, that is association between *SLC6A2* and ADHD in children stratified according to MSDP, the primary result of association (Z=3.74, P=0.0002) with rs36021 remains significant even if we apply the overly stringent Bonferroni correction (30 SNPs times two exposure strata, p = 0.002).

In addition, the widespread exploratory associations that are observed with behaviors relevant to ADHD measured by different observers (parents, teachers, and research staff) and in different settings (school, home, clinic) with rs36021 suggest that these associations are unlikely to be chance findings. We believe that this considerable consistency of results strengthens the overall credibility of the primary results and help to understand how genetic vulnerability to ADHD is mediated through the traits and endophenotypes underlying this disorder.

To our knowledge, this is the largest study (among family-based and case-control studies) testing the association between ADHD and *SLC6A2*, with such detailed genotype and phenotype characterization. While collaboration between multiple research groups in large consortia is vital for genetic studies of ADHD, it has been

shown that a significant amount of heterogeneity can be introduced in multicenter collaborative studies because of divergent clinical and evaluation practices (Muller et al., 2011). This underscores the value of the current study where a relatively large sample has been collected at a single center using a highly standardized approach. It is also the largest study worldwide to use a double-blind, placebo-controlled design for evaluation of treatment response, combining extensive evaluation of executive function and behavioral domains, with genetic and environmental data. Nonetheless, these results must be considered exploratory and independent replication is awaited.

If confirmed in independent studies, these results will help to disentangle the complex etiological pathways of ADHD. In the long term, this would very likely lead to development of therapeutics targeting specific biochemical pathways in specific sub-groups of children with ADHD.

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Author Contributions: R. Joober and N. Grizenko designed the study and acquired the data. G.A. Thakur, S.M. Sengupta, Z. Choudhry and R. Joober analyzed the data. G.A. Thakur, S.M. Sengupta, and R. Joober wrote the article. N. Grizenko and Z. Choudhry reviewed the article. All authors approved its publication.

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CHAPTER 5

Family-Based Association Study of ADHD and Genes Increasing the Risk for Smoking Behaviors

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Preface

Although studying genes identified in Genome-Wide Association Studies (GWAS) of ADHD will help to further our understanding of this complex disorder, a different approach for selecting candidate genes may be necessary. Given that ADHD and cigarette smoking are two highly comorbid phenotypes (35%-45%) and that maternal smoking during pregnancy (MSDP) plays an important role in the pathogenesis of ADHD, we decided to investigate genes identified through GWAS of disorders comorbid to ADHD, namely smoking behavior. Given that comorbidity among these two phenotypes could be due to shared genetic factors, we selected five single nucleotide polymorphisms (SNPs) located in different genes and loci highly associated with different dimensions of smoking behavior and studied them in ADHD.

In this chapter, we conducted family-based association tests to study transmission of risk alleles within these five SNPs in relation to clinical diagnosis of ADHD, and a number of behavioral and neurocognitive phenotypes relevant to the disorder. By using comorbid disorders to investigate ADHD genetics, we identified a novel association between the C* risk allele of rs1329650, a 10q25 SNP located in a non-coding RNA (*LOC100188947*), which may increase the risk for ADHD and smoking behavior through a common mechanism, possibly externalizing behaviors and specific cognitive deficits that manifest as ADHD in childhood and are the gateway to smoking behavior later in life. By investigating SNPs associated with disorders comorbid to ADHD, we may be able to further decipher the genetics of ADHD.

Abstract

Objective: To investigate five top single nucleotide polymorphisms (SNPs) located in different genes and loci (*CHRNA3*, *BDNF*, *DBH* and *LOC100188947*) that were highly associated with different dimensions of smoking behavior, in relation to ADHD. **Design:** Cohort study consisting of a clinical sample of children with ADHD. **Setting:** Douglas Institute ADHD Clinic in Montreal, Canada. **Patients:** Families of 454 children with ADHD aged 6-12 years old. **Interventions:** Family-based association tests used to study the transmission of risk alleles within these five genetic markers. **Main outcome measures:** Clinical diagnosis of ADHD, and a number of behavioral and neurocognitive phenotypes relevant to the disorder. **Results:** One SNP (rs1329650) from a non-coding RNA (*LOC100188947*) was significantly associated with overall ADHD diagnosis with the C* risk allele being over-transmitted from parents to children with ADHD ($p=0.02$). It was also over transmitted to children with higher scores on Conners' Parents ($p=0.01$) and Conners' Teacher ($p=0.002$) index scores, and Child Behavior Checklist withdrawn ($p=0.001$) and aggressive ($p=0.007$) behaviors. Children with poorer performances on executive and attention tasks were more likely to inherit the risk allele. **Conclusions:** The C* allele of rs1329650 may be increasing the risk for ADHD and smoking behavior through a common mechanism, possibly externalizing behaviors and specific cognitive deficits that manifest as ADHD in childhood and are the gateway to smoking behavior later in life. This exploratory study illustrates the use of comorbid disorders to investigate ADHD genetics. In spite of its relatively large sample size, replication in future studies is warranted.

Introduction

Attention-Deficit Hyperactivity Disorder (ADHD) is an etiologically complex, heterogeneous, highly heritable, neurobehavioral childhood disorder with 8-12% prevalence in the general population (Biederman, 2005). In spite of this high heritability, identifying genes in ADHD has been a challenging task.

Five genome-wide association studies (GWAS) of ADHD have been conducted (Lasky-Su et al., 2008, Lesch et al., 2008, Mick et al., 2010, Neale et al., 2008, Neale et al., 2010) and identified 85 top-ranked ADHD candidate genes ($p<0.0001$). However, none of the findings passed the GWAS significance threshold.

ADHD and cigarette smoking are two highly comorbid phenotypes with rates of comorbidity varying between 35% to 45% (Pomerleau et al., 1995). ADHD subjects begin smoking at an earlier age and are likely to smoke twice as much as controls. Evidence suggests that a history of childhood ADHD may predict worse smoking cessation outcomes (Humfleet et al., 2005). Although the underlying mechanisms of ADHD and smoking have yet to be fully understood, an integrated model of their comorbidity has been proposed (McClernon and Kollins, 2008) stating that both neurobiological and psychosocial factors may contribute to an increased risk of nicotine use and dependence in ADHD (McClernon and Kollins, 2008).

Neuropsychological theories suggest that children with ADHD may have deregulations of executive functions (EFs), including inhibitory control (Barkley, 1997, Quay, 1997), resulting in increased impulsivity, risk taking, and novelty seeking behavior, thereby increasing the risk for later substance/alcohol abuse and cigarette smoking (Stephens et al., 2011). ADHD may itself be a risk for smoking later in life, and early treatment with stimulant medication seems to have a protective effect in adolescence (Wilens and Morrison, 2011).

Converging data suggest that these two phenotypes may share underlying neurobiological mechanisms (McClernon and Kollins, 2008), related to monoaminergic transmission, specifically an altered dopamine/norepinephrine as well as cholinergic transmission. Thus, it is highly likely that genes implicated in smoking behavior may also increase the risk for ADHD and vice versa.

Smoking behavior is a complex phenotype with several genetic factors involved. Interestingly, genetic studies of smoking behavior have witnessed important advances in recent years due to the power of large scale GWAS. Meta-analytic results were reported from three GWAS smoking consortia: the Tobacco and Genetics (TAG) Consortium (16 studies, population-based and case-control), the European Network of Genetic and Genomic Epidemiology (ENGAGE), and the Oxford-GlaxoSmithKline (Ox-GSK) consortia, where a number of loci were identified and associated with different dimensions of smoking behavior, such as number of cigarettes smoked per day, smoking initiation and cessation (Tobacco and Genetics Consortium, 2010).

Among the five top associated markers, a synonymous single nucleotide polymorphism (SNP) in the nicotinic receptor gene *CHRNA3* (rs1051730) was associated with number of cigarettes smoked per day ($p<3x10^{-70}$), several SNPs in the *BDNF* gene were associated with smoking initiation ($p<5x10^{-8}$) and one SNP near the *DBH* gene (rs3025343) was associated with smoking cessation ($p<4x10^{-8}$). In addition, two 10q25 SNPs (rs1028936 and rs1329650), located in a non-coding RNA (*LOC100188947*), were very highly associated with number of cigarettes smoked per day ($p<2x10^{-9}$ and $p<6x10^{-10}$, respectively) (Tobacco and Genetics Consortium, 2010). Interestingly, two of these SNPs have previously been implicated in externalizing behaviors often seen in ADHD. More specifically, in an adolescent sample, an association with the *CHRNA5/CHRNA3/CHRNB4* locus and externalizing behaviors was reported (Stephens et al., 2011). Additionally, in a community-based cohort of 1,236 Swedish individuals, multivariate regression analysis showed that the Met allele of the Val66Met polymorphism in the *BDNF* gene was associated with ADHD, where the association was primarily driven by persistent hyperactivity-impulsivity symptoms (Bergman et al., 2011). Various polymorphisms within the *DBH* gene were linked with poorer cognitive performance in ADHD children (Barkley et al., 2006, Kieling et al., 2008), especially on tasks indexing cognitive impulsiveness. However, the two 10q25 SNPs have not yet been studied in ADHD or related phenotypes. In this study, we investigated transmission of risk alleles within these five SNPs in 454 ADHD families with respect to clinical diagnosis of ADHD and quantitative (behavioral and neurocognitive) phenotypes relevant to ADHD.

Methods

Subjects

Four hundred and fifty-four ADHD subjects were sequentially recruited from the Disruptive Behavior Disorders Program and the child psychiatry outpatient clinics at the Douglas Mental Health University Institute (DMHUI) in Montreal. They were referred to these specialized care facilities by schools, community social workers, family doctors and pediatricians.

Children were diagnosed with ADHD using DSM-IV criteria (Lahey et al., 1994) and based on clinical interviews of the child and at least one parent by a child psychiatrist (RJ or NG). A structured clinical interview of parents using the Diagnostic Interview Schedule for Children-IV (DISC-IV) (Shaffer et al., 2000) and school reports were used to corroborate the diagnoses. Mothers were primary informants in most cases. Details about diagnostic procedures have been described elsewhere (Grizenko et al., 2006).

Children with a history of Tourette's syndrome, pervasive developmental disorder, or psychosis were excluded. The research protocol was approved by the Research Ethics Board of the DMHUI. Parents provided written informed consent while children gave their verbal assent.

Maternal Smoking During Pregnancy

The Kinney Medical Gynecological Questionnaire (McNeil et al., 1994) was used to systematically evaluate pregnancy, delivery, and perinatal complications. Mothers retrospectively reported maternal smoking (yes/no) during pregnancy (MSDP). We had information pertaining to MSDP exposure for 394 of the families in the study: 171 were categorized as smoking and 223 as non-smoking families. In the families where mothers smoked during pregnancy, 76.6% of affected children were boys, whereas 81.6 % were boys in the unexposed group. The mean age of exposed children was 9.1 years (SD=1.7) and 8.9 years (SD=1.8) in unexposed children. Neither gender (χ^2=1.49, df=1, p=0.22) nor age ($F_{1,393}$=0.38, p=0.54) differed among the two groups of children.

Behavioral evaluations

The Child Behavior Checklist (CBCL) (Achenbach, 1991), which assesses children's behavioral and emotional problems, was completed by the parents and the child's overall behavior (without a specific timeframe) was evaluated.

The Conners' Global Index for parents (CGI-P) and teachers (CGI-T) (Conners, 1999) were used to assess behaviors relevant to ADHD in home and school settings, respectively. The CGI-P and CGI-T are subsets of the original Conners' Rating Scales, which are widely used to assess ADHD symptoms and other psychopathology in children between 3 and 17 years of age. The raw total scores are transformed into normalized T-scores. All assessments were completed while children were not taking any medication.

Neurocognitive assessment

A neuropsychological battery of tests was used to study attention and EFs in these children. Assessments were carried out at the end of a 1-week washout period if children were previously medicated. Full scale, verbal, and performance IQ were evaluated using the Wechsler Intelligence Scale (WISC) (Weschler, 1991). Children with IQ < 70 were excluded from the study. The Wisconsin Card Sorting Test (WCST) (Heaton et al., 1993), the Wide Range Assessment of Memory and Learning Finger Windows (FW) subtest (Sheslow and Adams, 1990), the Tower of London test (TOL) (Shallice, 1982), the Self-Ordered Pointing Task (SOPT) (Petrides and Milner, 1982), and the Conners' Continuous Performance Test (CPT) (Conners, 1995) were carried out.

These tasks are conceptually used to respectively assess mental flexibility, visual working memory, planning capacities, working memory and response inhibition and attention profile, respectively.

Genotyping and marker selection

DNA was extracted from a blood, buccal swab, or saliva sample from each affected child, parents, and unaffected siblings, whenever possible. Based on findings from the TAG study, five markers associated with different dimensions of smoking behavior were selected.

The panel of SNPs was genotyped using Sequenom iPlex Gold Technology (Ehrich et al., 2005). Every plate included duplicates of two reference samples to

estimate genotyping error and genotypes were read with 100% accuracy on each of the plates. The genotype distribution of all five markers did not depart from Hardy-Weinberg equilibrium: rs1051730 (p=0.96), rs1028936 (p=0.11), rs1329650 (p=0.12), rs3025343 (p=0.49) and rs6265 (p=0.23).

Family-Based Association Tests analysis

Single SNP tests of association were performed using family-based association tests (FBAT) to investigate the association between selected markers with ADHD diagnosis and quantitative phenotypes relevant to ADHD (v2.0.3 Harvard School of Public Health, Departments of Biostatistics and Environmental Health, Program for Population Genetics, Boston, MA, USA) (Laird et al., 2000).

First, the overall association between each of these markers and ADHD diagnosis was investigated. Second, association between a number of behavioral and cognitive quantitative traits was tested. Offsets used in the FBAT analysis were based on average scores found in the population (50 in the case of T-scores). All tests were performed under the assumption of an additive model, with a null hypothesis of no linkage and no association. Further details pertaining to the principle of FBAT have been described elsewhere (Choudhry et al., 2012).

Haploview v4.0 was used to determine linkage disequilibrium between the two 10q25 SNPs and haplotype analysis was then conducted in FBAT for all the ADHD phenotypes. Given the exploratory nature of this study, the significance level was set at $p = 0.05$.

To obtain a rough estimate of effect size, we calculated the effect size *F* as for a χ^2 test, using the following formula F = square root $[\chi^2/N\ (k - 1)]$, where N indicates sample number, and k indicates the number of rows or columns or 2 in the McNemar's test. This was based on the assumption that FBAT is an extension of McNemar's test used to calculate transmission disequilibrium in a pedigree, where $\chi^2_{TDT} = (T - NT)^2 / (T + NT)$. T and NT denote the number of transmissions and non-transmissions of a specific allele from the parent to the affected offspring. In a specific case of the FBAT (where both parents are known, and when the additive model is used), the Z^2 statistic can be considered equivalent to a χ^2_{TDT} statistic (N. Laird, personal communication, 5 January 2012).

The number of informative families was used to calculate N. Effect sizes of 0.1, 0.3, and 0.5 are considered small, medium and large, respectively.

Results

ADHD clinical diagnosis

Only one SNP (rs1329650) was nominally significantly associated with overall ADHD diagnosis (*p=0.02*) with a small effect size (ES) of 0.19, in the total sample with the C* allele over-transmitted from parents to children with ADHD (Table 5.1).

Haplotype analysis of the two 10q25 SNPs showed they are in strong linkage disequilibirum (D' = 0.98, r^2=0.59). FBAT analysis of the four haplotypes derived from these two SNPs showed that the A-C haplotype containing the A* allele from rs1028936 and the C* allele from rs1329650, was significantly over-transmitted in children (*p=0.02*, ES = 0.2), whereas none of the other haplotypes showed a significant association with ADHD, suggesting that the association is mainly driven by the C* allele in rs1329650.

When the total sample was stratified by MSDP, a marginally significant association between ADHD diagnosis and the risk variant in rs1329650 was distributed evenly between the two exposure subgroups (p=0.08 for both groups). Since none of the other genes were associated with ADHD diagnosis (before or after stratification with regard to MSDP, Supplementary Table), we further investigated the two 10q25 SNPs and their derived haplotypes with respect to a number of quantitative behavioral and cognitive traits in ADHD children.

Quantitative behavioral traits

A pattern of associations was formed with the two 10q25 SNPs and their derived A-C haplotype with several behavioral traits (Table 5.2). For rs1028936, the A* allele was significantly over-transmitted to children with higher T-scores on the CGI-P (*p=0.03*, ES = 0.22) and CGI-T (*p=0.02*, ES = 0.24), as well as the withdrawn (*p=0.01*, ES = 0.26) and aggressive (*p=0.008*, ES = 0.26) dimensions of the CBCL. For rs1329650, the C* allele was significantly over-transmitted to children with a higher total number of ADHD items (*p=0.04*, ES = 0.18), T-scores on the CGI-P (*p=0.01*, ES = 0.21) and CGI-T (*p=0.002*, ES = 0.27) and several dimensions of the CBCL behaviors T-scores: externalizing (*p=0.02*, ES = 0.21), withdrawn (*p=0.001*, ES = 0.3), attention (*p=0.01*, ES = 0.21) and aggressive (*p=0.007*, ES = 0.24). The A-C haplotype results mimicked those of the C* allele of rs1329650 with similar effect sizes (Table 5.2).

Cognitive traits

Several cognitive traits were significantly associated with both of the 10q25 SNPs and their derived A-C haplotype (Table 5.3). For rs1028936, the A* allele was associated with poorer performance on the WCST, more specifically, number of total (*p=0.02*, ES = 0.24) and non-perseverative (*p=0.01*, ES = 0.26) errors. For rs1329650, the C* allele was also associated with poorer performance on the WCST, number of total and non-perseverative errors (*p=0.03*, ES = 0.2), as well as SOPT total score (*p=0.02*, ES = 0.2), and the CPT omissions t-score (*p=0.04*, ES = 0.19). Again, the results and effect sizes obtained with the A-C haplotype mirrored those of the C* allele of rs1329650 (Table 5.3).

Tables

Table 5.1: FBAT analysis of genes increasing the risk for smoking behaviors in children with ADHD

Marker (Chromosome)	rs number	Alleles	Allele frequency	# of informative families	Z statistic	p-value	Effect size
CHRNA3 (15q25)	rs1051730	C	0.661	160	1.52	0.13	0.12
		T	0.339	160	-1.52	0.13	0.12
LOC100188947 (10q25)	rs1028936	A	0.827	108	1.44	0.15	0.14
		C	0.173	108	-1.44	0.15	0.14
LOC100188947 (10q25)	**rs1329650**	**A**	**0.252**	**141**	**-2.30**	***0.02***	**0.19**
		C	**0.748**	**141**	**2.30**	***0.02***	**0.19**
near *DBH* (9)	rs3025343	A	0.109	83	0.37	0.72	0.04
		G	0.891	83	-0.37	0.72	0.04
BDNF (11p13)	rs6265	A	0.184	119	-0.79	0.43	0.07
		G	0.816	119	0.79	0.43	0.07

Legend: ADHD, attention-deficit hyperactivity disorder; FBAT, family-based association tests; *CHRNA3*, cholinergic receptor, nicotinic, alpha 3; *DBH*, dopamine beta-hydroxylase; *BDNF*, brain-derived neurotrophic factor.

The only significant association observed between one of the genetic markers and overall ADHD diagnosis is highlighted in bold.

Table 5.2: Association between two *LOC100188947* SNPs and derived haplotypes with behavioral traits in a sample of children with ADHD

	Total sample					
	rs1028936	rs1329650	rs1028936 rs1329650	rs1028936 rs1329650	rs1028936 rs1329650	rs1028936 rs1329650
Alleles	**A**	**C**	**A-C**	**C-A**	**A-A**	**C-C**
ADHD						
Total DISC ADHD items						
Conners'-Parents						
Conners'-Teachers						
CBCL total						
CBCL internalizing						
CBCL externalizing						
CBCL withdrawn						
CBCL somatic complaints						
CBCL anxious/depressed						
CBCL social problems						
CBCL thought problems						
CBCL attention problems						
CBCL delinquent behavior						
CBCL aggressive behavior						

P values are provided according to this color scale:

	0.01-0.05
	0.001-0.009

Legend: ADHD, attention-deficit hyperactivity disorder; CBCL, Child Behavioral Checklist; DISC, Diagnostic Interview Schedule for Children; SNP, single nucleotide polymorphism. Solid color indicates under transmission of the rs1329650 risk allele (C*). Striped color indicates over transmission of the risk allele. Higher T-scores on the DISC, Conners'- parents and Conners'-teachers, and each of the CBCL dimensional scores indicate worse behavior. When risk alleles are over-transmitted (striped boxes) to the higher scores (positive Z statistic), children with this genotype have a more severe clinical presentation.

Table 5.3: Association between two *LOC100188947* SNPs and derived haplotypes with cognitive traits in a sample of children with ADHD

	Total sample					
	rs1028936	rs1329650	rs1028936 rs1329650	rs1028936 rs1329650	rs1028936 rs1329650	rs1028936 rs1329650
Alleles	**A**	**C**	**A-C**	**C-A**	**A-A**	**C-C**
WISC full scale IQ						
WISC verbal IQ						
WISC performance IQ						
WCST total errors						
WCST perseverative responses						
WCST perseverative errors						
WCST non-perseverative errors						
Finger Windows						
SOPT total						
TOL						
CPT omissions						
CPT commissions						
CPT hit RT						
CPT hit RT standard error						
CPT variability of standard errors						
CPT overall index						

P values are provided according to this color scale:

	0.01-0.05
	0.001-0.009

Legend: CPT, Continuous Performance Test; RT, reaction time; SNP, single nucleotide polymorphism; SOPT, Self-Ordered Pointing Task; TOL, Tower of London; WCST, Wisconsin Card Sorting Test; WISC, Wechsler Intelligence Scale.*Standard scores were used for all WCST and TOL measures, and T-scores were used for CPT measures (excl. overall index). Solid color indicates under transmission of the risk allele (C*). Striped color indicates over transmission of the rs1329650 risk allele. Risk alleles are under-transmitted (negative Z-score, solid boxes) to the higher scores (the higher standard scores imply a better performance on the test). Lower WCST standard scores indicate worse performance. Risk alleles were associated with poorer performance on the SOPT and CPT. Since these are not standardized scores, the higher SOPT total score and CPT T-scores indicate worse performance.

Supplementary Table:

FBAT analysis of genes increasing the risk for smoking behaviors in children with ADHD stratified by maternal smoking during pregnancy

Exposed to maternal smoking during pregnancy					
Marker	**Allele**	**Allele Frequency**	**Number of informative families**	**Z statistic**	**P value**
rs1051730	C	0,646	56	0,88	0,38
rs1051730	T	0,354	56	-0,88	0,38
rs1028936	A	0,829	39	0,41	0,68
rs1028936	C	0,171	39	-0,41	0,68
rs1329650	A	0,241	48	-1,77	0,08
rs1329650	C	0,759	48	1,77	0,08
rs3025343	A	0,117	35	0,73	0,46
rs3025343	G	0,883	35	-0,73	0,46
rs6265	A	0,186	44	-1,77	0,08
rs6265	G	0,814	44	1,77	0,08
Not exposed to maternal smoking during pregnancy					
rs1051730	C	0,671	103	1,25	0,21
rs1051730	T	0,329	103	-1,25	0,21
rs1028936	A	0,824	67	1,53	0,13
rs1028936	C	0,176	67	-1,53	0,13
rs1329650	A	0,264	91	-1,75	0,08
rs1329650	C	0,736	91	1,75	0,08
rs3025343	A	0,105	48	0	1
rs3025343	G	0,895	48	0	1
rs6265	A	0,18	77	0,49	0,62
rs6265	G	0,82	77	-0,49	0,62

Discussion

To the best of our knowledge, this is the first report of SNPs identified through GWAS of smoking behavior shown to be tentatively associated with ADHD. Two 10q25 SNPs (rs1028936 and rs1329650) from a non-coding RNA (*LOC100188947*) show a distinct pattern of association with respect to several behavioral and neurocognitive traits characteristic of ADHD. Risk variants of both SNPs, and their derived haplotype, were significantly over-transmitted from parents to affected offspring, and associated with a more severe ADHD phenotype, where children who inherited the risk variant from their parents had more externalizing symptoms and poorer performance on several neurocognitive tasks.

Using SNPs reliably associated through GWAS studies with somatic or behavioral disorders comorbid to ADHD may be an interesting approach to decipher the genetics of this complex psychiatric disorder. This strategy is now used by some other investigators in other complex disorders. For example, Hansen and colleagues investigated a number of SNPs associated with type II diabetes in patients with schizophrenia to identify risk variants for this disorder (Hansen et al., 2011). Here, we have used a similar approach relying on the observation that there is a strong link between ADHD and smoking. It has been suggested that this association may be due, at least in part, to shared genetic factors between these two phenotypes (Laucht et al., 2007).

Behavioral disinhibition/externalizing disorders are often comorbid with ADHD and smoking (Sousa et al., 2011). Thus, genetic factors predisposing to ADHD and smoking may act through an increased level of behavioral disinhibition (Sousa et al., 2011). Consistent with this hypothesis, we have observed in our sample that the risk variant associated with smoking in rs1329650 is also related to an increased risk of externalizing behaviors as seen on the CBCL.

In young adults, two theoretical models of tobacco use have been proposed: the self-medication and orbitofrontal/disinhibition model (Dinn et al., 2004). The latter predicts smokers will perform worse on neurocognitive tasks related to orbitofrontal dysfunction as compared to non-smokers. In the present study, poorer performance on neurocognitive tasks such as the WCST, SOPT, and CPT, was associated with the risk alleles of both 10q25 SNPs. This is in line with the disinhibition model, which also proposes that smokers obtain elevated scores on tasks that measure behavioral disinhibition and mirrors what we have seen. In our sample, children with the smoking risk variant seem to perform worse on tasks that measure response inhibition, such as the SOPT and CPT.

Given that smoking is a preventable behavior, studies identifying common genetic factors for ADHD and smoking may help in the earlier identification of subjects who are more prone to develop dependence to cigarette smoking. This genetic information would be crucial, once confirmed and furthered, to develop preventive strategies, especially since smoking in ADHD patients tends to start

earlier in life and once initiated, is much more severe and harder to curve down than in the general public (Humfleet et al., 2005).

There is an extensive body of literature associating MSDP with increased risk for ADHD. Although exposure to smoking during pregnancy could be increasing the risk for ADHD through direct neurobiological effects of cigarette smoke content on the developing brain, there is mounting evidence that the association between MSDP and ADHD might be due to factors shared by both conditions, including genetic variants shared by mothers and children, putting them at higher risk for developing smoking behavior and ADHD, respectively. In order to investigate this possibility, we stratified ADHD children according to whether or not their mothers had smoked during pregnancy (Supplementary Table). Our stratification revealed a marginally and equally significant pattern of transmission of the risk allele in both exposure groups suggesting that environmental exposure to smoking during pregnancy is not interacting with rs1329650 allelic variants to increase the risk for ADHD or to modify its clinical and cognitive dimensions. However, since this stratification reduced the sample size of each group, we cannot disregard that the absence of significant association, particularly in the group exposed to maternal smoking, is not due to a lack of statistical power.

Certain limitations should be kept in mind when interpreting these results. First, a larger sample size may have enabled us to have more statistical power to detect significant associations with other loci tested, as previous studies that investigated markers such as *CHRNA3*, had larger sample sizes (Stephens et al., 2011) and

identified significant associations with ADHD. It is also possible that we did not identify an association with these markers since our study was conducted with a clinical sample of ADHD children whereas previous studies investigated adolescents, adults and/or community-based samples (Winterer et al., 2010). However, given the heterogeneity among these associations, it is important to note that our negative results with respect to certain genes, such as BDNF, have also been reported by recent meta-analyses which do not support an association between BDNF and ADHD (Sanchez-Mora et al., 2010, Gizer et al., 2009). Also related to the problem of limited statistical power, the reported association with rs1329650 was only marginal and would not be significant had we applied the stringent Bonferroni correction (we tested five loci, thus corrected p = 0.01). However, the *a priori* plausibility of the involvement of these loci, highly associated with smoking justifies reporting these tentative results which warrant replication in larger samples.

Given the age range of children included in this study, none has (or declared) smoking behavior. Thus, it would be interesting to obtain future information about smoking behavior in these subjects, to investigate whether risk variant carriers are more likely to initiate smoking behavior in adolescence or later in life. It is also important to note that rs1329650 found to be associated with ADHD and its relevant behavioral dimensions is not located in any known gene.

Here, we have found an association between the *LOC100188947* marker and ADHD. A possible explanation may be that this variant influences regulatory

elements or tags a variant in a nearby gene. The *HECTD2* gene, located close to this marker, has been associated with prion disease (Lloyd et al., 2009a) and Alzheimer's disease (Lloyd et al., 2009b), but has not yet been investigated in ADHD. Thus, it may be worthwhile to study the *HECTD2* gene in the etiology of ADHD.

Future research will need to focus on understanding the functional consequences of these loci (Freedman et al., 2011) using fine mapping around these regions of interest to find out which gene(s) are close by and further dissect their relationship with smoking and ADHD.

In conclusion, we present preliminary evidence indicating that rs1329650 increases the risk for ADHD and we suggest a neuropsychological and behavioral mechanism that could underlie the link between this genetic risk locus, ADHD and smoking.

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Competing Interest: None

Contributorship: R. Joober and N. Grizenko designed the study and acquired the data. G.A. Thakur, S.M. Sengupta, Z. Choudhry, and R. Joober analyzed the data. G.A. Thakur and R. Joober wrote the article. S.M. Sengupta, N. Grizenko, and Z. Choudhry reviewed the article. All authors approved its publication.

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CHAPTER 6

Discussion

Attention-Deficit/Hyperactivity Disorder (ADHD) is a highly prevalent neurodevelopmental disorder affecting 8-12% of the general population (Faraone et al., 2003). Children with ADHD often experience trouble at home and at school, leading to low academic achievement (Faraone et al., 1993), as well as difficulties with peer and family relationships. When ADHD persists into adulthood, it often causes impairment in many spheres of an individual's life, including occupational and social impairment, and may lead to substance abuse, antisocial behavior and criminality (Thapar et al., 2007a). ADHD is a serious public health concern given that symptoms seen in childhood are one of the main reasons for follow-up services in child and adolescent mental health services (Thapar et al., 2007b) and that the resulting adverse consequences impact not only the affected child, but the family members as well.

With a heritability estimate of 77%, a significant genetic contribution has been established in the etiology of ADHD (Biederman, 2005). Thus, identifying genetic risk variants for ADHD has been the chief objective of geneticists over the years. Many genetic markers have emerged from candidate gene (Gizer et al., 2009) and genome-wide association studies (GWAS) of ADHD (Lasky-Su et al., 2008, Lesch et al., 2008, Mick et al., 2010, Neale et al., 2008, Neale et al., 2010a), but it has been difficult to consistently replicate results for these candidate genes (Neale et al., 2010b). More recently, important efforts have been directed towards genome-wide association studies, with the assumption that these studies will identify genetic variants with relatively small effects, including variants that are not located in or nearby genes. In contrast to many other complex disorders,

including psychiatric disorders, GWAS in ADHD have not yielded associations withstanding genome-wide correction, possibly indicating that ADHD has a more complex genetic structure. This is consistent with the fact that the relative risk for first-degree relatives, or λ siblings (ratio of the risk in first-degree relatives to the risk in the general population) in ADHD (λ = 5) is smaller than the one observed in other major psychiatric disorders (e.g. λ = 10 in schizophrenia and bipolar disorder). This genetic complexity may need multiple approaches to decipher the genetic architecture of ADHD.

Environmental risk factors play a considerable role (approximately 20%-30%) in the variable phenotypic expression of ADHD. While several risk factors related to environmental toxins and stressors have been investigated, a more complex picture has emerged suggesting that it may be more appropriate to use risk factors that *index* the effects of a particular environment and may share some of the same underlying pathways (Thapar et al., 2007a). Thus, overall findings have been widespread and mostly inconclusive to date, given the variability across studies with respect to environmental exposure, and lack of *reliably measured* environmental factors.

Psychiatric disorders also present an additional challenge, which is the phenotypic heterogeneity and complex nature of the disorder itself. In ADHD, there are three possible clinical subtypes (hyperactive, inattentive, and combined) which are categorized based on a myriad of clinical symptoms. It has previously been reported that simply considering the diagnosis of ADHD in genetic association

studies may in fact conceal potential effects between specific ADHD dimensions and risk variants. Therefore, it has become imperative to explore quantifiable intermediate constructs, termed *endophenotypes* that might capture some of the pathogenic pathways implicated in ADHD.

Research specifically examining the complex interplay, based on *strong biological hypotheses*, between genetic and environmental factors in ADHD has been lacking. Therefore, we have used gene-environment interplay to better understand the genetics of ADHD by using a dimensional approach and specifically examining a key environmental factor in ADHD, namely maternal smoking during pregnancy (MSDP).

<u>The main assumptions underlying this thesis are:</u>

(1) In addition to the categorical diagnosis, using several endophenotypes relevant to ADHD will help to identify robust associations between ADHD and candidate genes.

(2) Maternal smoking during pregnancy may help to identify a subgroup of patients with ADHD with a possibly more homogeneous genetic architecture.

Findings from this current study not only support the notion that studying both genetic and environmental risk factors is important, but also highlight the fact that dissecting the phenotype of interest (i.e. ADHD) into putatively simpler traits or endophenotypes related to behavioral and neurocognitive traits relevant to ADHD may enable a deeper understanding of the pathophysiology of this disorder.

We accomplished this goal by integrating detailed clinical and environmental data with extensive genetic information from a relatively large sample size and using a robust method of association study (family-based association tests) as our statistical method of choice.

First, we characterized children with ADHD based on their exposure to MSDP, second we reported an association between a well-known ADHD candidate gene and traits representative of the disorder in the group of children exposed to MSDP, and third we identified a novel association between a polymorphism originally linked to smoking behavior and ADHD.

Maternal Smoking During Pregnancy and ADHD: A Comprehensive Clinical and Neurocognitive Characterization

In Chapter 3, our objective was to determine whether children with ADHD exposed to MSDP showed a distinctive clinical and neurocognitive profile when compared to unexposed children. Although it is now well-established that an association does exist between smoking and ADHD, two comorbid phenotypes (McClernon and Kollins, 2008), a causal relation has not yet been confirmed.

By using an extensive battery of neurocognitive tests, in addition to ADHD diagnosis and clinical assessments of severity and comorbidity, we found that children who had been exposed to MSDP, even after controlling for key confounding factors, had a more severe clinical and neurocognitive profile as compared to unexposed children. To further explore this relation, we investigated

quantitative exposure by using the average number of cigarettes smoked per day and observed a dose-response effect with the dimensions that were associated with ADHD.

On the whole, children exposed to MSDP had more externalizing symptoms, greater comorbidity, particularly conduct disorder and oppositional defiant disorder, and a sluggish attention profile as measured by the Continuous Performance Test. Although conflicting evidence relating MSDP to ADHD does exist (Biederman et al., 2012), this result is in line with previous findings supporting an association between MSDP and attentional control in children with ADHD (Motlagh et al., 2011). Therefore, it is reasonable to state that MSDP is a strong environmental factor indexing a more homogenous subgroup of children with a particular phenotypic signature characterized by a more severe form of ADHD.

In addition to presenting comprehensive comparative profiles of children rated by three different observers in three different environments, we also identified a dose-response relationship between MSDP and ADHD.

Thus, given that MSDP is a preventable behavior, these findings are of particular interest to inform parents of the harmful effects of prenatal cigarette smoke exposure as well as other potentially negative behavioral outcomes. Furthermore, since the comorbidity between smoking and ADHD may be explained by shared genetic factors, studying genetic transmission from parents to offspring and

exploring a myriad of environmental factors, while controlling for potential confounders and parental psychopathology, may enable us to identify specific risk variants early on and further shed light on the role of MSDP.

Comprehensive Phenotype/Genotype Analyses of the Norepinephrine Transporter Gene (*SLC6A2*) in ADHD: Relation to Maternal Smoking During Pregnancy

With a mean heritability estimate of 77% (Biederman, 2005), ADHD is one of the most heritable psychiatric disorders. Yet, linkage and association studies have been unsuccessful in implicating a specific gene/chromosomal region beyond reasonable doubt. Environmental factors seem to account for the rest of the variance (Knopik et al., 2005). Therefore, the joint analysis of specific catecholaminergic candidate genes and measured environmental risk factors may be more effective in revealing their role in this complex disorder.

Given the pharmacological, imaging, and neuropsychological evidence implicating the norepinephrine transporter gene (*SLC6A2*) in ADHD, we tested its association with ADHD, in Chapter 4, by stratifying the sample of affected children into two groups based on MSDP.

Through family-based association tests of behavioral and neurocognitive traits, we identified a novel association between ADHD and rs36021 (and linked SNPs) only in those children whose mothers smoked during pregnancy, suggesting an

interaction between exposure to maternal smoking and carrying the risk allele(s) in the *NET1* gene.

Given that this association was only revealed after MSDP stratification, it is possible that the effect of a genetic polymorphism is masked in the total group when MSDP is not accounted for, giving additional credence to using environmental stratification to map genes implicated in ADHD.

Since the SNPs that showed the most significant association in this study (rs36021 and rs3785152, in particular) are within introns, it is possible that these intronic variants are either involved in gene regulation or are in linkage disequilibrium with a functional variant. Therefore, fine-mapping of this region will be needed to identify causal mutations.

This finding supports the fact that genetic variation in the NET is an important factor in a more severe subtype of ADHD and highlights the importance of conducting gene and environment studies in ADHD. By classifying patients in a particular subgroup, based on the presence of specific genetic markers and exposure to a certain environmental stressor, it will be possible to develop tailored treatment regimens targeting specific biochemical pathways in specific sub-groups of children with ADHD.

Family-Based Association Study of ADHD and Genes Increasing the Risk for Smoking Behaviors

In the final chapter, we used a novel approach to investigate SNPs located in different genes and loci highly associated with various dimensions of smoking behavior, in relation to ADHD.

In approximately 50-80% of ADHD cases, patients also present somatic or behavioral disorders comorbid to ADHD (Klimkeit et al., 2010). Since it is established that a strong link exists between ADHD and smoking (McClernon and Kollins, 2008), which may be explained by shared genetic factors, we selected genetic variants identified through GWAS of smoking behavior (Tobacco and Genetics Consortium, 2010) and explored them in relation to ADHD diagnosis and many behavioral and neurocognitive endophenotypes characteristic of ADHD.

By doing so, we identified a distinct pattern of association which emerged between the risk-variant associated with smoking in rs1329650, a SNP from a noncoding RNA (*LOC100188947*), and a more severe ADHD phenotype. Specifically, children who had inherited this risk-variant from their parents exhibited more externalizing symptoms and poorer performance on several neurocognitive tasks.

Since this polymorphism is not located in any known gene, it will be necessary to investigate whether this variant influences regulatory elements or tags a variant in

a nearby gene, such as the *HECTD2* gene, which has been implicated in other disorders (Lloyd et al., 2009a, Lloyd et al., 2009b).

This novel finding supports the fact that selecting SNPs related to phenotypes comorbid to ADHD, is an interesting way to further understand the genetic complexity of this disorder. It is also of particular interest since identifying common genetic factors for ADHD and smoking may enable an earlier detection of subjects prone to develop dependence to cigarette smoking.

Conclusions

Taken together, findings from the preceding chapters support the notion that ADHD is indeed a multifactorial psychiatric disorder. Results obtained in these studies highlight the importance of studying genetic and environmental contribution to ADHD and its component endophenotypes, since the combination of a particular gene (*SLC6A2*) with a certain exposure (MSDP) may affect distinct dimensions of ADHD (e.g. externalizing behavior and attention profile).

A certain commonality shared in these studies is that exposure to prenatal maternal smoking negatively affects behavioral and neurocognitive outcome in offspring with ADHD and that using stratification by MSDP may help to reveal, otherwise concealed, associations between genetic risk variants and ADHD.

Results presented in chapters 3-5 will need to be confirmed in a larger sample size to increase statistical power and draw firm conclusions. A further investigation of the association reported in Chapter 4 between the NET gene and ADHD would be essential and fine-mapping around regions of interest and molecular analysis may help to identify causal mutation(s) and determine if the mutation affects transcriptional regulation of the gene or structure and function of the protein, respectively. The novel finding reported in Chapter 5 illustrates the use of comorbid phenotypes to identify genetic risk variants in complex diseases, such as ADHD, an approach that may also be fruitful in other psychiatric disorders.

There are several clinical implications of this research. Firstly, it will be important to identify children with exposure to certain environmental factors, given that a phenotypic signature associated with MSDP helped us to identify a more homogenous subgroup of children characterized by a more severe form of ADHD. By doing so, it will be possible to develop tailor-made treatments for these specific sub-groups of children (Wallis, 2010). Second, by conducting detailed genotype and phenotype characterization, it will be possible to better classify the disorder and develop novel medications that can target particular neurotransmitter systems. And finally, by studying comorbid disorders in these children, it will be possible to understand the underlying complex and intersecting pathways.

Recent structural neuroimaging studies in ADHD have identified brain abnormalities in ADHD patients. Although there is significant heterogeneity across regions and studies, a consistent finding is a reduction in volume or area

within the prefrontal and other frontal lobe regions of interest (Valera et al., 2007). Overall, studies indicate that a dysfunction in one or more components of the cerebellar-prefontal-striatal network is involved in ADHD.

Another field that has most recently emerged is known as *imaging genetics*, where various techniques, such as magnetic resonance imaging (MRI) and positron emission tomography (PET) are used in order to discover how potential candidate genes (e.g. *DAT* and *DRD4* in the case of ADHD) may affect the different features of neural functioning (Durston, 2010). By detecting neural correlates related to brain structure and function, it may one day be possible to identify imaging-genetic biomarkers and further our understanding of the underlying pathophysiological mechanisms of ADHD, in order to better define the disorder.

Future studies will be better equipped to unravel the genetic and environmental contributions to this complex disorder by using a multi-disciplinary approach and integrating findings from psychiatry, molecular biology, genetics, and neuroimaging, as well as developing more comprehensive, genetically informed prospective study designs (Knopik, 2009, Purper-Ouakil et al., 2011).

In summary, findings reported in this thesis provide new evidence for the involvement of MSDP in the pathophysiology of ADHD by revealing highly significant associations with *SLC6A2* in children whose mothers smoked during pregnancy, as well as with genetic markers identified through comorbid smoking behavior.

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