Brain Morphology in Children with ADHD:

Investigating the Effects of Medication, Candidate Genes and Prenatal Smoking Exposure.

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In dedication to my husband, Steven. Since married, it has been him, me, and this PhD.

At times when the journey seemed interminable, he reminded me to have faith in my decision to pursue higher education. All that did was remind me I made the right decision in marrying him.

Abstract/Résumé

Abstract

Attention deficit/hyperactivity disorder (ADHD) is a prevalent neurodevelopmental disorder with heterogeneous clinical expression. Symptoms consist of age-inappropriate levels of inattention, hyperactivity and impulsivity beginning in childhood and often persisting into adulthood, causing significant impairments to daily functioning and well-being. ADHD medication, particularly psychostimulants, are effective in managing symptoms and are thought to function by regulating dopamine (DA) and norepinephrine (NE) concentrations in the brain. Given this, in addition to the high heritability of ADHD, many studies have sought to identify the underlying causes by investigating genetic variations within the DA and NE systems, as well as explore alterations in brain structure in affected individuals. Despite advancement in the field, ADHD is a complex disorder with multiple aetiologies that remain poorly understood.

The following thesis employs an emerging imaging-genetics approach to describe how the effects of specific genetic and environmental risks factors on brain structure can help characterize particular subgroups of ADHD, and therefore assist in disentangling pathways of the disorder. We began by exploring the effect of cumulative exposure to ADHD medication (CEM) on brain structure to account for potential confounding effects in our model. It was found that a higher CEM was significantly associated with smaller hippocampus subregional volumes. However, no effects were uncovered on cortical brain structures (cortical thickness and surface area).

Subsequently, we assessed the role of a previously associated single-nucleotide polymorphism (rs36021) within the norepinephrine transporter (NET) gene on brain structure. A significant effect of *NET* genotype was uncovered, where children homozygous for the risk-allele (TT) had reduced cortical surface area in attentional networks, notably prefrontal brain regions important for executive functioning. Furthermore, these differences in brain structure were significantly correlated to more disruptive behaviour, as indicated by higher externalizing disorder scores.

Finally, we investigated the effects of exposure to prenatal smoking on brain structure using two methods to categorize children into exposure groups. Significant reductions in cortical surface area within several regions in the right hemisphere, such as the orbitofrontal cortex, were observed when children's prenatal smoking exposure statuses were assigned through epigenetic markers. Moreover, these alterations in brain structure were significantly associated with poorer neuropsychological performance, as indicated by a higher rate of commission errors on the Continuous Performance Test. No effects were uncovered when exposure groups were generated according to maternal self-reports, suggesting that epigenetic markers associated with prenatal smoking may be more reliable in determining exposure status.

This work recognizes the effect of medication on brain structure and describes brain structure phenotypes associated with *NET* genotype and prenatal smoking exposure in a sample of children with ADHD. In addition, our findings highlight the benefit and potential of using

epigenetic markers to determine exposure status to environmental factors. Taken together, we provide further evidence in favor of using brain structure as an intermediate phenotype in ADHD genetic research to narrow the gap between genetic factors and clinical outcomes. This approach can help reduce the complexity of ADHD, by indexing subgroups with more homogeneous phenotypic profiles, and thus help delineate the pathophysiology of the disorder.

Résumé

Le trouble du déficit de l'attention / hyperactivité (TDAH) est un trouble neurodéveloppemental commun dont la présentation clinique est hétérogène. Les symptômes consistent en des niveaux anormaux et inadaptés d'inattention, d'hyperactivité et d'impulsivité. Le TDAH débute durant l'enfance, mais les symptômes persistent souvent à l'âge adulte, causant des difficultés marquées dans le fonctionnement quotidien et affectant le bien-être. Les médicaments utilisés pour traiter le TDAH, particulièrement les psychostimulants, réduisent les symptômes en régularisant les concentrations de dopamine et de noradrénaline dans le cerveau. Compte tenu de cette observation et de l'héritabilité élevée du TDAH, de nombreuses études ont cherché à identifier les causes de ce trouble en examinant les variations génétiques présentes au sein des systèmes dopaminergique et noradrénergique, ainsi qu'en explorant la structure du cerveau. Cependant, malgré les avancées scientifiques, le TDAH demeure un trouble complexe aux étiologies multiples qui restent à être élucidées.

Cette thèse présente une approche combinant l'imagerie-cérébrale et la génétique afin d'étudier les effets de certains facteurs génétiques et environnementaux sur la structure du cerveau. Cette méthode pourrait permettre l'identification de sous-groupes de TDAH et, par conséquent, réduire la complexité du désordre.

Nous avons d'abord exploré l'effet des médicaments traitant le TDAH sur la structure cérébrale. Nous avons remarqué qu'une durée et une dose plus élevées de médicament étaient associées à une réduction du volume de la sous-région de l'hippocampe CA1. Cependant, aucun effet n'a été observé sur les structures cérébrales corticales.

Par la suite, nous avons évalué l'impact d'un polymorphisme mononucléotidique (rs36021) situé dans le gène du transporteur de la noradrénaline (NET), précédemment associé avec le TDAH, sur la structure cérébrale. Un effet significatif du génotype *NET* a été observé chez des enfants homozygotes pour l'allèle a risque (TT). Chez ceux-ci, la surface corticale des régions cérébrales impliquées dans l'attention, notamment le cortex préfrontal, était réduite. De plus, ces différences dans la structure cérébrale étaient associées à un comportement plus perturbateur, représenté par les scores plus élevés d'un trouble extériorisant.

Enfin, nous avons étudié l'effet du tabagisme prénatal sur la structure cérébrale en utilisant deux méthodes différentes pour identifier les enfants exposés. Des réductions significatives de la surface corticale dans plusieurs régions de l'hémisphère droit, notamment le cortex orbitofrontal, ont été observées chez les enfants exposés au tabagisme prénatal, mais seulement lorsque l'identification des enfants exposés était déterminée par des marqueurs épigénétiques associés au tabagisme prénatal. Ces altérations de la structure cérébrale étaient associées à une plus faible performance lors d'un test neuropsychologique (Continuous Performance Test), et indiquent un taux d'erreur plus élevé au cours de l'évaluation. Aucun effet n'a été observé lorsque les sujets étaient divisés en fonction du témoignage de la mère, ce qui

suggère que les marqueurs épigénétiques associés au tabagisme prénatal pourraient être plus fiables pour déterminer le statut d'exposition des enfants atteints du TDAH.

Dans l'ensemble, ce travail démontre une association entre les médicaments pour le TDAH et la structure cérébrale, et décrit les phénotypes de structure cérébrale associés au génotype rs36021 et à l'exposition au tabagisme prénatal chez un échantillon d'enfants atteints du TDAH. De plus, nos résultats mettent en évidence l'intérêt et le potentiel d'utiliser des marqueurs épigénétiques pour déterminer l'exposition à certains facteurs environnementaux. De façon globale, nous fournissons des données supplémentaires supportant l'utilisation de la structure du cerveau comme phénotype intermédiaire dans la recherche génétique sur le TDAH, afin de réduire l'écart entre les facteurs génétiques et les phénotypes cliniques. Cette approche pourrait permettre de réduire la complexité du TDAH en identifiant des sous-groupes ayant des profils phénotypiques plus homogènes et ainsi pourrait contribuer à mieux comprendre la pathophysiologie de la maladie.

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List of Abbreviations

ADHD: Attention deficit/hyperactivity disorder

ANOVA: Analysis of variance

CA1: Cornu ammonis 1

CA2/3: Cornu ammonis 2/3

CBCL: Child behavioural check list

CEM: Cumulative exposure to ADHD medication

CIC: Cerebral imaging center

CpG: 5'-cytosine-phosphate-guanine-3'

CPT: Continuous performance test

CT: Cortical thickness

DA: Dopamine

DAT: Dopamine transporter

DG: Dentate gyrus

DMHUI: Douglas Mental Health University Institute

DSM: Diagnostic and Statistical Manual of mental disorders

EM: Epigenetic markers

+EM: Exposed to prenatal smoking according to epigenetic markers

-EM: Not exposed to prenatal smoking according to epigenetic markers

fMRI: Functional MRI

GWAs: Genome-wide association studies

IQ: Intelligence quotient

MPH: Methylphenidate

MRI: Magnetic resonance imaging

MSDP: Maternal smoking during pregnancy

+MSDP: Exposed to prenatal smoking according to maternal recall

-MSDP: Not exposed to prenatal smoking according to maternal recall

NE: Norepinephrine

NET: Norepinephrine transporter

PET: Positron emission tomography

PFC: Prefrontal cortex

ROFc: Right orbitofrontal cortex

RPHg: Right parahippocampal gyrus

RTc: Right middle temporal cortex

SA: Surface area

SD: Standard deviation

SLC6A2: Solute carrier family 6 member 2

SR/SL/SM: strata radiatum/lacunosum/moleculare

SHR: spontaneously hypertensive-rat

sMRI: Structural MRI

SNP: Single nucleotide polymorphism

WISC-IV: Wechsler Intelligence Scale for Children version IV

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I am immensely grateful to my dear parents, Zissis and Maria, who made sure I had everything I needed and more to complete this milestone. I share this accomplishment with them.

Contribution to Original Knowledge

The following dissertation employs neuroimaging as a tool to bridge the gap between risk factors of ADHD and clinical dimensions. A multidisciplinary approach is used that combines neuroimaging, environmental, behavioural, cognitive, genetic, and epigenetic data. As such, the strength of this work is founded on the in-depth characterization of the ADHD phenotype within our sample of children.

Objectives were addressed by recruiting children aged 6 to 12 years at the ADHD clinic located at the Douglas Mental Health University Institute. Children underwent a clinical evaluation by our team psychiatrist to confirm ADHD diagnosis. Parents were interviewed and children were administered neuropsychological assessments to obtain cognitive and behavioural profiles. Neuroimaging and genetic data were collected on site at the Cerebral Imaging Center and wet lab, respectively.

The first objective was addressed by collecting detailed pharmacological history of children with ADHD participating in our study through parental interviews and pharmacy logs. Cumulative exposure to ADHD medication was calculated using the dose and duration of medication, whilst considering medication breaks. This study is unprecedented in the breadth of medication exposure measurements, as it is the first study to use a continuous variable of medication exposure and provides novel information on the effects of medication on brain

structure in a clinical sample of children with ADHD. Many children diagnosed with ADHD within the population receive pharmacological treatment, and all the children with ADHD participating in our study were exposed to medication. Given this, our findings provide useful information to account for the confounding effects of ADHD medication on brain structure in subsequent models.

A manuscript summarizing the findings from objective 1 (Section III.1), entitled "Cumulative Exposure to ADHD Medication is Inversely Related to Hippocampal Subregional Volumes in Children", has been authored by ***Nellie H. Fotopoulos****, Gabriel A. Devenyi, Stéphanie Guay, Sherif Karama, Sarojini M. Sengupta, M. Mallar Chakravarty, Natalie Grizenko, and Ridha Joober.* The manuscript is currently under revision in the Journal: Progress in Neuropsychopharmacology & Biological Psychiatry.

The second objective was addressed by using the available genotypic data for children in our study collected through blood and saliva samples. Currently, there are no published reports investigating the effects of variations within *NET* on brain structure, and thus this study provides novel findings on the relationship of *NET* genotype and brain morphology. In addition, since previous data have associated variations in *NET* and behavioural measures, significant brain structure regions were investigated in relation to behavioural dimensions.

A manuscript summarizing the results from objective 2 (section III.2), entitled "Children with ADHD Homozygous for the *NET* (rs36021) Risk-Allele have Reduced Cortical Surface Area

in Executive Brain Regions", has been authored by ***Nellie H. Fotopoulos****, Sarojini M. Sengupta, Gabriel A. Devenyi, Sherif Karama, M. Mallar Chakravarty, Natalie Grizenko, and Ridha Joober*. The manuscript has been reviewed and approved by all authors and ready for submission.

The third objective was addressed through whole-methylome analysis to characterize children into groups according to exposure to prenatal cigarette smoking. This is the first study comparing retrospective reports of maternal smoking during pregnancy and epigenetic markers associated with prenatal smoking exposure on brain structure. A novel approach combining neuroimaging and epigenetics is used to investigate the effects of an environmental risk factor for ADHD on brain structure. Moreover, significant brain structure regions were assessed in relation to cognitive dimensions.

A manuscript has been generated, entitled "Maternal Smoking During Pregnancy and Cortical Structure in ADHD Children: Enough to Rely on Recall?" authored by ***Nellie H. Fotopoulos****, Sarojini M. Sengupta, Boris Chaumette, Gabriel A. Devenyi, Sherif Karama, M. Mallar Chakravarty, Aurelie Labbe, Natalie Grizenko, Norbert Schmitz, and Ridha Joober.* The manuscript has been reviewed and approved by all authors and ready for submission.

Thesis Format

The following is a traditional dissertation in accordance with the Thesis Preparation Guidelines stated by the Department of Graduate and Postdoctoral Studies. Research was conducted under the supervision of Dr. Ridha Joober and co-supervision of Dr. Sherif Karama. The thesis is comprised of six chapters.

Chapter I contains the introduction and pertinent background information, as well as the hypotheses and objectives. Chapters II consists of the relevant materials and methods required to conduct the research presented in this dissertation. Chapter III presents the results of three main analyses; (1) the effects of cumulative exposure to ADHD medication on brain structure, (2) the effects of *NET* genotype on brain structure and behavioural measures, and (3) the effects of prenatal exposure to cigarette smoking on brain structure and cognitive dimensions. Three independent manuscripts summarizing these findings have been completed and are in the submission and revision process to publishing bodies.

Although not included in the following dissertation, a previous imaging-genetics analysis conducted by Nellie H. Fotopoulos using similar methodology has been combined to a report entitled "Dissecting genetic cross-talk between ADHD and other neurodevelopmental disorders: Evidence from behavioural, pharmacological and brain imaging investigations", published in *Psychiatry Research* in 2018, by Sengupta, S. M., **Fotopoulos, N**., Devenyi, G. A., Fortier, M. È.,

Ter-Stepanian, M., Sagliker, S., Karama, S., Chakravarty, M. M., Labbe, A., Grizenko, N., & Joober, R. (PMID: 30216917).

Chapter IV contains the discussion that places main findings in the context of the ADHD neuroimaging-genetics field, the specific limitations from each independent analysis, as well as the general limitations of the research overall. Chapter V contains the conclusions and possible future directions. References are listed in Chapter VI. Additional material (publications, permissions, and Ethics approval) are summarized in the Appendix section.

Contributions of Authors

The work presented in this thesis was carried out by Nellie H. Fotopoulos under the supervision of Dr. Ridha Joober and Dr. Sherif Karama. The contributions of authors for each section are presented in the following paragraphs.

The analyses in Chapter III, section III.1: Cumulative Exposure to ADHD Medication is Inversely Related to Hippocampus Subregional Volume in Children, were carried out by ***N. Fotopoulos***. The conception and study were designed by ***N. Fotopoulos,*** *R. Joober,* and *S. Karama.* Clinical evaluations were administered by the ADHD team's psychiatrists; *R. Joober and N. Grizenko*. Participant recruitment and consenting for the MRI-Project were performed by *R. Deguzman* and ***N. Fotopoulos***. Data for exposure to medication (with cross-referencing pharmacy logs), and brain imaging were collected by ***N. Fotopoulos*** and *R. Deguzman*. Neuropsychological and behavioural assessments were administered by ***N. Fotopoulos***. Data was formatted by ***N. Fotopoulos*** and *S. Guay.* Statistical analyses were conducted by ***N. Fotopoulos*** with technical assistance from *G. A. Devenyi* at the Cerebral Imaging Center. Findings were interpreted by ***N. Fotopoulos*** with the guidance of *R. Joober, S. Karama,* and *M. M. Chakravarty.* Analytic recommendations were provided by *R. Joober, S. Karama, M. M. Chakravarty, Y. Trakadis,* and *G. A. Devenyi.* The text and generation of figures and tables were written and designed by ***N. Fotopoulos***.

The analyses in Chapter III, section III.2: Children with ADHD Homozygous for the NET (rs36021) Risk-Allele have Reduced Cortical Surface Area in Executive Brain Regions, were carried out by ***N. Fotopoulos***. The conception and study were designed by *R. Joober, N. Grizenko, S. Karama,* and *S. M. Sengupta.* Clinical evaluations were administered by the ADHD team's psychiatrists; *R. Joober and N. Grizenko*. Genotypic data was previously collected by the ADHD team at the DMHUI. Participant recruitment and consenting were performed by ***N. Fotopoulos*** and *R. Deguzman.* Neuroimaging data were collected by ***N. Fotopoulos*** and *R. Deguzman*. Neuropsychological and behavioural assessments were administered by ***N. Fotopoulos***. Data was formatted by ***N. Fotopoulos***. Statistical analyses were conducted by ***N. Fotopoulos*** with technical assistance from *G. A. Devenyi* at the Cerebral Imaging Center. Findings were interpreted by ***N. Fotopoulos*** with guidance from *R. Joober, S. Karama,* and *M. M. Chakravarty.* Analytic recommendations were provided by *R. Joober, S. Karama, M. M. Chakravarty,* and *Y. Trakadis.* The text and generation of figures and tables were written and designed by ***N. Fotopoulos***.

The analyses in Chapter III, section III.3: Maternal Smoking During Pregnancy and Cortical Structure in ADHD children: Enough to rely on Recall?, were carried out by ***N. Fotopoulos***. The conception and study were designed by *R. Joober, N. Grizenko, S. Karama, S. M. Sengupta,* and *B. Chaumette.* Clinical evaluations were administered by the ADHD team's psychiatrists; *R. Joober and N. Grizenko*. Data for prenatal exposure to smoking were collected by ***N. Fotopoulos,*** *B. Chaumette, W. Fageera* and *S. Sengupta.* Neuroimaging data were collected by ***N. Fotopoulos*** and *R. Deguzman*. Neuropsychological and behavioural assessments were administered and collected by ***N. Fotopoulos***. Epigenetic data was formatted by ***N. Fotopoulos,*** *B. Chaumette, W. Fageera and S. Sengupta.* Analysis of epigenetic data was performed by *B.*

Chaumette. Statistical analyses were conducted by ***N. Fotopoulos*** with technical assistance from *G. A. Devenyi* at the Cerebral Imaging Center. Findings were interpreted by ***N. Fotopoulos*** with the guidance of *R. Joober, S. Karama, M. M. Chakravarty,* and *A. Labbé.* Analytic recommendations were provided by *R. Joober, S. Karama, M. M. Chakravarty,* and *A. Labbé.* The text and generation of figures and tables were written and designed by ***N. Fotopoulos***.

Chapter I: Introduction

The World Health Organization estimates that 1 in 5 individuals will experience mental illness during their lifetime, and that psychiatric, neurological and substance use disorders will become the second-leading cause of death in Canada by 2040 (Patel et al., 2016; Steel et al., 2014). The most common childhood-onset psychiatric disorder is attention-deficit/hyperactivity disorder (ADHD), with a prevalence of 5.3% in school-aged children (S. V. Faraone et al., 2015). Although ADHD can be misconceived as a behavioural consequence of the modern life-style, its first description as "Hyperkinetic Disorder" dates to the 19th century. In the mid-20th century, psychostimulants were serendipitously discovered as an effective treatment for hyperactivity. ADHD-like symptoms were later described to result from "minimal brain damage", which implicated the brain in the pathophysiology of the disorder (S. V. Faraone et al., 2015; Taylor, 2011). This initiated the search to uncover the biological basis for ADHD that continues to this day.

I.1 Attention-Deficit/Hyperactivity Disorder

ADHD is a global health concern affecting individuals, families, and society. The economic burden of ADHD is estimated at 7 billion dollars annually, straining the educational, health-care and judicial systems (Daley, Jacobsen, Lange, Sorensen, & Walldorf, 2019). Upon adjustment for cultural and diagnostic differences, the prevalence of ADHD does not significantly differ among countries in the Americas, Europe, Asia and Africa, as well as in Australia (Polanczyk, Willcutt, Salum, Kieling, & Rohde, 2014). ADHD is associated with negative life outcomes such as academic underachievement, social dysfunction, low self-esteem, substance-use disorders, unemployment, traffic accidents, criminality and suicide (Franke et al., 2018; R. G.

Klein et al., 2012). Individuals with ADHD are highly susceptible to develop psychiatric comorbidities (70%) and are three times more likely to die prematurely from unnatural causes (Dias et al., 2013; Sun et al., 2019).

ADHD is a neurodevelopmental disorder characterized by atypical development of the central nervous system. Symptoms generally arise in early childhood and persist throughout the lifespan, causing functional impairment in 2.5% of adults (S. V. Faraone et al., 2015). In children, boys are more frequently diagnosed than girls (4:1) however in adults, the diagnostic sex-ratio is equal (1:1) (Thapar & Cooper, 2016). This may be attributable to a biased clinical representation of ADHD symptoms in childhood or different sex-specific effects of ADHD over time. In either case, girls tend to go undiagnosed, posing additional serious risk, as girls with ADHD have been associated with higher rates of suicide attempts and self-injury in comparison to girls without the disorder (Hinshaw et al., 2012).

I.2 Clinical Features

The third version of the Diagnostic and Statistical Manual of Mental Disorders (DSM) introduced the term "Attention-Deficit Disorder (ADD) with or without hyperactivity" and shaped the first operational diagnostic criteria for health-care providers. The DSM-IV further refined these criteria and characterized three subtypes of ADHD: primarily inattentive (20-30%), hyperactive-impulsive (15% or less) and combined (50-75%) (Klimkeit, Rinehart, May, & Bradshaw, 2010). Indeed, ADHD is heterogeneous in its clinical expression and is characterized by core symptoms

of inattention, hyperactivity, and impulsivity. A diagnosis is given when symptoms reach a clinically defined threshold of 6 or more items, in either or both domains, and cause significant daily impairments in at least two areas of life (e.g. school, home, activities). While academic difficulties are usually the primary motive for medical consultation, ADHD is best described as a deficit in executive functioning (e.g. planning, inhibition, etc.) accompanied by emotional dysregulation, and thus can impact all areas of life across the lifespan (S. V. Faraone & Larsson, 2018). DSM-IV diagnostic items are listed in Table I.1.

The revised and current edition, DSM-V, uses the same diagnostic criteria apart from making some subtle modifications. These modifications consist of raising the age of onset of ADHD symptoms from 7 to 12 years (as functional impairments may be less obvious in younger children), subtypes are now referred to as "presentations", and Autism Spectrum Disorder is no longer part of the exclusionary criteria. Moreover, terms were added to describe the severity of ADHD (i.e. mild, moderate, and severe) (Epstein & Loren, 2013). In acknowledgement that ADHD-like symptoms can occur transiently when exposed to environmental stressors, symptoms must persist over a minimum period of 6 months for diagnosis. The children participating in the current research project were recruited from a phase-IV clinical trial, ongoing for the past two decades at the ADHD clinic. For consistency between participants, ADHD diagnosis continues to be based on DSM-IV criteria in our sample.

Although the current categorical system for ADHD has provided a reliable and standardized approach for diagnosis, it encourages a dichotomous perception of the disorder.

Table I. 1: Summary of DSM-IV diagnostic criteria (American Psychiatric Association, 1994)

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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 another 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
</td>
<td>
Impulsivity
(g) often blurt out answers before questions have been completed
(h) often has difficulty awaiting turn
(i) often interrupts or intrudes on others (e.g. 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 a 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).
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Code based on subtype:

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
314.03 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.
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</table>

ADHD is more accurately reflected as a complex trait found at the extreme end of a continuum, with a normal distribution in the population (Heidbreder, 2015). Importantly, determination of ADHD criteria is based on historically-defined clinical measures, as biological tools for diagnosis are currently unavailable. Therefore, the pathophysiology of ADHD remains to be fully recognized and depends upon understanding the underlying mechanisms of the disorder.

I.3 ADHD endophenotypes

The current nosology of ADHD is built upon behavioural symptoms, and although this has benefited clinical practice, the etiology of the disorder remains unknown. This illustrates the large and complex gap between risk factors and clinical outcomes. To overcome this challenge, the use of endophenotypes have been proposed to help delineate the pathophysiology of ADHD (M. Klein, Onnink, et al., 2017).

An endophenotype is described as an intermediate measure between genes and clinical symptoms, and can provide a more simple, heritable, stable, specific and quantifiable trait for study (Castellanos & Tannock, 2002; Doyle et al., 2005). Given the high heterogeneity of ADHD, the same endophenotype is unlikely to be expressed across all patients. Therefore, the search for endophenotypes holds promise in reducing the complexity of ADHD by characterizing more homogeneous subgroups of the disorder that may arise via different aetiological pathways.

I.3.1 Neuropsychological

Compared to typically developing children, individuals with ADHD perform relatively poorer on neuropsychological assessments and two potential endophenotypes have been steadily discussed: deficits in response-inhibition and higher reaction-time variability (Crosbie, Perusse, Barr, & Schachar, 2008; Doyle et al., 2005). First, inhibitory control is a major executive function often compromised in ADHD individuals. Response-inhibition is a proxy measure for inhibition regulation and represents the ability to withhold a premature and incorrect response on a neuropsychological test (e.g. Continuous Performance Test). Several studies have reported lower response-inhibition scores in ADHD patients, inferring a higher degree of impulsivity. Second, reaction-time is a measure of time consistency across responses in a neuropsychological assessment and is one of the most replicated neuropsychological deficits in ADHD (Doyle, 2015). A higher degree of variability in reaction-time indicates more difficulty in sustaining attention over the course of the test. Even with their strong association to ADHD symptoms, these endophenotypes have not been universally observed in all cases and purportedly represent pointers to subgroups of ADHD.

I.3.2 Brain Imaging

While behavioural and cognitive studies in ADHD have provided important information about clinical outcomes, our understanding of the neurobiology of ADHD remains incomplete. Neuroimaging offers great potential in elucidating the pathophysiology of ADHD by identifying brain endophenotypes (Bednarz & Kana, 2018). Magnetic Resonance Imaging (MRI) has become

increasingly available in recent decades and enables *in vivo* quantification of brain structure and function in a non-invasive fashion. As such, MRI has been a ground-breaking tool for pediatric brain research (Barkovich, Li, Desikan, Barkovich, & Xu, 2019). Both structural and functional abnormalities have been reported in ADHD. Although, studies implicating brain endophenotypes mainly involve structural neuroimaging.

Structural MRI (sMRI) provides grey matter (neuronal cell bodies) and white matter (myelinated axons) measurements, which are used to compute total brain, cortical and subcortical volumes. More specific measurements, such as cortical thickness and surface area are also quantifiable with sMRI. Children and adolescents with ADHD have demonstrated significantly smaller grey matter volume in the precentral gyrus, paracingulate cortices, medial cortex and orbitofrontal cortex (Bralten et al., 2016). These cortical brain regions are involved in executive functions such as decision making, motivation, motor functioning and cognitive control, wherein deficits have been related to ADHD. Furthermore, smaller volumes have also been observed in the unaffected siblings of ADHD probands relative to controls in the paracingulate cortices, medial and orbitofrontal cortex, thereby providing additional support in favour of cortico-structural endophenotypes in ADHD (Bralten et al., 2016).

Briefly, functional MRI (fMRI) provides measures of relative activity in brain regions over time. It uses a blood-oxygen level dependent (BOLD) signal to record blood flow during task-performance and at rest. fMRI can also provide information on the functional connectivity between various brain regions by assessing activation patterns. Decreased functioning of the prefrontal and

anterior cingulate cortices has been reported in ADHD. Specifically, a meta-analysis of task-based functional MRI studies in ADHD reported under-activation of fronto-striatal, fronto-parietal and ventral attentional networks, as well as hyperactivation in somatomotor and visual systems (Cortese et al., 2012). The activation pattern of inhibitory networks has been proposed as a complementary tool for ADHD diagnosis. However, findings have been reported in opposite directions regarding response-inhibition related activity (Albajara Saenz, Villemonteix, & Massat, 2018). Therefore, the existence of functional brain endophenotypes in ADHD has not been established.

I.4 Brain Structure and Development in ADHD

A widely discussed theory in ADHD is delayed brain maturation, brought forth from a landmark study by Shaw and colleagues (Shaw et al., 2007). Children with ADHD have shown significant delays in cortical thinning of fronto-temporal regions in comparison to non-ADHD children (Cubillo, Halari, Smith, Taylor, & Rubia, 2012; Shaw et al., 2007). Typically, peak cortical thickness occurs around 7.5 years of age. However, children with ADHD attain this neurodevelopmental milestone when approximately 10.5 years old, demonstrating an average 3-year delay in cortical development (Shaw et al., 2007; Shaw et al., 2012). The delays in cortical development were shown to be most prominent in prefrontal regions, important for executive functions, thereby fitting the current framework of ADHD pathophysiology (Rubia, 2007; Shaw et al., 2007; Shaw et al., 2006). Moreover, although these findings deduce slower brain maturation, ADHD does not seem to be associated with maldevelopment of the cortex or divergent cortical trajectories.

Meta-analyses of sMRI studies have generally reported global reductions in total brain volume (3-5%) in ADHD cases relative to controls (Castellanos et al., 2002; Valera, Faraone, Murray, & Seidman, 2007). A multi-site mega-analysis ($n \geq 3000$) found smaller volumes in subcortical structures, particularly the caudate nucleus, putamen, accumbens, amygdala and hippocampus (Hoogman et al., 2017). Moreover, abnormalities in the fronto-temporo-parietal and fronto-cerebellar structural networks have also been observed in ADHD (Castellanos, 2002; Shaw et al., 2006; Silk et al., 2016).

Variations in brain structure between ADHD cases and controls are more distinguishable in children than adults, further supporting a brain maturation delay in ADHD (Barkovich et al., 2019; Franke et al., 2018; Hoogman et al., 2017). However, more than half of the patients with ADHD do not remit from the disorder and continue to have occupational impairments throughout life. This suggests that while some brain structure delays may dissipate with age, others may be pervasive (Krain & Castellanos, 2006). Indeed, significant differences in cortical thickness and basal ganglia volumes have been observed in adult cases of ADHD (Frodl & Skokauskas, 2012; Shaw et al., 2014). Therefore, age is not only an important factor for ADHD diagnosis, but for relative timing of brain development as well.

I.5 Treatment of ADHD

The opportune discovery of ADHD symptom relief through the use of Ritalin® in 1944 pioneered the concept that ADHD is a neurobiological disorder. Indeed, the effective treatment of

ADHD symptoms with pharmacological agents is tightly linked to our current understanding of its pathophysiology and forms the basis of ADHD research. As such, the following sections summarize the recent literature on the treatment of ADHD, followed by a description of the proposed pathophysiology.

In comparison to other psychiatric disorders, ADHD is well-managed with pharmacotherapy. Both stimulant and non-stimulant medications are licensed for the treatment of ADHD symptoms. The most widely prescribed are psychostimulants, notably methylphenidate (MPH) and amphetamine. Psychostimulants have a high clinical efficacy (approximately 75%), making them the first-line treatment for ADHD (Atkinson & Hollis, 2010). Non-stimulant medications have also been shown to be effective in reducing ADHD symptoms, and are prescribed in lieu of psychostimulants in cases where they are ineffective, cause adverse effects or are not recommended. These include atomoxetine and guanfacine, which have a treatment response rate estimated at 50-60% and 37%, respectively (Clemow & Bushe, 2015; Strange, 2008).

Psychostimulants such as methylphenidate (Ritalin®, Concerta® and Biphentin®) and amphetamines (Vyvanse® and Adderall®), as well as NE-specific therapeutic agents such as atomoxetine (Strattera®) increase DA and NE synaptic concentrations, and have been shown to successfully alleviate ADHD core symptoms (Atkinson & Hollis, 2010; Briars & Todd, 2016; Rubia et al., 2014). It is estimated that 5.2% of children are currently prescribed ADHD medication, representing a five-fold increase from 1994 to 2010, as reported by the Center for Disease Control and a 2018 study (Danielson et al., 2018; Visser et al., 2016). The prominent increase in the pharmacological treatment for ADHD may be attributable to the high clinical

efficacy of psychostimulants and their well-established positive effects on behavioural, occupational and clinical outcomes (Atkinson & Hollis, 2010). Moreover, ADHD is a treatable yet chronic condition, where 50-60% of children diagnosed with ADHD will have persistent symptoms into adulthood, and thereby continue to require ADHD medication across the lifespan (S. V. Faraone et al., 2015). As such, the typical course of treatment for ADHD involves constant administration of medication. Although the long-term outcomes of ADHD medication on behavioural outcomes are recognized, there is a scarcity of information regarding the downstream effects of medication use on brain structure and development.

I.5.1 Pharmacological Mode of Action

Under physiological conditions, dopamine (DA) and norepinephrine (NE) are released into synaptic clefts through their respective neurons and bind to receptors located on post-synaptic neurons, initializing a cascade of cellular events. Subsequently, the dopamine and norepinephrine transporters (DAT, NET) recycle DA and NE from synapses into pre-synaptic neurons. Psychostimulants block DAT and NET, preventing the re-uptake of DA and NE into pre-synaptic neurons. This results in an increase of synaptic DA and NE concentrations, thereby increasing the availability for post-synaptic receptor binding. The non-stimulant atomoxetine similarly increases synaptic concentrations of these neurotransmitters. However, it selectively inhibits NET, which has a higher binding affinity for both DA and NE relative to DAT (Bymaster et al., 2002). Figure I.1 illustrates the mode of action of NET.

Finally, the non-stimulant guanfacine does not directly act on DA and NE transporters. Rather, this agent specifically binds and activates alpha2A adrenergic receptors located in post-synaptic neurons, leading to higher post-synaptic signaling (Sikirica et al., 2013). Higher synaptic levels of DA and NE and post-synaptic receptor binding appear to counteract ADHD symptoms. Therefore, pharmacological treatment of ADHD entails the equilibration of DA and NE levels (Hohmann et al., 2015), and infers the disorder arises, at least partly, from dysregulation within these neurotransmitter systems (Del Campo, Chamberlain, Sahakian, & Robbins, 2011).

I.5.2 Medication and Brain imaging

Some MRI studies have investigated the effects of medication on brain structure and function. However, a prospective, randomized-controlled study would comprise unethical means, either by unnecessarily medicating typically-developing children or by withholding treatment from children with ADHD over an extended period to estimate medication effects on brain structure and development. Therefore, human neuroimaging studies have primarily reported on the acute effects of ADHD medication between groups of treatment-naïve and medicated children with ADHD. Moreover, the majority of medication studies have been conducted via single-dose designs and have assessed brain function (fMRI) in children with ADHD, thereby further contributing to the gap in the literature regarding the downstream effects of medication on brain structure. Consequently, the mechanisms and biological impacts of long-term medication use on brain development have yet to be determined (Oakes et al., 2018; Schmitz et al., 2017).

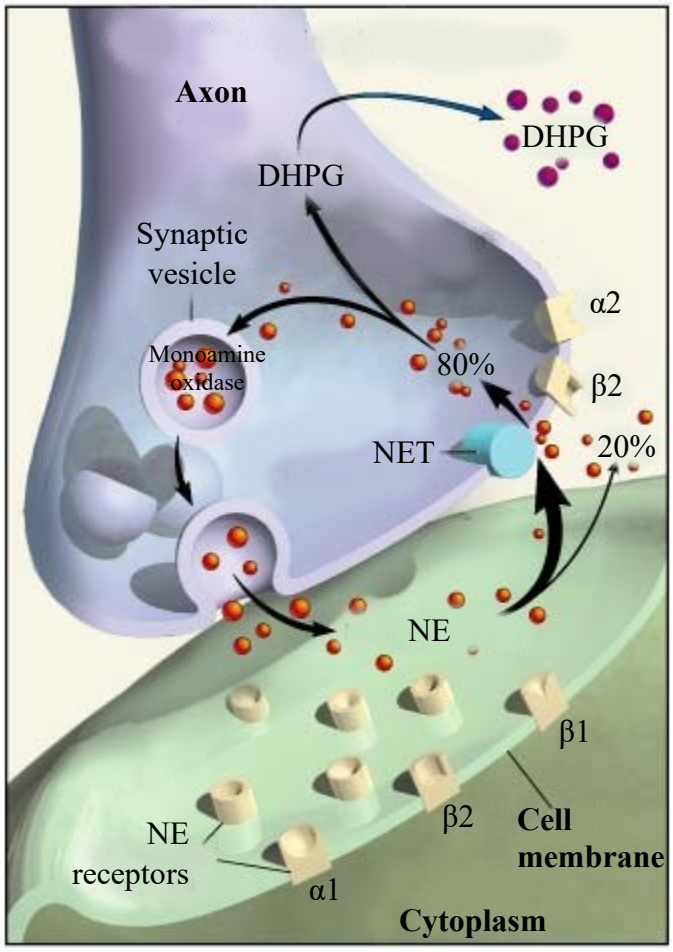

Figure I. 1: Norepinephrine Mode of Action at Synapse.

Reproduced with permission from (Shannon et al., 2000), Copyright Massachusetts Medical Society.

Functional magnetic resonance imaging (fMRI) studies have provided some evidence for the acute effects of ADHD medication on brain function. Single-dose MPH studies have observed upregulation of functional connectivity in fronto-striatal, fronto-parietal and fronto-cerebellar networks following psychostimulant administration (Arnsten, 2006; Spencer et al., 2013). The most consistently reported medication effect is an increased activation of the right inferior frontal gyrus and striatum (Shaw et al., 2009; Spencer et al., 2013). The available findings provide some evidence in support of a short-term neuroprotective effect of medication. However, there is a shortage of information regarding the effects of continued use of ADHD medication on brain function as well. This is a concerning gap in the literature, as proper management of ADHD symptoms is primarily achieved through long-term pharmacological treatment, often beginning in early childhood. Further research using suitable and ethical study designs are promptly required.

The limited neuroimaging studies that have investigated the effects of ADHD medication on brain structure (sMRI) are summarized in the following paragraph. These studies typically compared three groups of children: medication-naïve with ADHD, chronically-treated with ADHD and unmedicated children with neurotypical development (control). One study reported that cortical thickness measurements did not significantly differ between the group of children with ADHD receiving pharmacological treatment and the control group of typically-developing children. However, a higher rate of cortical thinning was detected in the group of treatment-naive children with ADHD in comparison to the two other groups (i.e. medicated with ADHD and control) (Shaw et al., 2009).

An earlier study by Castellanos et al. 2002 found that treatment-naive children with ADHD had significantly smaller frontal, temporal and total white matter volumes in comparison to the groups of medicated children with ADHD and control children. In line with the abovementioned study by Shaw et al., no significant differences in white matter volumes within these regions were observed between the medicated children with ADHD and control children. Nevertheless, the authors further reported that both groups of children with ADHD, regardless of their medication exposure status, had a significant reduction in their total volume of cortical grey matter relative to the control group. It was concluded that as opposed to white matter, grey matter may not be susceptible to medication effects (Castellanos et al., 2002). These findings suggest that while some brain regions may be affected by medication, other structures may not be. Indeed, additional studies have observed significantly smaller white matter, anterior cingulate cortex, cerebellar and thalamic volumes in treatment-naïve children with ADHD relative to medicated children with ADHD and typically-developing children (Ivanov et al., 2010; Schweren, de Zeeuw, & Durston, 2013; Semrud-Clikeman, Pliszka, Bledsoe, & Lancaster, 2014).

Taken together, these studies do not provide evidence for abnormal brain development following exposure to ADHD medication. Rather, they suggest an acute normalizing effect of medication exposure on specific brain regions (Bledsoe, Semrud-Clikeman, & Pliszka, 2009; Loureiro-Vieira, Costa, de Lourdes Bastos, Carvalho, & Capela, 2017; Nakao, Radua, Rubia, & Mataix-Cols, 2011).

As mentioned in Section I.4, some ADHD case-control neuroimaging studies investigating brain structure have identified significant differences in cortical and subcortical brain measurements. However, there is considerable variability in findings across studies, and thus brain structure phenotypes for ADHD children have not been reliably established. One meta-analysis on ADHD neuroimaging studies demonstrated that only 25-50% of published reports had reproducible findings (Frodl & Skokauskas, 2012). Since pharmacological agents are commonly used to treat ADHD symptoms, it is important to assess their impact on brain structure. If exposure to ADHD medication significantly alters brain structure measurements, it might provide partial explanation for the varying results across ADHD neuroimaging studies.

The limited evidence from the available retrospective studies comparing brain structure between treatment-naïve children with ADHD, medicated children with ADHD and typically-developing control children suggest that medication is associated with more normative measurements in brain regions relevant to ADHD (Loureiro-Vieira et al., 2017). Nevertheless, two important caveats should be considered. First, studies assign a categorical designation (naïve vs. treated) to investigate ADHD medication effects on the brain. Therefore, the effects of ADHD medication on brain structure in chronically-treated children have not yet been investigated. Indeed, neither of the two longitudinal studies assessing the effects of ADHD medication on brain structure considered the duration of medication use within the treated group of ADHD children (Castellanos et al., 2002; Shaw et al., 2014). Therefore, if a normalizing effect is occurring, the duration and dose required to achieve this effect remains unknown. Studies using an accurate and continuous value for cumulative exposure to ADHD medication would address this gap in the literature and shed light on the long-term effects of ADHD medication on brain structure.

Second, there is increasing awareness that head and breathing motions during MRI acquisition lead to underestimation of brain structure measurements (Reuter et al., 2015; Weinberger & Radulescu, 2016). Since children with ADHD tend to be hyperactive and pharmacological treatment reduces hyperactivity, it is possible that unmedicated children with ADHD have a significantly higher degree of motion during scanning and accrue more motion artifacts on raw brain images compared to medicated children with ADHD and control children. If this is the case, motion may confound the structural findings cited above and partly explain the observations of normalization (Pardoe, Kucharsky Hiess, & Kuzniecky, 2016). In combination to an appropriate quality control, restricting the sample to children undergoing pharmacological treatment for ADHD can address this issue by removing the heterogeneity of motion existing between treatment groups.

It is also worth mention that pharmacological treatment of ADHD often improves attention and reduces hyperactivity, which is beneficial in having children comply with the research protocol and scanning process (Barkovich et al., 2019). Treatment-naive children with ADHD may have more difficulty following instructions and remaining still during the scan. Therefore, the behavioural effects of medication surrounding the scanning research protocol cannot be fully discounted from the neurobiological findings across ADHD studies. It has been recommended to recede from treatment-group comparisons and recruit participants with varying degrees of medication exposure in order to better extrapolate “true” medication effects on the brain (Bednarz & Kana, 2018).

I.6 Pathophysiology of ADHD

In addition to the pharmacological evidence highlighted above, there is converging evidence that ADHD symptoms are associated with reduced activity in dopamine (DA) and norepinephrine (NE) systems (S. V. Faraone, 2018; S. V. Faraone et al., 2015). Studies have proposed that changes in these neurotransmitter systems affect the functioning of brain structures relevant for ADHD (S. V. Faraone, 2018; S. V. Faraone et al., 2015), and are discussed in the following section.

I.6.1 Dopamine System

Neuroimaging and animal studies have garnered convincing evidence that dysregulation within the DA system is involved in the pathogenesis of ADHD. MRI case-control studies have found volumetric reductions in dopamine-rich brain regions, notably the caudate nucleus and globus pallidus, as well as decreased activation of the DA pathway in probands (Castellanos & Tannock, 2002; Durston, 2003). Opposing findings have also been observed, where an augmented striatal dopaminergic response was reported in the treatment-naïve group with ADHD (Cherkasova et al., 2014). In either case, exaggerated phasic dopamine release may contribute to ADHD symptoms (i.e. impulsivity) (Cherkasova et al., 2014). A meta-analysis of positron emission tomography (PET) studies reported a 14% increase of striatal DAT density in ADHD cases, albeit some studies have contradicted these findings (Fusar-Poli, Rubia, Rossi, Sartori, & Balottin, 2012). Moreover, an exploratory analysis between cortical thickness and striatal dopamine transmission found that lower baseline DA receptor availability was associated with thinner cortical

measurements in the treatment-naïve ADHD group relative to controls (Cherkasova et al., 2017). Therefore, evidence suggests that dopamine transmission is associated with cortical measurements, and that this association may be aberrant in ADHD individuals.

Research using a validated ADHD animal model, the spontaneously hypertensive-rat (SHR), has demonstrated that a hypo-dopaminergic state is accompanied by hyperactivity, and that psychostimulants that increase DA concentrations alleviate hyperactive behaviour (Li et al., 2007; van der Kooij & Glennon, 2007). SHRs have been reported to have decreased dopamine D4 receptor (DRD4) gene expression and protein synthesis in the prefrontal cortex, as well as reduced volumes in the cerebellum, caudate and putamen (Li et al., 2007).

Given this, and since DA is important for executive functioning, genetic studies have largely focused on candidate genes within the DA system to study ADHD pathophysiology. Indeed, ADHD diagnosis has been steadily associated with variations in DAT1 and DRD4 (Brookes et al., 2006). Nevertheless, alterations within the DA system may not exclusively explain ADHD pathogenesis, as psychostimulants act on both DAT and NET, and non-stimulant therapeutic agents that specifically target the NE system are also effective in reducing ADHD symptoms.

I.6.2 Norepinephrine System

The combination of strong pharmacological evidence and the well-established NE's role in sustaining attention implicates this neurotransmitter in ADHD pathophysiology. NE is ubiquitously expressed throughout the cortex, especially in prefrontal regions (Arnsten & Pliszka, 2011; Greene, Bellgrove, Gill, & Robertson, 2009; Vanicek et al., 2014). The neurotransmission of NE originates in the locus coeruleus and innervates neurons in the thalamus, cerebellum, and prefrontal cortex (PFC). The prefrontal cortex is important for regulation of higher-order functions such as attention, behaviour and emotion, where tight modulation of DA and NE via NET plays a supporting role (Hohmann et al., 2015). NE neuronal projections are dense in the PFC, and lesions within regions of the PFC negatively impact concentration, inhibition control and motor control (Klimkeit et al., 2010). Please refer to Figure I.2 for the NE pathway.

Indeed, sMRI studies have reported reduced cortical thickness in the PFC, as well as smaller volumes in NE rich brain regions (i.e. thalamus and cerebellum) (Hoogman et al., 2017; Shaw et al., 2018). Moreover, decreased activation in the PFC, basal ganglia and thalamus, and increased activation in the cerebellum have been observed in ADHD (Albajara Saenz et al., 2018).

Due to a lack of suitable NE ligands, almost no PET studies quantifying the NE system in ADHD are available (Arakawa et al., 2008). Findings from one existing study reported no changes in NET distribution in ADHD participants relative to controls. However, the authors stated that NET availability cannot be reliably measured in the PFC using the currently available radioligands, and thus findings are inconclusive (Sigurdardottir et al., 2016).

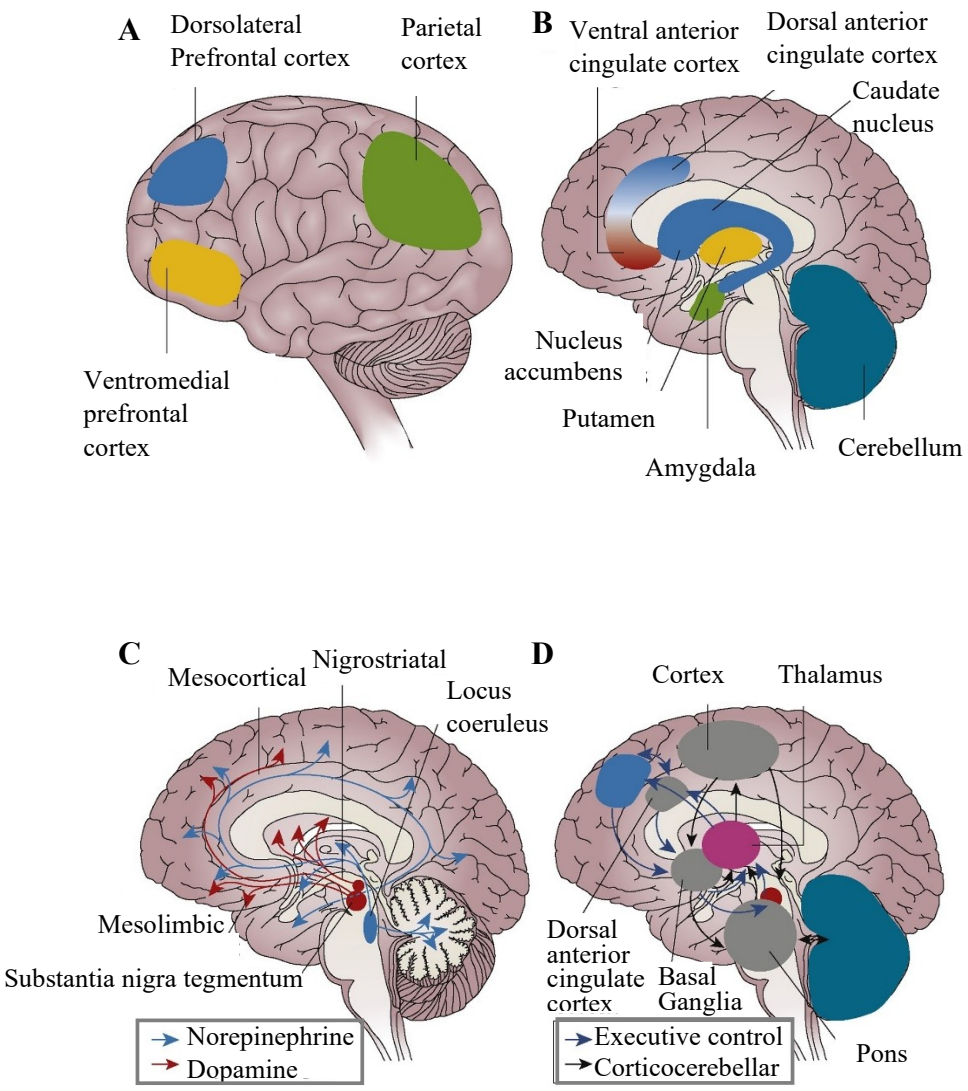

Figure I. 2: Brain mechanisms involved in ADHD by (S. V. Faraone, 2018). (A) Lateral view of cortical regions relevant in ADHD. (B) Medial view of subcortical regions relevant in ADHD. (C) Dopamine and norepinephrine (i.e. noradrenergic) neurotransmitter pathways in the brain. (D) Executive control and cortico-cerebellar networks involved in ADHD. Image licensed under Creative Common http://creativecommons.org/licenses/by/4.0/. Reprinted with permission from Macmillan Publishers Ltd: [NAT REV DIS PRIMERS] (Faraone et al., 2015), copyright 2015.

At present no animal models of ADHD with selective disruptions within the NE system exist. However, findings from rodent models implicate that disturbances in both DA and NE neurotransmission cause hyperactivity and distractibility in ADHD (V. A. Russell, 2002). Moreover, stimulation of the NE system has been reported to increase attention and cognitive functioning in non-human primates (Borodovitsyna, Flamini, & Chandler, 2017).

Despite the relatively limited focus on the NE system in ADHD genetic research, evidence from pharmacological and clinical studies support a role for NET in ADHD pathophysiology. Indeed, association between single-nucleotide polymorphisms (SNPs) within NET and ADHD has been reported in the literature, as well as by our team (Bobb et al., 2005; Sengupta et al., 2012; Thakur, Sengupta, Grizenko, Choudhry, & Joober, 2012).

I.7 ADHD Etiology

ADHD is a complex disorder believed to have multiple etiologies. A combination of various unidentified genetic and environmental factors, as well as their interactions, contribute to the pathogenesis of the disorder.

I.7.1 Genetics

Family, twin, and adoption studies have consistently reinforced a strong genetic background for ADHD. With an estimated average heritability of 74%, ADHD is one of the most

heritable psychiatric disorders (S. V. Faraone & Larsson, 2018). Heritability estimates from twin studies are shown in Figure I.3. Furthermore, a recent review on genetic studies in ADHD continues to support a strong genetic background for ADHD (S. V. Faraone & Larsson, 2018). Several genes, each with a small to moderate effect, are suspected to play a role in the disorder. Researchers have sought to discover the genetic underpinnings of ADHD using different approaches, namely linkage analysis, genome-wide association studies (GWAs) and candidate-gene studies. However, given its polygenic nature and heterogeneity, the genetic etiology of ADHD remains to be fully elucidated.

Linkage studies

Linkage studies have reported over 100 different regions in the genome linked to ADHD, though with very low to no replicability (S. V. Faraone et al., 2008). The most promising loci were reported in regions 16p13 and 17p11 (Ogdie et al., 2003; Smalley et al., 2002). Moreover, a meta-analysis combining seven genome-wide linkage studies in ADHD (n = ~2000) found a significant link on chromosome 16 (between 64 and 83 Mb), as well as suggestive evidence of linkage in nine other genomic regions (Zhou et al., 2008). Linkage analysis is a powerful tool to identify high-penetrance variants in complex trait disorders, but less optimal in finding genetic variants with small effect sizes (S. V. Faraone & Larsson, 2018; S. V. Faraone et al., 2005). As mentioned above, the genetic variants suspected to play a role in ADHD have a small contribution individually. Given prior evidence pointing towards the DA and NE systems, a candidate gene approach may be more suitable to uncover the latent genetic risk factors of ADHD.

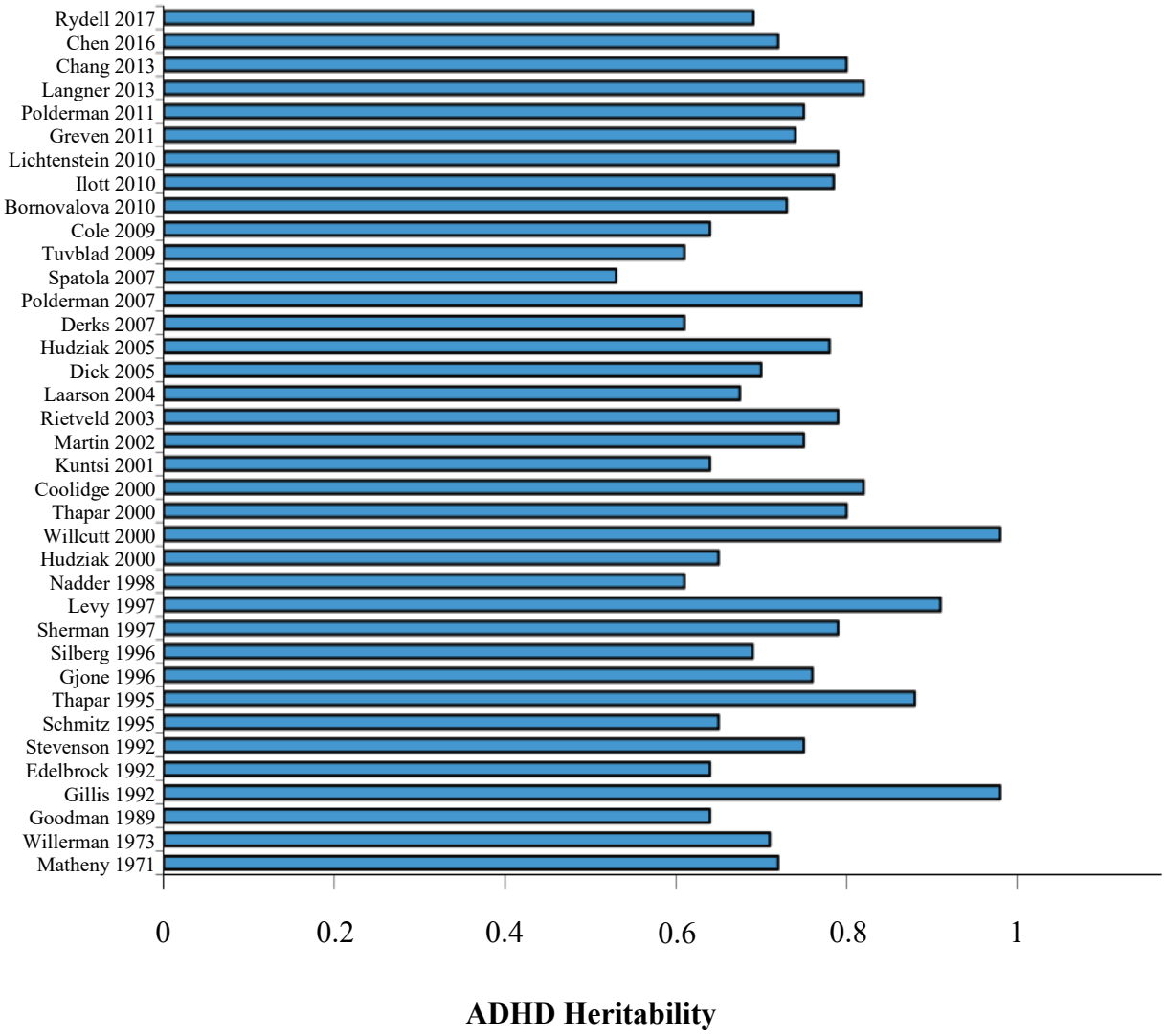

Figure I. 3: Heritability estimates from twin studies in ADHD

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Genome-Wide Association Studies

Following a series of GWAs in ADHD with no significant findings, the largest and most recent study (ADHD n = 20 183; controls n = 35 191) successfully identified 12 loci in association to ADHD (Demontis et al., 2019). Several of the significant loci were located within or proximal to genes involved in neuronal development, notably FOXP2, SEMA6D and DUSP6. More specifically, FOXP2 has been previously associated with ADHD and regulates DA levels, SEMA6D plays a role in neuronal fetal development and DUSP6 modulates DA synaptic concentrations (Demontis et al., 2019; S. V. Faraone & Larsson, 2018). This was a momentous step forward in understanding the genetic etiology of ADHD. Nonetheless, the authors reported that these findings capture but a third of the common genetic risk variants for ADHD, and further research is required to disclose the remaining heritability (Demontis et al., 2019).

Candidate gene studies

Based on pharmacological evidence implicating the DA and NE systems in ADHD pathogenesis, candidate-gene studies have largely focused on genes within these neurotransmitter systems. Six genes have been significantly associated with ADHD across several studies; DAT1, DRD4, DRD5, 5HTT (serotonin transporter), HTR1B (serotonin receptor) and SNAP25 (synaptic vesicle regulator) (Gizer, Ficks, & Waldman, 2009). Moreover, a meta-analysis combining all candidate gene studies found a significant association between ADHD and BAIAP2, a gene

involved in neuronal proliferation and morphogenesis of dendritic spines (Bonvicini, Faraone, & Scassellati, 2016).

I.8 Norepinephrine Transporter Polymorphisms

As mentioned, NE is the main substrate for NET. However, NET is also central for the reuptake of DA, as NET has higher binding affinity relative to DAT (Del Campo et al., 2011). Considering this, in addition to the enrichment of NET in the PFC where DAT density is low, highlights the central role of NET in PFC function (Arnsten, 2009; Arnsten & Pliszka, 2011). Furthermore, the drug atomoxetine has proven to be effective in treating ADHD symptoms by specifically targeting NET, thus inferring an even greater role for NET in ADHD pathophysiology (Sigurdardottir et al., 2016; Thakur et al., 2012). It has been suggested that optimal synaptic functioning requires adequate level of DA and NE, where both too low and too high concentrations are associated with deterioration in executive functioning (Arnsten & Pliszka, 2011; Cools & D'Esposito, 2011; Hohmann et al., 2015). In other words, cognitive functioning may be modulated according to an inverted-U-shape curve of NE (Arnsten & Pliszka, 2011; Cools & D'Esposito, 2011). As such, polymorphisms within *NET* is an important focus for ADHD genetic studies.

NET is a solute carrier family 6 member 2 (SLC6A2) transmembrane glycoprotein spanning 45 kb and containing 617 amino acids in 14 exons (Sengupta et al., 2012; Thakur et al., 2012). The encoding gene is *SLC6A2* located on chromosome 16q12.2 (Hohmann et al., 2015; Thakur et al., 2012). Some genetic studies have investigated the association between various

single-nucleotide polymorphisms (SNPs) and ADHD however, findings have been difficult to replicate. The most associated SNPs of *SLC6A2* and ADHD are rs3785143 and rs11568324, which were initially identified in a large-scale ADHD genetic study (International Multisite ADHD Geneproject), and subsequently replicated in two independent studies (Brookes et al., 2006; J. W. Kim et al., 2008; Xu et al., 2008). A previous Family-based Analysis Test (FBAT) conducted by Thakur et al. tested a panel of 30 SNPs in *NET* and found that rs36021 was associated with ADHD diagnosis, behavioural and cognitive dimensions, and treatment-response. Interestingly, rs36021, located within an intron, is in complete linkage disequilibrium (LD) with rs3785143 and rs11568324. The FBAT study showed an over-transmission of the rs36021 T-allele, which was proposed as the risk-allele as per its association with more severe ADHD symptoms, behavioural disorders (internalizing, externalizing and aggression scores), and cognitive deficits (sustained attention, response-inhibition, response variability and reaction-time). It was concluded that rs36021 and linked SNPs are significant genetic determinants of behavior, cognition, and treatment response in children with ADHD, especially in children exposed to maternal smoking during pregnancy (Thakur et al., 2012).

I.8.1 Imaging-genetics in ADHD

The burgeoning field of imaging-genetics holds potential in breaking down the complexity of ADHD by identifying subgroups of ADHD founded on the neurobiological effects of specific candidate genes on brain structure, and thus can reveal ADHD endophenotypes (Bednarz & Kana, 2018; M. Klein, Onnink, et al., 2017). Variations within DAT1 have received much attention in genetic studies, and one of the polymorphisms, the 10-repeat allele, has been associated with

childhood ADHD (Cornish et al., 2005). Interestingly, a sMRI study investigating the relationship between the 10-repeat allele and brain structure found that homozygous children with ADHD were associated with decreased cortical thickness in the right lateral prefrontal cortex in comparison to children with a single or no copy of the allele (Fernandez-Jaen et al., 2015). These results suggest that combining a candidate gene approach with neuroimaging can help to find ADHD endophenotypes and thus tease apart genetic aetiological pathways.

Imaging-genetics studies applying structural MRI are rare, and despite the high relevance of *NET* to ADHD pathophysiology, investigated genes primarily belonged to the dopaminergic and serotonergic neurotransmitter systems (SLC6A3/DAT1, DRD2, DRD4, SLC6A4/5-HTT/SERT) (M. Klein, Onnink, et al., 2017). Remarkably, there are no studies using an imaging-genetics approach to examine the effects of polymorphisms within *NET* on brain structure in children with ADHD. This thesis aims to expand on these previous findings by our team and model the effects of rs36021 genotype on brain structure and clinical dimensions.

I.9 Environmental Risk Factors

Although the estimated heritability of ADHD is quite high, approximately 26% of the conferred risk for the disorder is attributed to environmental factors (S. V. Faraone & Larsson, 2018). Therefore, environmental factors, as well as their interactions with genetic risk factors, play a substantial role in ADHD susceptibility. The calculated heritability of ADHD may also comprise gene-environment interactions, and gene expression can be modulated via epigenetic

modifications brought on by environmental factors (Walton et al., 2017). Genetic studies in ADHD are generally limited in accounting for environmental risk factors, as well as for their effects on genetic variants. Therefore, in order to delineate the remaining genetic etiology of ADHD, genetic studies must also consider the environmental contributions to the disorder.

Epidemiological studies have investigated exposure to a range of prenatal, perinatal, toxic, dietary, and psychosocial factors in ADHD. Given that ADHD is a neurodevelopmental condition, the highest contribution of environmental risk factors are believed to occur predominantly during fetal brain development (Banerjee, Middleton, & Faraone, 2007). There is considerable evidence associating increased risk for ADHD with prenatal exposure to alcohol, environmental toxins, maternal stress and maternal smoking during pregnancy (Froehlich et al., 2011). Furthermore, the earlier the environmental insult occurs during fetal brain development, the more widespread the effects are presumed to be (Tremblay, 2010).

Maternal Alcohol Use during Pregnancy

Maternal alcohol use during pregnancy has been suggested as an environmental risk factor for development of ADHD in offspring (Banerjee et al., 2007). A recent study reported a weak but potentially causal association with ADHD symptoms. However, no effects were uncovered with regards to ADHD diagnosis (Eilertsen et al., 2017). Therefore, further work is needed to determine the relationship between fetal alcohol exposure and clinically-relevant ADHD symptoms.

Environmental Toxins

Exposure to environmental neurotoxins, particularly lead and mercury, impact the development of the nervous system and have been associated with distractibility, hyperactivity and lower intellectual ability (A. Chen, Cai, Dietrich, Radcliffe, & Rogan, 2007; S. Huang et al., 2016). Population safety measures put in place over the past decades, especially concerning lead contamination, have diminished incidences of hazardous exposure to neurotoxins in children and pregnant women. However, the prevalence of ADHD remains high, suggesting that either exposure below the recommended threshold can still lead to ADHD symptoms or that other environmental risk factors are also involved in the disorder (Froehlich et al., 2011).

Maternal Stress during Pregnancy & Obstetrical Complications

Moderate to extreme stress experienced by the mother during pregnancy, as rated through objective measures, has been associated with more severe ADHD symptoms in children (Grizenko, Shayan, Polotskaia, Ter-Stepanian, & Joober, 2008). It has been proposed that the association between maternal stress during pregnancy and severity of ADHD symptoms is mediated by genetic risk factors (Choudhry et al., 2012; Grizenko et al., 2012). Moreover, obstetrical complications such as preterm delivery, eclampsia, prolonged labor, hypoxia, low Apgar scores and antepartum hemorrhage have been more frequently reported in ADHD cases (Ben Amor et al., 2005; Schwenke et al., 2018). Additional research is required to determine the causality of these factors in the development of ADHD.

Maternal Smoking during Pregnancy

In addition to the well-established adverse effects of exposure to maternal smoking during pregnancy (MSDP), such as spontaneous abortion, low-birth weight and preterm delivery, MSDP has also been associated with more disruptive behaviour and poorer cognitive functioning in exposed children (Banerjee et al., 2007; Sabra, Gratacos, & Gomez Roig, 2017). Several studies have found an association between MSDP and an increased risk of ADHD in offspring, which remained significant even after adjustment for socioeconomic status and parental psychopathology (Braun, Kahn, Froehlich, Auinger, & Lanphear, 2006; Linnet et al., 2005). A recent meta-analysis of studies investigating the relationship between MSDP and ADHD calculated a pooled estimate of 60% increased risk of ADHD in exposed children (L. Huang et al., 2018). The authors further reported a dose-dependent effect, where mothers who smoked heavily had children with more severe ADHD symptoms relative to children whose mothers smoked lightly or moderately during pregnancy.

Several confounding factors were considered, including the possibility that MSDP and ADHD may be associated through common genetic risk factors. By grouping studies using a sibling design, the authors failed to show a relationship between exposure to MSDP and ADHD. This suggests that genetic factors and family context are difficult to disentangle, although this sub-analysis was limited by a small sample. However, the increased risk of ADHD imparted by MSDP remained significant when parental ADHD was controlled for, inferring that the relationship between MSDP and ADHD is not entirely based on common genetic risk factors (L. Huang et al., 2018). This observation is in line with another study, which reported an increased risk for ADHD

associated with exposure to MSDP after adjustment for parental ADHD (Mick, Biederman, Faraone, Sayer, & Kleinman, 2002).

Taken together, MSDP is one of the most significant and frequently associated environmental risk factors of ADHD and further research aimed in understanding the biological basis of this relationship is warranted (Langley, Rice, van den Bree, & Thapar, 2005).

I.9.1 Prenatal Smoking Exposure

According to the Public Health Agency of Canada, 13-24% and 23.4% of women reported smoking and passive smoking during pregnancy, respectively. Children with ADHD are consistently shown to have higher prenatal smoking exposure relative to the general population (Holbrook, 2016). Indeed, smoking behaviour and ADHD are highly comorbid (35-45%) and likely result in part, from shared genetic risks factors (Obel et al., 2011; Thapar et al., 2009). Along this line, prenatal smoking exposure may be acting as a marker for shared risk factors with ADHD, and thus can help shed light on one of the pathways leading to the manifestation of the disorder.

Fetal Exposure to Nicotine

Studies in animal models have shown that nicotine, the major psychoactive ingredient in cigarettes, readily crosses the placenta and affects fetal growth and brain development, as well as

placental organization (Z. Y. Chen & Yao, 2017; Muhammad et al., 2012; Zhu et al., 2017). Nicotine binds to cholinergic receptors and impacts the programming of neurotransmitter systems in the developing brain (Pistillo, Clementi, Zoli, & Gotti, 2015). More specifically, during fetal development, nicotine inhibits the widespread functions of acetylcholine, thus interfering with multiple catecholamine systems (Zhu et al., 2017). These effects can disrupt the proliferation and differentiation of neuronal progenitor cells, promote apoptosis, produce deficits in the number of neural cells (Alkam et al., 2017), including pyramidal neurons in the somatosensory cortex, and decrease hippocampal cell size (Roy, Seidler, & Slotkin, 2002).

Moreover, a dose-dependent effect of prenatal exposure to nicotine has been reported in animal studies, where reduced activation of DA and NE systems was associated with smaller birth weight, hyperactivity and poorer cognitive performance (Alkam et al., 2017; Banerjee et al., 2007). In human studies, prenatal exposure to cigarettes has been associated with increased symptom severity, comorbidities (i.e. externalizing disorders) and cognitive deficits (Keyes, Davey Smith, & Susser, 2014; Thakur et al., 2013).

Maternal Smoking during Pregnancy and Brain Structure

Human neuroimaging studies have mostly investigated the effects of active cigarette smoking on brain structures and functions in adult populations. Studies have generally reported a thinner cortex in adult smokers compared to non-smokers (Akkermans et al., 2017; Karama et al.,

2015). Less is known about the effects of exposure to prenatal cigarette smoking and cortical structures.

Evidence in favour of an association between MSDP and ADHD, as well as the reported impact of MSDP on fetal brain development, has motivated some investigations of MSDP exposure on brain structure. A review of sMRI studies reported decreased cortical thickness and volume in the cerebellum and corpus callosum in adults with ADHD exposed to MSDP versus non-exposed (Bublitz & Stroud, 2012). Additionally, a 25-year longitudinal study found decreased volume in the several brain regions, notably the right frontal gyrus, in adult with ADHD exposed to MSDP (Holz et al., 2014). Likewise, De Zeeuw et al. found smaller cerebellar volumes in adults with ADHD exposed to MSDP in comparison to non-exposed (de Zeeuw, Zwart, Schrama, van Engeland, & Durston, 2012). Almost all studies investigating exposure to MSDP on cortical brain structure have focused on adults with ADHD, where personal exposure to cigarette smoking during adolescence and adulthood, regardless of foetal exposure, may be a major confounding factor (Akkermans et al., 2017).

Regarding sMRI pediatric studies, cortical thinning in the superior frontal and superior parietal cortices have been observed in typically-developing children exposed to MSDP (El Marroun et al., 2014). Taken together, there is evidence that exposure to MSDP is associated with brain structure alterations in regions relevant for ADHD. However, neuroimaging studies investigating the downstream effects of MSDP on brain structure in children diagnosed with ADHD are limited to none. Given the relatively high incidence of MSDP, as well as its increasing

prevalence in developing countries, further research is required to uncover the downstream effects of MSDP on brain development in ADHD (Abdullah & Husten, 2004; Lange, Probst, Rehm, & Popova, 2018).

Epigenetic Modifications and Prenatal Smoking Exposure

One of the major limitations in epidemiological and brain imaging studies investigating the relationship between MSDP and ADHD is the retrospective assessment of the child's prenatal exposure to smoking, which may be fraught by various problems. First, there is a possibility of non-disclosure of MSDP since this behaviour is socially negatively valued (T. Russell, Crawford, & Woodby, 2004; Shipton et al., 2009). Second, the effects of passive smoking (second-hand) are generally unaccounted for in many research studies and may go underreported for the same reason as direct exposure to MSDP or if perceived as non-important (R. Chen, Clifford, Lang, & Anstey, 2013). Third, there is a higher prevalence of smoking in males than in females, and by focusing mainly on maternal smoking, paternal smoking effects on the fetus have largely been ignored (Langley, Heron, Smith, & Thapar, 2012). Consequently, identifying a more reliable method of diagnosing MSDP may represent a major improvement in research design.

Knowledge on the biological mechanisms through which exposure to MSDP affects brain structure, and ultimately behaviour, is unknown. However, there are robust data showing that the exposure to smoking alters gene expression by way of epigenetic modifications across the genome (Zhang, Florath, Saum, & Brenner, 2016). Whole-methylome studies have reported that active

smoking induces a replicable and specific differential methylation pattern across thousands of CpG sites (Joehanes et al., 2016). Interestingly, this "epigenetic signature" has also been observed in children exposed to MSDP, suggesting that in-utero exposure can affect gene expression during critical periods in development (Joubert et al., 2012; Ladd-Acosta et al., 2016). Moreover, the altered CpG sites that were found to be associated with smoking are implicated in various neurological pathways pertaining to embryogenesis and brain development. These findings further supports the notion that epigenetic mechanisms are involved in the pathogenesis of ADHD (Sengupta, Smith, Grizenko, & Joober, 2017; Vaiserman, 2015).

Importantly, the epigenetic markers associated with prenatal smoking exposure bring forth the possibility of determining smoking exposure status based on objective molecular markers, which can circumvent some of the limitations cited above. Indeed, using epigenetic markers may be more reflective of the "true" prenatal smoking exposure in children, regardless of its source (Ladd-Acosta, 2015). This approach provides us with the unique opportunity to use a methylation signature as a surrogate for retrospective self-reported MSDP. The meta-analysis supporting an association between MSDP and ADHD by Huang, et al. (described in section I.9) called for using molecular indicators of exposure to MSDP to avoid misclassification through retrospective reports, although none of the studies included in their meta-analysis used such approaches (L. Huang et al., 2018). Therefore, research investigating the relationship between the epigenetic markers associated with MSDP and brain structure holds promise in disentangling one of the several potential underlying mechanisms giving rise to ADHD.

Hypothesis

The overarching goal of this dissertation is to investigate the effects of genetic and environmental risk factors, previously associated with ADHD, on brain structure and clinical outcomes in a sample of children diagnosed with ADHD. The central hypothesis is that the genetic and environmental risk factors are associated with significant structural alterations in brain regions relevant for ADHD, and that these morphological differences are correlated to poorer cognitive and behavioural dimensions. Characterizing brain structure phenotypes as an intermediate measure between genetic and epigenetic risk factors and clinical dimensions may help construe more homogeneous subgroups of ADHD, which may lead to a better understanding of the aetiological pathways of this complex disorder.

Specific objectives

1. **Assessment of the effects of cumulative exposure to ADHD medication (CEM) on brain structures.**

The first objective of this dissertation is to examine the effects of cumulative exposure to ADHD medication (duration × dose) on cortical and subcortical brain structures in a clinical sample of children being pharmacologically treated for ADHD. We set to investigate the relationship between medication used for the treatment of ADHD symptoms, and cortical thickness and surface area. Since medication has been proposed in some studies to normalize brain structure measurements, we hypothesize that significant increases in mean cortical thickness and surface area are associated with CEM. Next, we explore the effects of medication on the volume of 51 subregions within five subcortical structures (i.e. cerebellum, hippocampus, striatum, thalamus and globus pallidus). To keep in line with the concept of normalization, we hypothesize that CEM is significantly correlated to subcortical volumes. Establishing these effects within our sample would allow us to account for the cofounding effects of medication in our subsequent models, and thus lays the initial foundation for this dissertation. Finally, the changes in brain structure associated with CEM in relation to cognitive and behavioural measures are explored.

To the best of our knowledge, this is the first structural MRI study moving away from group comparisons to investigate the effects of medication in children with ADHD. Moreover,

this study is unique in using continuous and detailed estimates for medication exposure to investigate the effects of CEM (duration × dose) on brain morphology in a clinical sample of children being treated for ADHD.

2. Investigation of the effects of a polymorphism within *NET* (rs36021) on brain morphology and cognitive/behavioural dimensions.

Remarkably, there are no studies using an imaging-genetics approach to examine the effects of polymorphisms within *NET* on brain structure in children with ADHD. The following work aims to address this gap by expanding on the previous findings by our team and model rs36021 genotype effects on brain structure. Structural MRI is used to assess rs36021 genotype effects on cortical structures (thickness and surface area) and 51 subcortical volumes within 5 regions (cerebellum, hippocampus, striatum, thalamus and globus pallidus) in a sample of children with ADHD. We hypothesize that significant morphological differences exist between groups of rs36021 genotypes in children with ADHD. Under the assumption of an additive model, it is suspected that children with ADHD homozygous for the risk-allele (TT) would have the largest reductions in cortical and subcortical measurements, followed by heterozygous children (AT), in comparison children homozygous for the A allele (AA). However, given the proposed inverted-U-curve of NE, the possibility of an overdominance model exists, in which it would be expected to find significant morphological differences between the rs36021 heterozygous (AT) and both homozygous groups (AA, TT) of children with ADHD.

Next, we explore the relationship between the most significant brain structure finding (vertex, t_{max}) and clinical measures. We hypothesize that the group of ADHD children homozygous for the risk-allele (TT) is associated with reduced structural measurements in regions relevant for ADHD, and that these reductions are correlated to more behavioural problems (Child Behavioural Checklist; CBCL) and poorer performance on cognitive measures (Continuous Performance Test; CPT).

To the best of our knowledge, this is the first imaging-genetics study to investigate the effects of *NET* rs36021 genotype on cortical and subcortical structures in a clinical sample of children with ADHD. Further, it is the first study exploring the relationship between *NET* genotype, brain structure and clinical dimensions.

3. Investigation of the effects of prenatal smoking exposure on brain morphology and cognitive/behavioural dimensions through the use of epigenetic markers and maternal self-reports.

The third and final objective of this dissertation is to research the effects of prenatal exposure to cigarette smoking on cortical structures in a clinical sample of children with ADHD. First, children are grouped according to maternal recall for exposure to cigarette smoking during pregnancy (+MSDP and –MSDP). We hypothesize there would be significant differences in cortical thickness and surface area in brain regions relevant for ADHD between exposure groups. Second, the epigenetic markers associated with exposure to prenatal smoking are used to separate children into two groups (+EM and −EM). We investigate whether this

molecular assignment of prenatal smoking exposure is more valid than maternal self-reports in designating children into "true" exposure status groups. Third, the cortical differences observed between exposure groups in relation to cognitive/behavioural dimensions are explored.

The main goals are to determine whether epigenetic markers are better predictors of cortical measurements in comparison to retrospective reports, and to investigate the effects of exposure to prenatal smoking on cortical structures in children with ADHD. To the best of our knowledge, this is the first neuroimaging study using epigenetic markers associated with an ADHD environmental risk factor in order to explore the effects of prenatal smoking exposure on cortical brain structures in children diagnosed with ADHD.

Chapter II: Materials and Methods

For over the past two decades, the ADHD research team at the Douglas Mental Health University Institute (DMHUI) has been conducting a double-blind, randomized, placebo-controlled, crossover, pharmacogenetic, phase-IV clinical trial funded by the Canadian Institute of Health Research (CIHR). Children are referred to the ADHD clinic for psychiatric evaluation by their primary care physician. In the cases where ADHD diagnosis is confirmed, the research team offers families to participate in the medication trial and neuroimaging study. The medication trial consists of two randomized weeks; one week on placebo and one week on methylphenidate (active week), for which the children, families, teachers and research team are fully blinded.

Interviews are administered to parents to collect data on demographics, family history, exposure to environmental risk factors during pregnancy and their child's behavioural profile. Over the course of the trial, a combination of cognitive tests and behavioural assessments are administered to participants during both weeks in order to determine response to treatment. This provides a unique opportunity for families to try a pharmacological intervention in a controlled setting and obtain feedback on their child's response to treatment from clinicians, researchers, and teachers. Concurrently, the research team collects extensive data from children, parents, and teachers to generate a comprehensive phenotypic characterization for every child in our study (n ~1000). Genetic data are collected from blood or saliva samples of probands and parents. In 2013, a neuroimaging sub-study was initiated at the ADHD clinic, where children who completed the medication trial were invited back to the DMHUI for an MRI scan and neuropsychological assessment. The overarching goal of the MRI project is to model the effects of genetic, epigenetic, and environmental factors on brain structure.

II.1. Participants

One-hundred and forty-four unrelated children aged between 6 and 12 years (mean = 9.3 years, SD = 1.8) were recruited for the MRI study at the ADHD clinic of the Douglas Mental Health University Institute (DMHUI) in Montreal. The research protocol was approved by the Research Ethics Board of DMHUI. The study was explained to parents who provided written consent and children gave verbal assent. Demographic data was collected through general information questionnaires completed by parents. Household income was determined through salary ranges (<10k, 10-40K, >40K) and used to approximate socioeconomic status. Sample characteristics are available in Table III.1.

Out of the total sample (n = 144), 109 children had a confirmed diagnosis of ADHD based on a clinical evaluation by a psychiatrist according to Diagnostic and Statistical Manual of Mental Disorder version 4 (DSM-IV) criteria and corroborated with the Diagnostic Interview Schedule for Children version IV (DISC-IV) administered to parents (Kasius, Ferdinand, van den Berg, & Verhulst, 1997). The DISC-IV is a highly structured diagnostic interview administered for screening and research purposes. It is designed to assess approximately 30 psychiatric conditions affecting children and adolescents, and does not require an extensive training period (Shaffer, Fisher, Lucas, Dulcan, & Schwab-Stone, 2000). Further details about diagnostic procedures can be found in (Grizenko et al., 2006).

Parents completed the child behavioural checklist (CBCL) to acquire behavioural dimensions related to ADHD (e.g. anxiety, aggression, etc.). The CBCL is a comprehensive rating scale consisting of a 113-itemed questionnaire. Raw scores are transformed into standardized T-scores, where the average score is 50 and the clinical threshold is 65 (Achenbach, 1991). Information on the child's behaviour in home and school settings was collected via the Conners' Global Index (CGI) scale from parents (CGI-P) and teachers (CGI-T), respectively (Conners, Sitarenios, Parker, & Epstein, 1998). The CGI is widely used to assess ADHD symptoms and psychopathology in children and adolescents. Emotional liability and restless-impulsive behaviour are the two main components assessed in the CGI. Similar to the CBCL stated above, the GCI scale transforms the total raw scores into normalized T-scores. Moreover, genetic factors have been reported to account for up to 78% of the variance observed in CGI scores (Sengupta et al., 2012).

Children completed the Continuous Performance Test (CPT) to acquire measures of attention, impulse-control and response-inhibition (Conners, 1985). The CPT is a computerized neuropsychological assessment aimed to measure response-time, response-time variability, overall index and two types of errors: omission and commission. Omission and commission errors consist of a failure to respond on target, and an incorrect response to a nontarget, respectively.

Children with an intelligence quotient (IQ) less than 70 according to the Weschler Intelligence Scale for Children IV (WISC-IV), a diagnosis of Tourette syndrome, pervasive

developmental disorder and/or psychosis, were excluded from the study. The WISC-IV was used to measure full-scale IQ, where the average standard score is 100 and a higher score indicates better performance on the test (Weschler, 1991). A subgroup of matched typically-developing children was used as a control group for complementary analyses (n = 35).

II.2 Image Acquisition

All children (n = 144; ADHD n = 109; control n = 35) were scanned on site at the Cerebral Imaging Center. Image acquisition was carried out on a 3T Siemens Trio MRI scanner (MAGNETOM Tim Trio, Siemens AG, Erlangen, Germany) with a 12-channel head coil. A 3D sagittal magnetization-prepared rapid gradient echo (MP-RAGE) with a sequence of TR/TE/TI/FA = 2300 msec/2.96 msec/900 msec/9 degrees, were used to acquire T1-weighted structural images (1 mm isotropic voxels; 192 slices). Scanning time consisted of two rounds of nine minutes, totalling 18 minutes. The protocol was tailored to a pediatric population to improve compliance, minimize anxiety, and reduce motion during scanning. Specifically, all children practiced on a mock scanner prior to MRI scanning, a cartoon was shown, and sandbags were placed over their extremities. Children were invited to choose between three preselected cartoons to maximize engagement and encourage remaining still during the scan. The scanning process was reinitiated in participants with considerable head motion. In cases where multiple scans were collected per child, a single optimal scan was selected for processing. Non-usable scans with motions artifacts were discarded.

II.3 Image Processing

An initial quality control of the raw scans was carried out to select a single optimal scan for every child. Two participants with ADHD were excluded due to motion (n = 107). Pre-processing of raw scans was conducted to minimize downstream failures via the minc-bpipe-library pipeline (https://github.com/CobraLab/minc-bpipe-library). Pre-processed scans were input to CIVET-1.1.12 and MAGeT-Brain for cortical and subcortical analysis, respectively.

Images were registered into a common 3D space using the corticometric iterative vertex-based estimation thickness pipeline (CIVET). CIVET is an automated imaging software tool used to obtain corticometrics (version 1.1.12, Montreal Neurological Institute, McGill University, Montreal Quebec, Canada) (Ad-Dab'bagh, 2006; Collins DL, 1995; Sled, Zijdenbos, & Evans, 1998). White and gray matter surfaces were produced using the Constrained Laplacian Anatomical Segmentation using Proximities method (CLASP) and used to compute cortical surface area (J. S. Kim et al., 2005).

Cortical thickness and surface area were calculated at roughly 82 000 points across the cortex and data was blurred using the default surface-based diffusion kernel of 20 mm for thickness and 40 mm full-width at half-maximum for surface area. MAGeT-Brain was used to extract volumes from 51 subregions of the cerebellum, hippocampus, striatum, thalamus and globus

pallidus (Chakravarty, Bertrand, Hodge, Sadikot, & Collins, 2006; Chakravarty et al., 2013; Park et al., 2014). The hippocampus was subdivided into 5 subregions (CA1, CA2/CA3, CA4/DG, SR/SL/SM and subiculum) (Pipitone et al., 2014; Winterburn et al., 2013). A final quality control was carried out on the processed images, and one more participant with ADHD was removed due to failure (n = 141; ADHD = 106; control = 35).

II.4 Methodology for the Investigation of CEM on Brain Structure

II.4.1 Determination of Cumulative Exposure to ADHD Medication

Lifetime pharmacological history of ADHD medication was collected retrospectively as reported by the parents, and subsequently corroborated against pharmacy prescription logs. All children with ADHD participating in the MRI study were exposed to medication for a minimum of one week prior to scanning (range: 0.02 to 4.69 years, median = 0.25 years). Medication breaks (i.e. holidays, weekends, and summer) were considered and subtracted from the total duration from the date of initial exposure to date of scanning. ADHD medications were prescribed by the treating psychiatrist at different doses for various durations depending on the clinical needs of the child. For each period of treatment at a given dose, the exposure to medication was calculated as the product of duration and dose (days × mg/day). The cumulative exposure to ADHD medication (CEM) was then calculated by summing all the exposures (range: 0.075 to 108.75 grams, median = 1.5). Supplemental Figure SIII.1 shows the untransformed distribution of CEM.

In general, children were first exposed to MPH during the medication trial, followed by a prescription of an ADHD medication best suited to their clinical needs. Indeed, the majority of children participating in the MRI study tried different types of ADHD medication before selecting an optimal brand for their treatment management. Therefore, most children were each prescribed a variety of ADHD brands, preventing the feasibility of subgrouping children according to specific medication type. Nevertheless, dosage equivalencies between psychostimulant brands are comparable, with the exception of Adderall® (dextroamphetamine) which has double the potency of Ritalin® (methylphenidate). Indeed, dextroamphetamine is typically prescribed at half the dosage relative to other psychostimulant brands. A total of five dextroamphetamine prescriptions were found in our total sample, and dosage was adjusted in a supplemental analysis. Moreover, a small subset of prescriptions was for the non-psychostimulant NE-specific agent, Strattera® (atomoxetine). A supplemental analysis controlling for atomoxetine exposure was conducted. Typically-developing children belonging to the control group had no exposure to ADHD medication.

II.4.2 Statistical Analysis

Children with ADHD

The cumulative exposure to ADHD medication data was log-transformed to generate a normal distribution (supplemental Figure SIII.2) and RMINC was used to perform linear modelling (https://github.com/Mouse-Imaging-Centre/RMINC/). Age and sex were used as

covariates, and cortical thickness and surface area were the main outcome measures. Similarly, a linear model was generated for analysis of 51 subcortical volumes and CEM, where age, sex and total brain volume were included as covariates, and subcortical volumes as main outcome measures. As there is a possible collinearity between severity of ADHD and medication dose, an additional analysis was performed including a measure for ADHD symptom severity. The Conners' Global Index scale (CGI) is an estimate of the child's ADHD symptom severity at baseline (i.e. before treatment interventions), and thus was included as a covariate. Finally, to explore whether the effect of medication on brain structure changes as a function of age, an interaction analysis between age and CEM was performed. Multiple-testing correction using false discovery rate (FDR) of was performed.

To investigate the relationship between brain structure and ADHD cognitive measures, the significant brain regions affected by CEM were correlated to the Continuous Performance Test (CPT) dimensions. Four outcome measures (omissions, commissions, variability and reaction-time) were selected based on a meta-analysis associating them to ADHD (Huang-Pollock, Karalunas, Tam, & Moore, 2012). Correction for multiple comparisons was performed using Bonferroni for non-independent variables by considering the correlation between the four CPT measures ($r = 0.43$). The p-value cut-off was stated at 0.021.

Group Comparison between ADHD and Control Children

All analyses regarding CEM were conducted in a clinical sample of children being treated for ADHD, as typically-developing children were unmedicated. To explore whether the brain regions significantly associated with CEM were independent of ADHD diagnosis or age, two complimentary analyses using a control group (n = 35) were conducted. Subcortical volumes within 51 subregions were compared between ADHD cases and controls. Demographics were assessed between groups and measures that significantly differed were included as covariates in the model (i.e. sex and full-scale IQ). Specifically, age, sex, total brain volume, IQ and CEM were used as covariates, and subcortical volumes as main outcome measures. Moreover, an interaction analysis between age and diagnosis was performed. Since a control group of typically-developing children exposed to ADHD medication is not ethically feasible, a 3-way interaction (age, ADHD diagnosis and CEM) was not possible to examine.

II.5 Methodology for Investigation of rs36021 Genotype on Brain Morphology

II.5.1 Participants

Seventy-six out of the 109 children with neuroimaging data had genetic information available, collected through blood and saliva samples at the DMHUI ADHD clinic prior to this study. Parents consented to the inclusion of their child's genetic data in the current study.

A panel of thirty preselected tag SNPs within the *NET* gene were genotyped and rs36021 genotype group (n = 76; AA = 24, AT = 28, TT = 24) was determined. Briefly, our team used the Sequenom iPlex Gold Technology (Ehrich, Bocker, & van den Boom, 2005), where reference samples were included to estimate genotype error. Genotypes were read at high accuracy (>99%) and in Hardy-Weinberg equilibrium. Through the use of Haploview v4.0, our team showed in a previous report that there are three major haplotype blocks, and that rs36021 was in LD with rs3785143 and rs11568324 (Thakur et al., 2012). Genotype (rs36021) sample characteristics are found in Table III.2.

II.5.2 Image Acquisition and Processing

Raw scans were reviewed for motion and a single optimal scan was selected per child by a researcher blind to the identity and genotype. Two children had no viable scans available and were removed from the analysis (n = 74). Selected scans were pre-processed via the minc-bpipe-library pipeline (https://github.com/CobraLab/minc-bpipe-library) and used as inputs for CIVET-1.1.12 and MAGeT-Brain. CIVET-1.1.12 is an automated neuroimaging software tool used to estimate cortical thickness and surface area measurements. Cortical thickness and surface area were calculated at every vertex point across the brain and data was blurred using a surface-based diffusion kernel of 20 mm and 40 mm full-width at half-maximum respectively to preserve cortical topology. MAGeT-Brain was used to extract volumes from 51 subregions of the cerebellum, hippocampus, striatum, thalamus and globus pallidus. A final quality control was carried out on the CIVET-1.1.12 and MAGeT-Brain outputs to assure no computational errors occurred.

II.5.3 Statistical Analyses

Demographic and clinical measures were compared among the three *NET* genotype groups using chi-square and ANOVA for categorical (sex, income, and ethnicity) and continuous variables (age, cumulative exposure to ADHD medication, full-scale IQ, Conner's, CBCL and DISC) respectively. Genotype demographics are represented in Table III.2.

Vertex-wise comparisons were made in 81 924 vertices across the whole brain and independent linear modelling was performed using RMINC to asses rs36021 genotype effects on brain structure (https://github.com/Mouse-Imaging-Centre/RMINC/). The first model tested for association between *NET* genotype group and cortical structures (cortical thickness and surface area). Age, sex, and cumulative exposure to ADHD medication (CEM) were included as covariates, and cortical thickness and surface area measurements as main outcome measures. The second model tested for association between *NET* genotype group and subcortical volumes in 51 subregions. Age, sex, CEM, and total brain volume were included as covariates and subcortical volumes as main outcome measures. Multiple-testing correction using an FDR was performed.

To explore the relationship between *NET* genotypes on brain structure and behavioural/cognitive measures, the most significant cortical vertex (t_{max}) was tested for association with CBCL (total, internalizing, externalizing and aggression scores) and CPT

cognitive dimensions (omissions, commissions, variability and reaction-time). Selection of these outcomes was based a previous study conducted by our team and a meta-analysis (Huang-Pollock et al., 2012; Thakur et al., 2012). Correction for multiple comparisons was performed using Bonferroni correction ($p \leq .0096$), adjusted for the correlation ($r = 0.21$) between measures.

II.6 Methodology for the Investigation of Prenatal Smoking Exposure on Brain Morphology

II.6.1 Assignment of MSDP Exposure Status

Determination of children's prenatal exposure status to cigarette smoking was carried out through two methods. The first method relied on maternal interviews to assign children into smoking exposure groups, which were based entirely on the mothers' recall and disclosure of maternal smoking during pregnancy. The Statistics Canada Canadian Community Health Survey Cycle 3.1 questionnaire was administered to mothers and used to collect information on MSDP (Sengupta et al., 2015). Mothers reported on past and current smoking behaviour, including MSDP and the number of cigarettes consumed per day (n = 109). Children were designated as "+MSDP" if the mother reported smoking (yes/no) during any trimester of pregnancy (1st trimester n = 24; 2nd trimester n = 17 and 3rd trimester n = 17). The number of reported cigarettes ranged from two to 40 per day.

A second method was used in a subsample of the children in the MRI study (n = 35) to group children according to epigenetic markers (EM) previously associated with prenatal smoking exposure in the literature, as well as in our current study (+EM = 14, −EM = 21). Children participating in our MRI study provided either blood or saliva samples. Given that there may be variation in methylation across different tissues, the analysis was restricted to the children who provided whole blood samples. DNA was extracted from the blood samples and sent to the Genome Quebec Innovation Center for treatment with sodium bisulfite and genome-wide DNA methylation analysis. The Infinium MethylationEPIC BeadChip was used to determine presence of methylation at approximately 850K CpG sites across the genome. Moreover, the Illumina GenomeStudio software was used to acquire signal intensities. Computational analyses were conducted using the Chip Analysis Methylation Pipeline (ChAMP) in R version 3.3.2 (http://www.r-project.org). An association analysis, correcting for age and sex, was performed to identify the significant differentially methylated probes between the children exposed to prenatal smoking and non-exposed. Multiple-testing correction using FDR was performed. Significant results were retained (corrected p-value < 0.05, n = 46 DMP) and submitted for cluster analysis in R. The number of clusters was set to 2 in order to dichotomize children into exposed (+EM = 14) or non-exposed (−EM = 24) groups. CpG sites most significantly associated with prenatal smoking exposure are listed in appendix section.

II.6.2 Image Acquisition and Processing

Quality control was carried out on raw images, by a researcher blind to the exposure status of prenatal smoking of the child. A single scan was selected for each child for pre-processing via minc-bpipe-library pipeline (https://github.com/CobraLab/minc-bpipe-library). Pre-processed images were reviewed and selected as inputs for CIVET-1.1.12. A third quality control was carried out on the CIVET-1.1.12 outputs. Two subjects had unviable raw scans and one failed CIVET quality control, and thus were excluded from the final analysis (n = 106; +MSDP: n = 23; −MSDP: n = 83).

II.6.3 Statistical Analyses

Sample characteristics are found in Tables III.2 and III.3. Chi-square and analysis of variance (ANOVA) were applied to test for differences between categorical (sex, income, and ethnicity) and continuous variables (age, medication, full-scale IQ, Conner's, CBCL and DISC) between exposure groups, respectively. Independent linear modelling was performed using RMINC (https://github.com/Mouse-Imaging-Centre/RMINC/) at 81 924 vertices across the cortex. The first model tested for association between self-reported MSDP and cortical structures. Age, sex, and full-scale IQ were included as covariates, and cortical thickness and surface area measurements as main outcome measures. The second model tested for association between prenatal smoking exposure, according to epigenetic markers, and cortical structures. Out of the total 35 participants who had both epigenetic and imaging data available, one failed quality control and four were girls. For optimal use of general linear modelling, each predictor should have a minimum of 10 observations (i.e. subjects). As such, the analysis was restricted to boys (n = 30;

+EM = 10; −EM = 20). Age was included as a covariate and cortical thickness and surface area measurements as main outcome measures. Supplemental analysis correcting for IQ scores was carried out. FDR was used to account for multiple-comparisons. To explore the relationship between brain structure and cognitive measures, the most significant (t_{max}) brain morphology finding was correlated to CPT dimensions (four outcome measures: omissions, commissions, variability, and reaction-time). As previously stated, selection of these outcomes was based on a meta-analysis linking CPT dimensions to ADHD (Huang-Pollock et al., 2012). Correction for multiple comparisons was performed using Bonferroni ($p \leq .021$), which considered the correlation ($r = .43$) between the four CPT measures.

Chapter III: Results

III.1 Cumulative Exposure to ADHD Medication is Inversely Related to Hippocampus Subregional Volume in Children.

III.1.1 Cumulative Exposure to ADHD Medication

All children with ADHD participating in the MRI project were exposed to ADHD medication for a minimum of one week, and thus none were treatment-naïve (apart from the control group of non-ADHD children). Our ADHD sample consisted of children with variable medication prescriptions to treat their symptoms. The distribution of the number and type of prescriptions is described here. Five children with ADHD were concurrently prescribed anti-psychotics and were excluded from the final analysis (n = 101). The number of independent prescriptions for ADHD medication per child (n) was one (n = 7), two (n = 34), three (n = 21), four (n = 18) and five prescriptions (n = 21). A total of 315 prescriptions were included for a total of 101 children with ADHD: Ritalin® (35.2%), Biphentin® (32.4%), Concerta® (22.6%), Vyvanse® (5.7%), Strattera® (2.5%) and Adderall® (1.6%). The cumulative exposure to ADHD medication values were log transformed to produce a normal distribution and enable linear modelling.

III.1.2 Cumulative Exposure to ADHD Medication and Cortical Structure

A linear model was generated to assess the effects of CEM on cortical thickness and surface area, which included age and sex as covariates. No global effects of CEM on cortical thickness or surface area were detected in either brain hemispheres. CEM did not significantly predict cortical

thickness and surface area measurements in the vertex-wise comparison. Likewise, no effects of CEM on cortical structures were observed when controlling for ADHD severity.

III.1.3 Cumulative Exposure to ADHD Medication and Subcortical Volumes

A linear model was generated to assess the effects of CEM on 51 subcortical structures, which included age, sex, and total brain volume as covariates. Significant effects of CEM were found in two out of the five subregions of the hippocampus, the left Cornu Ammonis 1 (CA1; df = 95; q = 0.003) and the left strata radiatum/lacunosum/moleculare (SR/SL/SM) (df = 95; q = 0.003). Moreover, trends were found in the right CA1 (df = 95; q = 0.06), right SR/SL/SM (df = 95; q = 0.08), right dentate gyrus (DG; df = 95; q = 0.08) and left CA2/3 (df = 95; q = 0.08). Specifically, an inverse relationship was uncovered, where a higher CEM value was significantly associated with decreased volumes within significant subregions (Figure III.1). Post-hoc analysis revealed an effect size of 38.5% at 99% power (predictors = 3; R-squared = 0.385; $\alpha = 0.05$; n = 101). Supplemental analyses controlling handedness and dosage equivalencies between medication types yielded the same findings (i.e. dextroamphetamine; Adderall®). Results remained significant after controlling for ADHD symptom severity (CA1 and SR/SL/SM; df = 94; q = 0.0147) as well as when controlling for exposure to the non-psychostimulant, atomoxetine (i.e. Strattera®) (CA1 and SR/SL/SM; df = 94; q = 0.006). Moreover, the interaction analysis between CEM and age yielded no significant findings, and no significant effects were detected between hippocampal CA1 volumes and neuropsychiatric assessments (CPT performance).

III.1.4 Control Children and Subcortical Volume

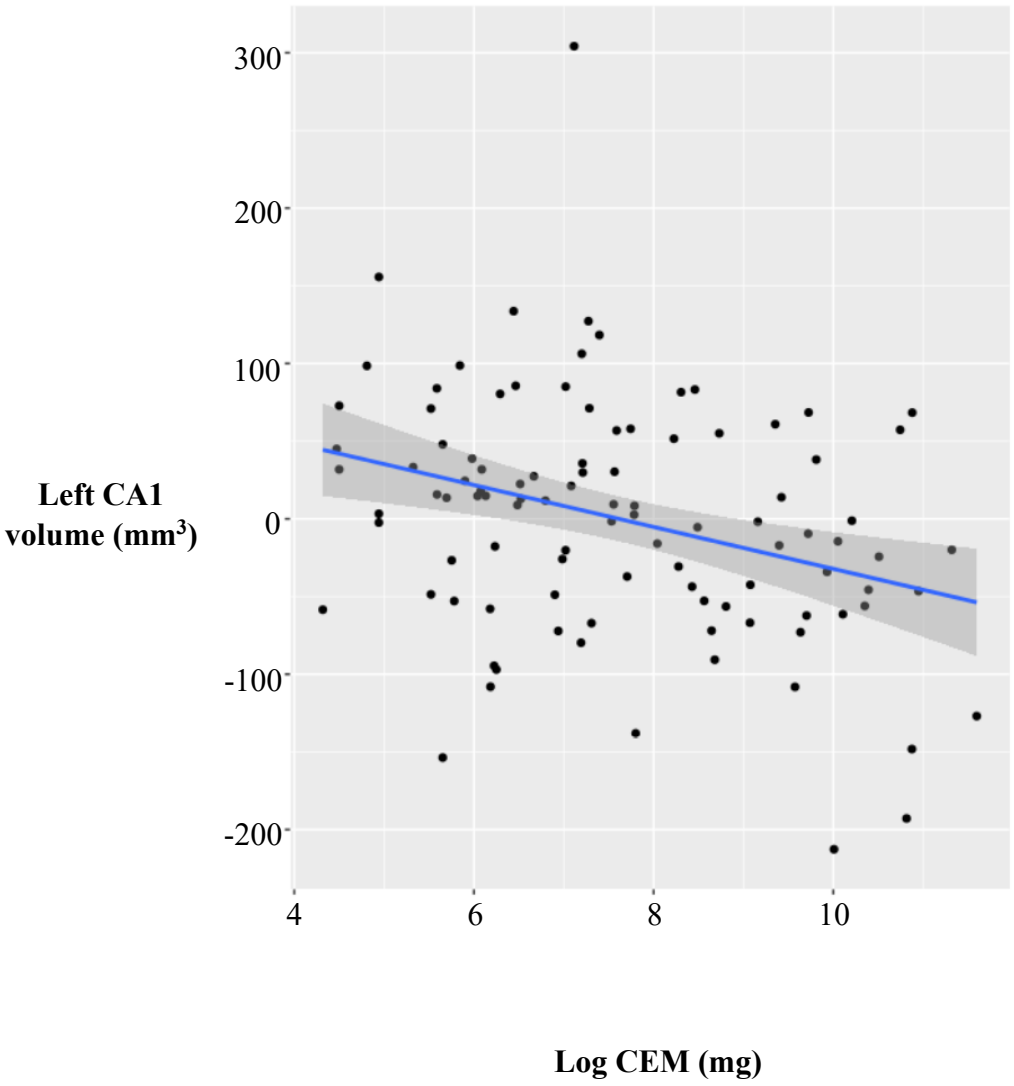
Group demographics regarding age, income, ethnicity, and handedness did not significantly differ between ADHD and control children. Significant group differences were found for sex and full-scale IQ, and thus were included as covariates in the analysis. As expected, significant differences in ADHD symptomatology and behavioural measures were detected between ADHD and control children (CBCL, Conner's, ADHD total items; Table I.1). Group comparison between ADHD and control children revealed no significant volumetric differences within any of the 51 subregions. No significant effects were uncovered in the age-by-diagnosis interaction analysis.

Table III. 1: Sample Demographics for ADHD and Typically-Developing Children.

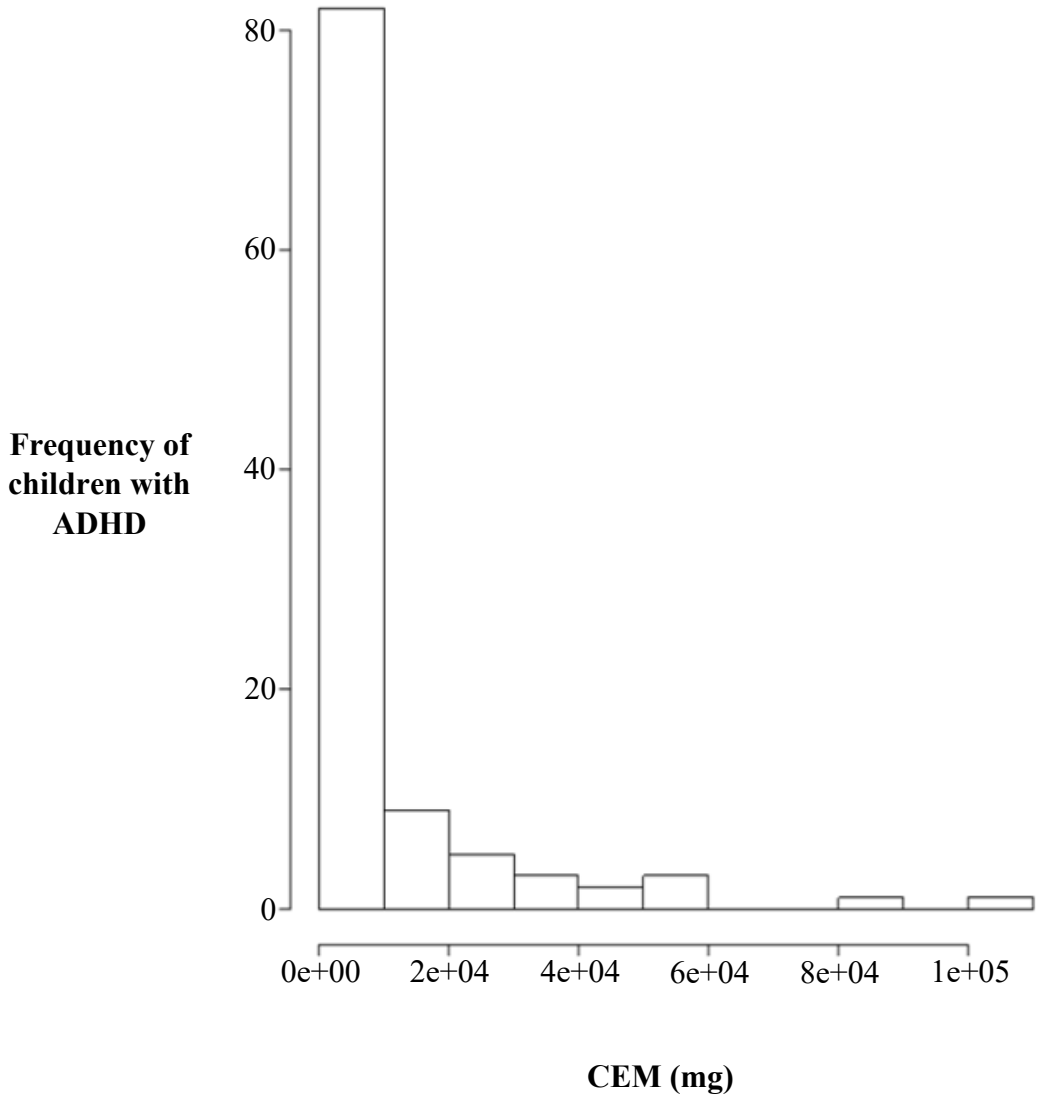
	ADHD (n=101)	**Control (n=35)**	**Total (n=136)**	**Stats**
Age yrs. (SD)	9.51 (1.67)	8.83 (2.1)	9.34 (1.8)	$F_{1,135}$=3.88; *p*=.051
Full Scale IQ (SD)	97.26 (13.52)	109.94 (14.31)	100.52 (14.76)	$F_{1,131}$=21.57; ***p*=.000**
Sex (% male)	76/101 (75)	16/35 (46)	92/136 (68)	X^2=10.38; df=1; ***p*=.001**
Income (%)				
<10K	4/97 (4)	0/33 (0)	4/130 (3)	X^2=1.69; df=2; *p*=.430
10-40K	21/97 (22)	6/33 (18)	27/130 (21)	
40K +	72/97 (79)	27/33 (82)	99/130 (76)	
Ethnicity (% Caucasian)	89/101 (88)	26/34 (77)	115/135 (85)	X^2=2.74; df=1; *p*=.098
Handedness (%)				
Right	84/100 (84)	33/35 (94)	117/135 (87)	X^2=2.39; df=2; *p*=.303
Left	7/100 (7)	1/35 (3)	8/135 (6)	
Ambidexter	9/100 (9)	1/35 (3)	10/135 (7)	
Conner's Total Baseline Parent (SD)	72.37 (10.89)	48.09 (5.16)	66.03 (14.45)	$F_{1,133}$=160.77; ***p*=.000**

Conner's Total Baseline Teacher (SD)	66.87 (11.56)	N/A	N/A	N/A
CBCL Total T-Score (SD)	67.70 (7.52)	44.15 (8.78)	61.71 (12.93)	$F_{1,133}$=228.10; ***p*=.000**
DISC Total ADHD items (SD)	12.92 (3.32)	1.71 (1.92)	10.01 (5.78)	$F_{1,134}$=355.34; ***p*=.000**

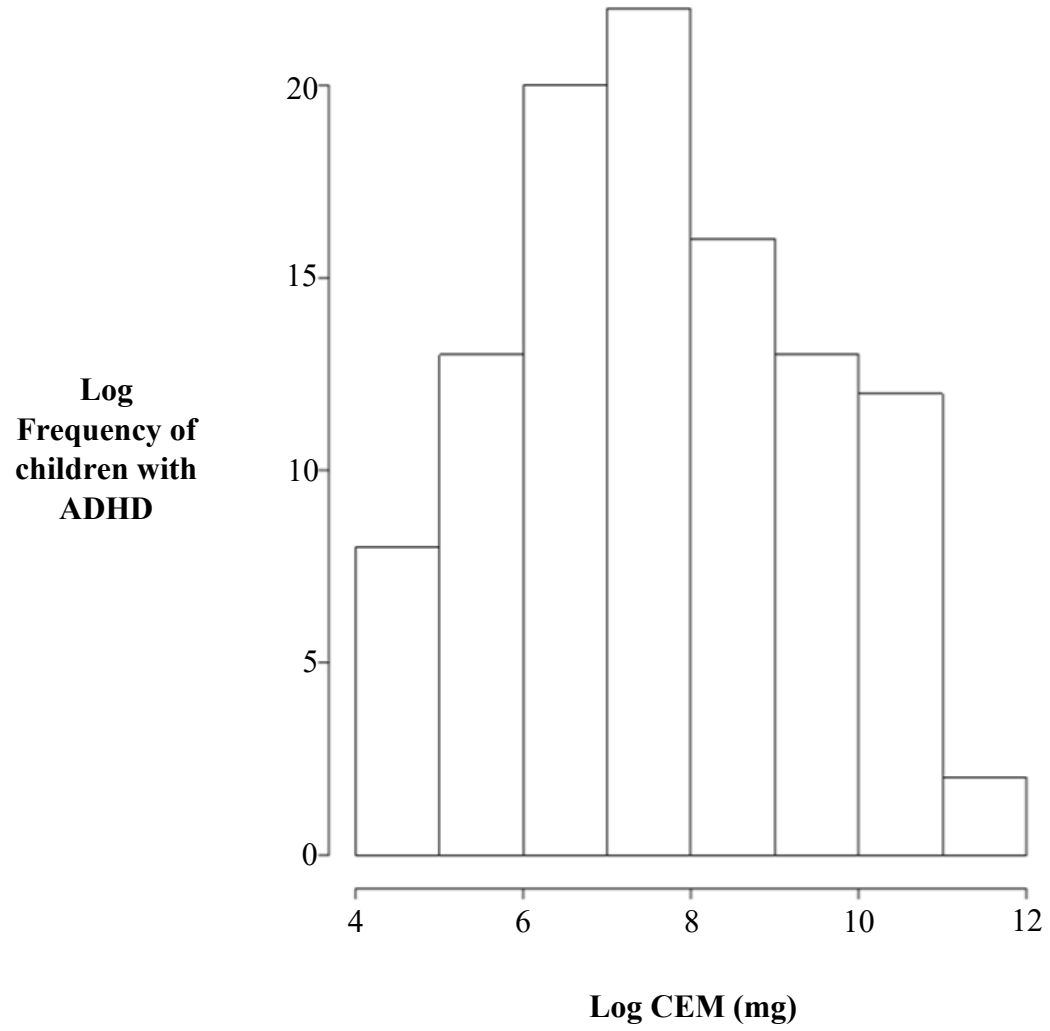
Figure III. 1: Graph Representing Association between CEM and Hippocampus CA1 volume.



Supplemental Figure SIII. 1: Histogram representing CEM distribution in children with ADHD (untransformed).



Supplemental Figure SIII. 2: Histogram representing transformed CEM distribution in children with ADHD (Log-transformed).



Preface: *NET* polymorphism and Brain Structure

We uncovered a significant association between cumulative exposure to ADHD medication and volume in subregions of the hippocampus. However, no medication effects were observed on cortical thickness or surface area. In the following section, we base ourselves on previous findings from our team reporting an association between a *NET* polymorphism (rs36021) and ADHD. We build a model to investigate the effects of *NET* genotype on cortical thickness and surface area. Genetic information used to determine *NET* genotype was previously collected in the medication trial and is applied here to investigate effects on cortical structures. Moreover, neuropsychological assessments conducted on the day of scan were used to acquire data on cognitive and behavioural dimensions. These measures are intended to assess whether differences in cortical measurements across genotype groups are associated with cognitive performance and behavioural scores.

III.2 Children with ADHD Homozygous for the *NET* (rs36021) Risk-Allele have Reduced Cortical Surface Area in Executive Brain Regions.

III.2.1 Cortical and Subcortical Analysis

In comparison to the AT genotype, mean cortical thickness was significantly smaller in the AA group (left hemisphere df = 4, p = 0.04; right df = 3; p = 0.03) and TT group (left df = 4; p = 0.04; right df = 4; p = 0.04) (Figure III.2). Total surface area was also significantly reduced in the TT group (left df = 4; p = 0.004; right df = 4; p = 0.004) relative to the AT group (Figure III.3). Post-hoc analysis revealed an effect size of 22% at 93% power (predictors = 3; R-squared = 0.22; $\alpha = 0.05$; n = 74). Vertex-wise comparison between AT and TT genotype groups yielded significant reductions in surface area (left $t_{max} = 3.26$; 15% FDR; right $t_{max} = 3.37$; 10% FDR) in the TT group. Surface area reductions were located in several regions, notably the orbitofrontal (OFC), ventromedial prefrontal (vmPFC), posterior inferior parietal lobe (piPL), frontal and temporal cortices (Figure III.4 provides q-map statistics of significant regions at 15% FDR). No significant effects of *NET* rs36021 genotype groups on subcortical volumes were detected.

III.2.2 Cortical Surface Area and Clinical Measures

Linear modelling was used to test whether the most significant brain region finding (t_{max}), localized in the left frontal lobe, was associated with CBCL and CPT dimensions. Out of the eight items assessed, higher aggression (df = 70; p = 0.03) and CBCL externalizing scores (df =

70; $p = 0.008$) were associated with smaller surface area measurements. However, only the latter survived correction for multiple comparisons. Post-hoc analysis revealed an effect size of 12.4% at 76% power (predictors = 3; R-squared = 0.124; $\alpha = 0.05$; $n = 74$).

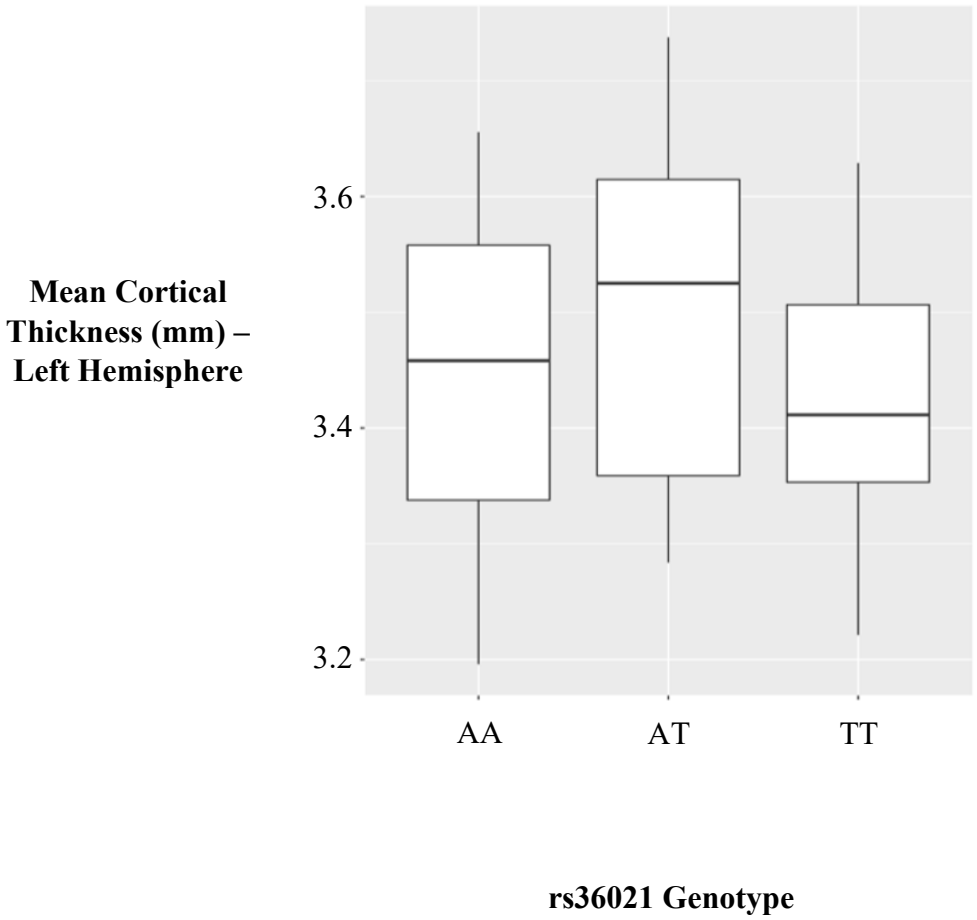
Table III. 2: Demographic and Clinical Measures according to *NET* rs36021 Genotype Groups.

	AA (23)	**AT (28)**	**TT (23)**	**Total (74)**	**Stats**
Age (SD)	9.23 (1.58)	9.60 (1.48)	9.69 (1.80)	9.51 (1.61)	$F_{2,73}$=.53; p=.59
IQ (SD)	97.26 (13.3)	93.89 (15.0)	97.41 (11.5)	96.01 (13.4)	$F_{2,70}$=.56; p=.57
Biological sex					
(% male)	21/23	21/28	15/23	57/74	X^2=4.53; df=2; p=.10
Income					
< 10 K	0/21	1/27	2/23	3/71	X^2=6.24; df=4; p=.18
10 – 40 K	2/21	4/27	7/23	13/71	
40 K +	19/21	22/27	14/23	55/71	
Ethnicity					
(% Caucasian)	21/23	26/28	18/23	65/74	X^2=2.89; df=2; p=.24
Medication AUC (SD)	16688.2 (26832.2)	4076.5 (5863.2)	11526.3 (20311.1)	10311.8 (19550.1)	$F_{2,73}$=2.83; p=.07

CBCL Total (SD)	69.1 (5.6)	66 (8.1)	69 (6.5)	67.9 (7.0)	$F_{2,73}$=1.69; p=.19
ADHD items (SD)	14 (3.1)	12.3 (3.5)	13.4 (3.5)	13.2 (3.4)	$F_{2,73}$=1.81; p=.17
Conner's Parent BL (SD)	74.3 (9.8)	74 (11)	73.3 (11.2)	73.9 (10.6)	$F_{2,72}$=.04; p=.97
Conner's Teacher BL (SD)	68.3 (10.9)	62.8 (12.4)	71.7 (11.1)	67.4 (11.9)	$F_{2,68}$=3.72; **p=.03***

Figure III. 2: Box Plots for Mean Cortical Thickness in the A) Left and B) Right Hemispheres according to *NET* rs36021 Genotype Groups.

A)



B)

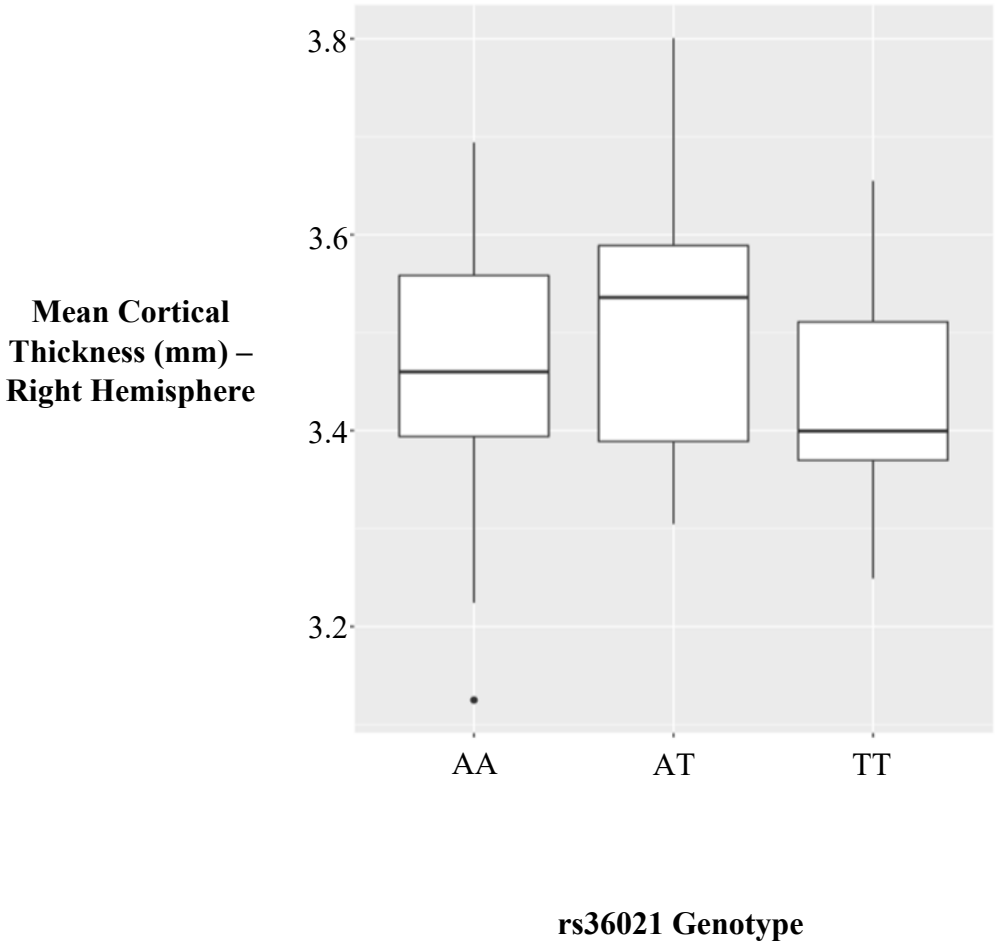
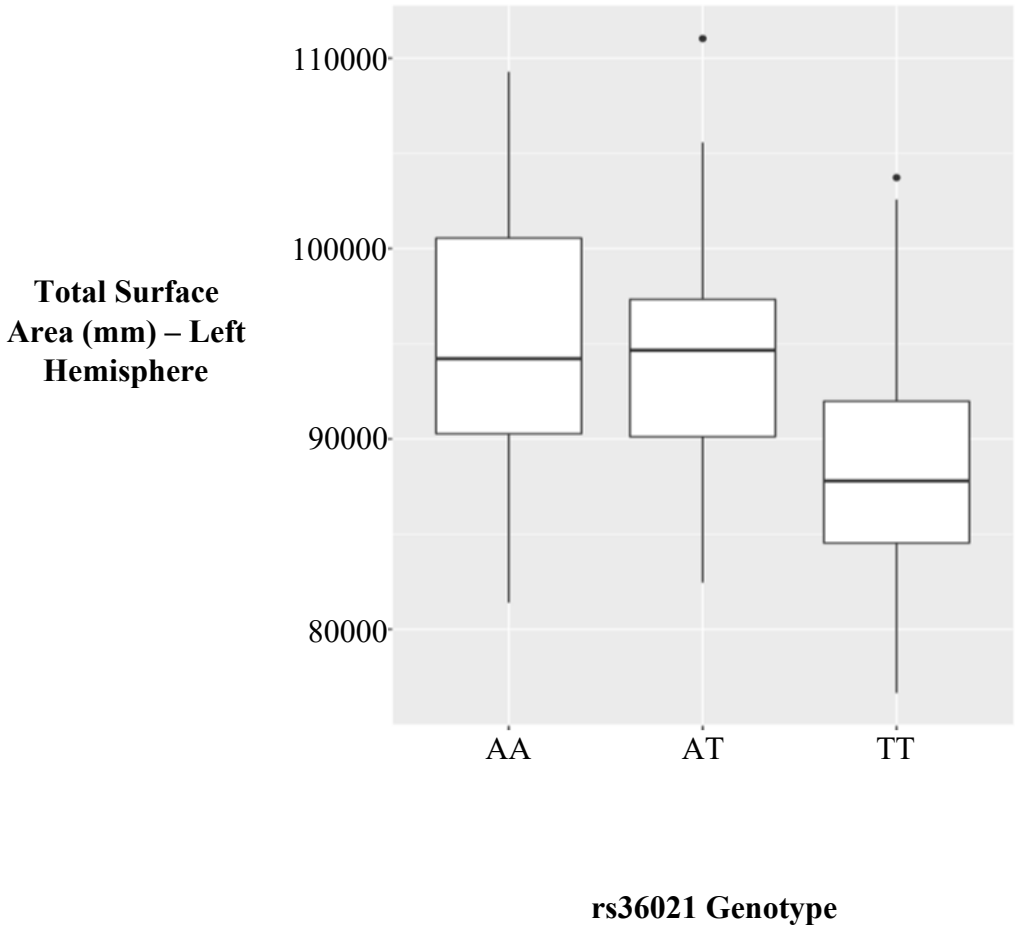

Mean Cortical Thickness (mm) – Right Hemisphere
3.8
3.6
3.4
3.2
AA
AT
TT
rs36021 Genotype

Figure III. 3: Box Plots for Total Cortical Surface Area in the A) Left and B) Right Hemispheres according to *NET* rs36021 Genotype Groups.

A)



B)

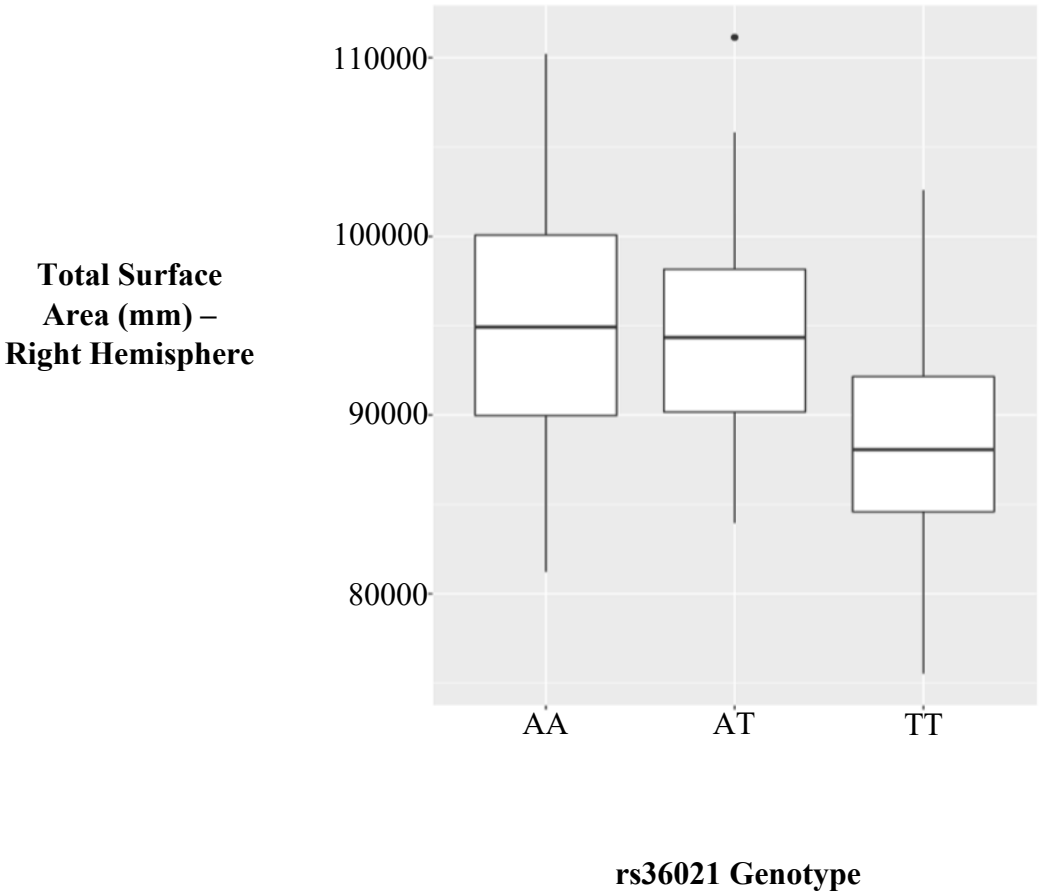
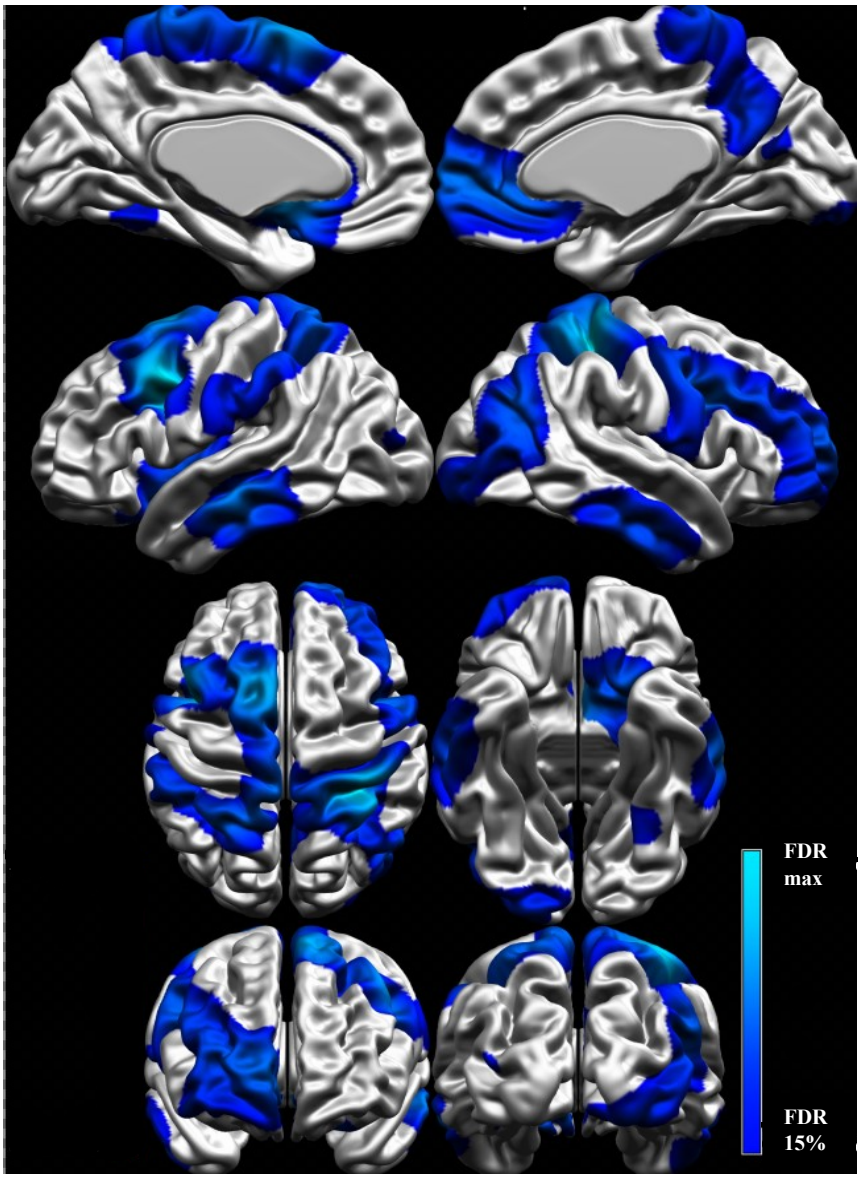

Total Surface Area (mm) – Right Hemisphere
110000
100000
90000
80000
AA
AT
TT
rs36021 Genotype

Figure III. 4: q-statistics map representing vertices with significant reductions in cortical surface area measurements (blue) in children with the TT in comparison to the AT genotype (rs36021). In sequence, left and right medial and lateral views, dorsal and ventral views.



Preface: Prenatal Exposure to Smoking and Brain Structure

The above findings describe a novel and significant effect of *NET* genotype on cortical surface area. An association between cortical surface area measurements and behavioural scores was also observed. The following work is based on evidence from the literature, as well as a previous report by our team showing an association between exposure to prenatal smoking and ADHD (Thakur et al., 2012). Specifically, our team found that the association between the *NET* SNP (rs36021) and ADHD became highly significant when the sample was stratified according to exposure to maternal smoking during pregnancy. Therefore, the following aim was to investigate the effects of prenatal exposure to cigarette smoking on cortical thickness and surface area within a sample of children with ADHD. Two methods were used to determine prenatal exposure status. First, mothers were interviewed to collect retrospective data on their smoking behaviour during pregnancy, including the number of cigarettes consumed per day throughout each trimester. The second method used epigenetic markers robustly associated with prenatal smoking to segregate children into exposure groups. Possible discrepancies across retrospective reports and epigenetic markers, and whether epigenetic markers may more accurately reflect prenatal smoking exposure were also explored. Finally, cognitive, and behavioural data collected on the day of scan were analyzed to observe potential association with cortical measurements within significant regions.

III.3 Maternal Smoking During Pregnancy and Cortical Structure in ADHD children: Enough to Rely on Recall?

III.3.1 Demographics

Demographic characteristics are summarised in Table III.3 and III.4. No significant differences across measures were found between exposure groups, apart from full-scale IQ in the self-reported MSDP model.

III.3.2 Self-reported MSDP Model

Linear modeling was used to investigate effects of prenatal exposure to smoking on cortical structures, whilst correcting for age and sex effects. Since full scale IQ score significantly differed between exposure groups, it was included as a covariate in the model. No significant differences in cortical structures (cortical thickness or surface area) were detected at a threshold ≤ 15 % FDR between children who are +MSDP (n = 23) and −MSDP (n = 83). Likewise, no effects of MSDP were found when the analysis was restricted to boys (n = 81) nor when IQ was removed from the model as a covariate. Moreover, the reported number of cigarettes per day did not yield any effects on cortical structures.

III.3.3 Epigenetic Markers

To explore the possible discordance between the two methods (MSDP and EM), a fisher exact test was used to compare exposure status as determined through self-reports versus epigenetic markers. A significant difference of exposure status was identified ($p = 0.03$). Indeed, approximately 30% of children who were −MSDP were found to be +EM. Supplemental table SIII.3 depicts the number of children in each group. Notably, nine children who were –MSDP were found to have methylation changes compatible with exposure to smoking and assigned +EM.

As depicted in boxplots of Figures III.5, boys who were in the exposed group (+EM) had smaller total surface area in both hemispheres relative to boys who were -EM ($t = -2.14$, $df = 27$, $p = 0.04$). Post-hoc analysis revealed an effect size of 15% at 50% power (predictors = 2; R-squared = 0.15; $\alpha = 0.05$; n = 30). Vertex-wise comparison uncovered significantly smaller surface area in the right orbitofrontal cortex (ROFc), middle temporal cortex (RTc) and parahippocampal gyrus (RPHg) in the exposed group of boys. Refer to Figure III.6 for visualization of q-statistics ($t_{max} = -3.78$; 15% FDR). IQ was subsequently included in the EM model to maintain consistency with the previous model (MSDP) and results remained unchanged. Out of the four CPT dimensions, higher commissions-error T-scores were significantly associated with reduced cortical surface area at t_{max}, located within the RPHg ($df = 27$; $p = 0.006$), surviving correction for multiple-comparisons. Post-hoc analysis revealed an effect size of 40% at 97% power (predictors = 2; R-squared = 0.40; $\alpha = 0.05$; n = 30).

Table III. 3: Demographics and Clinical Characteristics of +MSDP and -MSDP Groups.

	-MSDP (n=85)	**+MSDP (n=24)**	**Total (n=109)**	**Stats**
Age yrs. (SD)	9.62 (1.67)	8.93 (1.62)	9.47 (1.68)	$F_{1,104}$=3.19; *p*=.077
Medication AUC (SD)	9041.77 (18663.46)	9325.52 (13264.05)	9106.01 (17528.84)	$F_{1,104}$=.005; *p*=.95
Full Scale IQ (SD)	98.53 (13.45)	91.30 (12.58)	96.90 (13.54)	$F_{1,100}$=5.29; ***p*=.024**
Sex (%male)	65/82 (79)	16/24 (67)	81/106 (76)	X^2=1.63; df=1; *p*=.27
Income				
<10K	2/80 (3)	2/22 (9)	4/102 (4)	X^2=3.76; df=2; *p*=.15
10-40K	16/80 (20)	7/22 (32)	23/102 (23)	
40K +	62/80 (76)	13/22 (60)	75/102 (74)	
Ethnicity (% Caucasian)	72/82 (88)	20/24 (83)	92/106 (88)	X^2=.32; df=1; *p*=.57
Conner's Total Baseline Parent (SD)	72.47 (11.7)	74.58 (7.63)	72.97 (10.85)	$F_{1,78}$=.54; *p*=.46
Conner's Total Baseline Teacher (SD)	65.54 (11.61)	71.78 (11.18)	67.04 (11.74)	$F_{1,74}$=4.01; *p*=.05
CBCL Total T-Score (SD)	67.20 (7.4)	69.63 (4.5)	67.78 (6.9)	$F_{1,79}$=1.84; *p*=.18

DISC Total Items (SD)	12.76 (3.51)	13.83 (2.3)	13.00 (3.3)	$F_{1,105}$=2.00; *p*=.16

*Medication AUC = area under the curve, calculated by the product of duration (days) by dose (mg/day)

*Full scale IQ = intelligence quotient according to the WISC-IV.

Table III. 4: Demographic and Clinical Characteristics of +EM and -EM Groups.

	-EM (n=21)	**+EM (n=14)**	**Total (n=35)**	**Stats**
Age yrs (SD)	9.47 (1.48)	9.82 (1.82)	9.60 (1.60)	$F_{1,32}$=.36; *p*=.56
Medication AUC (SD)	10179.33 (19635.77)	12587.65 (18221.82)	11100.16 (18863.34)	$F_{1,32}$=.13; *p*=.72
Full Scale IQ (SD)	94.75 (12.97)	99.46 (15.01)	96.61 (13.78)	$F_{1,31}$=.92; *p* =.35
Sex (%male)	20/21 (95)	10/13 (77)	30/34 (88)	X^2=2.59; df=1; *p*=.11
Income				
<10K	1/20 (5)	0/13 (0)	1/33 (3)	X^2=.71; df=2; *p*=.70
10-40K	5/20 (25)	3/13 (23)	8/33 (24)	
40K +	14/20 (71)	10/13 (77)	24/33 (73)	
Ethnicity (% Caucasian)	20/21 (95)	11/13 (85)	31/34 (91)	X^2=1.12; df=1; *p*=.29
Conner's Total Baseline Parent (SD)	71.71 (12.1)	76.60 (10.24)	73.46 (11.5)	$F_{1,27}$=1,17; *p*=.29

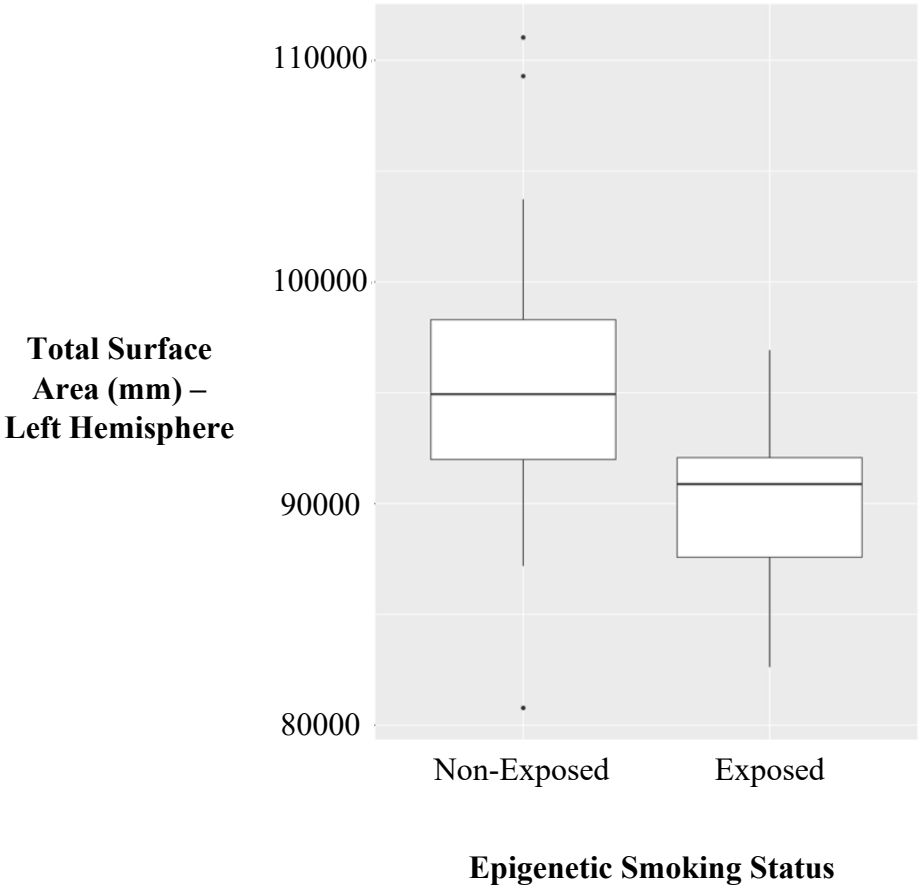
Conner's Total Baseline Teacher (SD)	66.79 (11.18)	69.25 (13.73)	67.52 (11.77)	$F_{1,26}$=.24; *p*=.63
CBCL Total T-Score (SD)	69.74 (6.26)	69.20 (5.25)	69.55 (5.84)	$F_{1,28}$=.05; *p*=.82
DISC ADHD Items (SD)	12.95 (3.89)	14.00 (3.24)	13.35 (0.63)	$F_{1,33}$=.665; *p*=.42

*Medication AUC = area under the curve, calculated by the product of duration (days) by dose (mg/day)

*Full scale IQ = intelligence quotient according to the WISC-IV.

Figure III. 5: Box Plots for Total Cortical Surface Area in the A) Left and B) Right Hemispheres comparing +EM (n = 10) to -EM (n = 20) Boys with ADHD (n = 30).

A)



B)

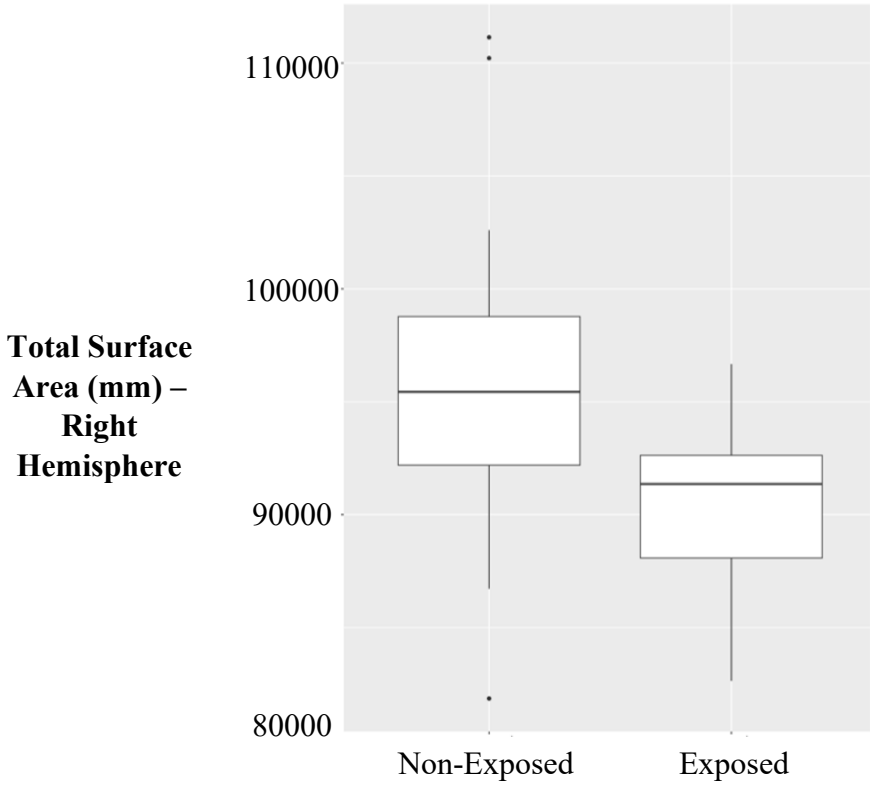
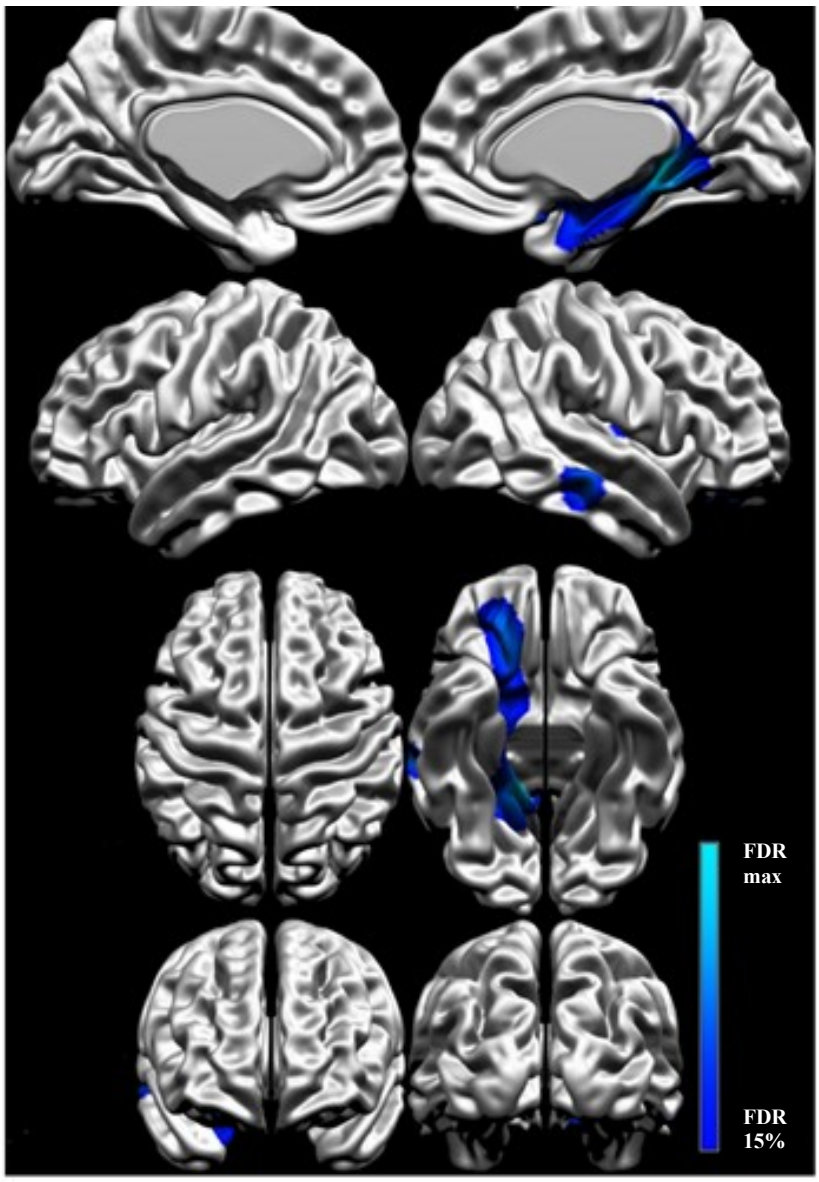

Total Surface Area (mm) – Right Hemisphere
110000
100000
90000
80000
Non-Exposed
Exposed
Epigenetic Smoking Status

Figure III. 6: q-statistics map representing brain regions (blue) with smaller cortical surface area measurements in +EM compared to -EM boys with ADHD. In sequence, left and right medial and lateral views, dorsal and ventral views, and front and back views. FDR 15%.



Supplemental Table SIII. 1: Concordance between Self-Reported MSDP and Epigenetically Determined Prenatal Smoking Exposure.

	+MSDP (n=6)	**-MSDP (n=29)**	**Total (n=35)**
+EM	5	9	14
-EM	1	20	21

Chapter IV: Discussion

ADHD is a neurodevelopmental disorder believed to arise through a complex interplay between genetic and environmental risk factors. Despite extensive research, current understanding of the aetiological mechanisms of ADHD remains limited. Neuroimaging case-control studies have garnered evidence of brain structure altercations in ADHD, especially in regions involved with executive functioning, such as the prefrontal cortex. Moreover, dysregulations within neurotransmitter systems (dopamine and norepinephrine) as well as exposure to prenatal cigarette smoking are strongly suspected to play a role in ADHD pathophysiology. However, findings in the literature are inconsistent and a causal relationship has yet to be established. Research using a combined genetic and neuroimaging approach has the potential to delineate the various pathways of ADHD and characterize more homogeneous subgroups of the disorder (Durston, 2010; M. Klein, van Donkelaar, Verhoef, & Franke, 2017), and as such, formed the foundation of this dissertation.

The purpose of this thesis was to contribute to the collective understanding of ADHD pathophysiology by investigating brain structure phenotypes in relation to risk factors and clinical dimensions. We have presented novel findings on the effects of ADHD medication, *NET* genotype and exposure to maternal smoking during pregnancy on brain structure, as well as associated behavioural and cognitive measures to brain regions that were found to be significant. The ensuing chapter begins by discussing the relevance and implications of each of these findings independently, followed by presenting the overall strengths and limitations that are common to each chapter.

IV.1 Cumulative Exposure to Medication is Inversely Associated with CA1 Volume in Children being Treated for ADHD.

The effects of medication on cortical measurements and subcortical volumes were investigated in a sample of children being treated for ADHD. Cumulative exposure to ADHD medication (CEM) was defined by the product of duration (days) and dose (mg/day). To the best of our knowledge, this is the first neuroimaging study using a continuous variable for medication exposure (CEM) and investigating the effects on subcortical volumes within 51 subregions. It was found that higher cumulative exposure to ADHD medication was negatively correlated to left hippocampus CA1 and SR/SL/SM volumes. Trends were also found in the same direction within the right hippocampus CA1, SR/SL/SM, DG, and left CA2/3 regions. However, no medication effects were detected at the cortical level (i.e. cortical thickness and surface area). The contribution of these findings is two-fold. First, the use of a quantitative measure for medication exposure that considered duration as well as dosage is unprecedented. Therefore, we addressed an important gap in the literature regarding the downstream effects of cumulative exposure to ADHD medication on brain structure in children. Second, since all children in our sample were exposed to psychostimulants, our findings provided the information required to account for confounding effects of medication in subsequent research models.

Preceding neuroimaging studies have provided contradictory results concerning differences in hippocampus volume between ADHD and typically-developing children. The most recent subcortical meta-analysis combining over 3000 scans from multiple sites reported smaller

global hippocampus volumes in children with ADHD (Hoogman et al., 2017). This analysis was based on a mixed sample of treatment-naïve and chronically-treated children and concluded that medication was not a contributing factor to the observed differences in subcortical volumes. However, the authors stated in their limitations that interpretation of results warrant some caution, as the study design was not optimal to test for medication effects. On the other hand, opposite findings have been reported, where children with ADHD were found to have larger global hippocampus volumes compared to control children (Plessen et al., 2006). Sixty-nine percent of children were taking psychostimulants, and medication exposure was corrected for in the analysis. The authors specified that the volumetric increase was driven by enlargement of the anterior region of the hippocampus, specifically the CA1, CA2/3 and DG subregions. Interestingly, the authors also observed contraction in the posterior portion of the hippocampus, indicating smaller volumes in the underlying tissues for the ADHD group within this region. These results suggest that the various hippocampus subregions may be differentially affected in ADHD pathophysiology and in parallel, differentially impacted by medication. However, few studies investigating the different hippocampus subregions in relation to ADHD exist in the literature. A study by Al-Amin et al. reported reductions in several hippocampus regions, including CA1, between ADHD and control children (n = 860; ADHD = 327; control = 533) (Al-Amin, Zinchenko, & Geyer, 2018), though correction for medication exposure was not performed. Since a high proportion of children diagnosed with ADHD receive pharmacological treatment, it is possible that medication effects are confounding the findings on hippocampus subregion volumes between diagnostic groups (Al-Amin et al., 2018). The authors proposed that the volumetric reductions were caused by neuronal atrophy within the subregions (Al-Amin et al., 2018). Moreover, chronic methylphenidate (MPH)

exposure at high doses has been demonstrated to induce neuronal cell death in the rat hippocampus CA1 regions (Carvallo et al., 2018; Schmitz et al., 2017).

In the analysis Chapter III section III.1, the hippocampus was divided into five subregions (CA1, CA2/CA3, CA4/DG, SR/SL/SM, and subiculum) and tested for association with CEM. Three supplemental analyses were conducted to help extrapolate medication effects: 1) case-control group comparison, and interaction analyses of 2) age-by-diagnosis and 3) age-by-CEM. Volumes within hippocampus subregions did not significantly differ between cases and controls in our sample, suggesting that the findings were not unduly influenced by diagnostic effects. Previous case-control imaging studies that identified differences in hippocampus volumes used case groups either consisting solely of treatment-naïve ADHD children, or mixed exposure (i.e. never exposed and exposed children, where status of medication exposure was not always considered) (Al-Amin et al., 2018; Hoogman et al., 2017). All the ADHD children in the current study have been exposed to medication for a minimum of one week, thereby controlling, at least partly, for heterogeneity of treatment exposure status in the analysis. Studies that investigated medication effects on brain structure compared groups of treatment-naïve, treatment-exposed and typically-developing children, and failed to detect significant hippocampus volume differences between the treatment-exposed and control group (Nakao et al., 2011; Spencer et al., 2013). Although it can not be determined if the children with ADHD in our sample initially had larger hippocampus CA1 volumes prior to medication exposure, the lack of significant volumetric differences between treated and control children is in line with the literature. As such, our findings do not contradict the theory of medication-induced normalization of brain structure. Furthermore,

evidence exists for a delay in developmental trajectories in children with ADHD, with a larger magnitude of brain volume differences observed in children relative to adults (Hoogman et al., 2017; Shaw et al., 2018). The age-by-diagnosis analysis did not reveal any significant findings, suggesting that diagnostic effects of ADHD on hippocampus subregional volumes, if existing in the first place, do not change as a function of the age range in our sample. Second, no significant effects were uncovered in the age-by-CEM interaction, suggesting that the effects of CEM on hippocampus volumes remain stable with age. Taken together, current findings propose that the association between CEM and hippocampus subregion volumes is independent of age and ADHD diagnosis.

Pharmacological studies conducted in rodents have found that psychostimulants increased synaptic levels of DA and NE in several brain regions, notably the prefrontal cortex, a region robustly associated with ADHD, and the hippocampus (Berridge & Devilbiss, 2011; Carvallo et al., 2018; Kuczenski & Segal, 2001). Changes in the hippocampus induced by psychostimulants have been implicated in the therapeutic response and as a potential side-effect (Britton & Bethancourt, 2009). MPH has been shown to increase DA and NE concentrations in a dose-dependent fashion within the hippocampus of adolescent rats, and thus MPH exposure has been proposed to impact hippocampal development (Kuczenski & Segal, 2002; Schmitz et al., 2017). Santos et al. reported that chronic MPH treatment administered to control rats caused synaptic remodelling within the hippocampus, which led to memory and cognitive deficits (Coelho-Santos et al., 2019). A study comparing effects of low versus high doses of chronic MPH treatment on hippocampal cell proliferation and survival found that administration at both doses increased

neurogenesis. However, the maintenance and integration of newly-formed neurons were only observed in the low-dose group. Authors concluded that chronic exposure to MPH at high doses initially increased neurogenesis but that hippocampal atrophy ensued as newly-formed neurons failed to survive long-term (Oakes et al., 2018). A review on the neurotoxic effects of psychostimulants reported similar findings, where young rats repeatedly administered MPH displayed significant decreases in the number of neurons and astrocytes in the hippocampus. However, neuronal proliferation was not affected by MPH (Goncalves, Baptista, & Silva, 2014). Furthermore, chronic MPH treatment at high doses has been linked with oxidative stress, neuroinflammation and neurodegeneration in the hippocampus of rats (Motaghinejad, Motevalian, & Shabab, 2016). Indeed, early and chronic administration of MPH was shown to ultimately cause deformations in the shape of the rat hippocampus (Coelho-Santos et al., 2019; van der Marel et al., 2015).

Nevertheless, the studies highlighted above employed non-ADHD animal models with typical catecholamine functioning. Since the current model for ADHD pathophysiology consists of DA and NE dysregulation, medication is likely to differently impact the brain of ADHD individuals (Biezonski et al., 2016). Convergent evidence exists of a dose-dependent U-shape therapeutic response from MPH, where lower doses improve cognitive performance and higher doses induce neurotoxic effects and cognitive impairment by bringing DA and NE above optimal concentrations (Cheng et al., 2014; Coelho-Santos et al., 2019; Devilbiss & Berridge, 2008). In the current study, smaller hippocampus volumes were associated with higher CEM. Future studies

are required to determine whether the nature of these alterations are therapeutic benefits or side-effects induced by continual exposure to ADHD medication use in humans.

Although it is uncertain if deficits in neurogenesis and long-term memory are involved in ADHD pathophysiology, the hippocampus is also involved in motivation and emotional regulation, which are functions impaired in ADHD individuals. Although the underlying cause for the volumetric reductions in the present study cannot be confirmed, it is reasonable to hypothesize, given the considerable evidence from animal studies, that alterations in neurogenesis and synaptic modelling following chronic exposure to ADHD medication are driving the structural changes observed. In addition, no significant associations with omissions, commissions, variability, and reaction-time CPT scores were detected, suggesting that selective and sustained attention was not related to the CA1 volume reduction in our sample. However, further research is required to determine whether these volumetric decreases are directly associated with long-term memory function, motivation, emotional regulation, or other behaviours mediated by the hippocampus in humans.

The major strength of this study is the detailed variable created for CEM. The structured interview with the parents in conjunction with access to the child's prescription history provides the information required to generate a precise and quantitative value of medication exposure, which considers dose, duration, multiple prescriptions, and medication breaks. External validation of parental reports was also made possible through prescription pharmacy logs, which has not been

performed in previous studies (Shaw et al., 2009). The third strength is the specificity of the subcortical regions under study, which were subdivided into 51 regions to explore more localized effects in the hippocampus. Moreover, restricting the sample to medicated children shifted the focus from comparing groups of treatment-naïve to treatment-exposed children with ADHD, to investigating medication effects within chronically-treated children with ADHD. This enabled the possibility of researching the effects of various cumulative exposures to ADHD medication, as opposed to group differences of medication exposure, as this has already been reported in the literature.

This work should be viewed in light of its limitations. First, the cross-sectional design prevented the assessment of medication effects over time on brain development. As such, longitudinal studies investigating brain development as a function of CEM are warranted. Differences in brain structure are prominent in children, and while the effects of age were accounted for in the model, our sample was limited to a pediatric population limiting the generalizability of findings to other age groups. Similarly, sex was included in the model, though it is plausible that medication effects on brain structure are different between boys and girls. As such, it would be valuable to explore sex-specific effects of CEM on brain structure in future studies. Third, although most prescriptions in our cohort were psychostimulants (97.5%), we cannot entirely discount the potential differences between the various medication brands. Nevertheless, dosage equivalencies among psychostimulant brands are relatively similar, apart from dextroamphetamine (i.e. Adderall®). Dosage was adjusted for dextroamphetamine in a supplemental analysis and yielded the same findings. Furthermore, a supplemental analysis

controlling for atomoxetine exposure (i.e. Strattera®), a non-psychostimulant medication specific for NE, was conducted and CEM effects on left CA1 and SR/SL/SM volumes remained significant (df = 94; q = 0.006). Fourth, while the sample size (n = 101) was sufficiently powered to uncover medication effects on hippocampus subregions, repeating the analysis in a larger independent cohort is required to confirm these findings, as well as increase power to detect potential smaller medication effects in other brain regions. This can be extended to the modest size of the typically-developing control sample in our study, which produced no significant results against the ADHD treatment group. Regardless, our negative findings remain in line with the literature, as similar hippocampus volumes have been reported between typically-developing and treatment-exposed children with ADHD.

The therapeutic response of ADHD medication and associated side-effects on behaviour are well documented in the literature. However, the effects of prolonged ADHD medication use and dosage on human brain structure remain elusive. This knowledge gap is relevant to both researchers and healthcare providers and raises important concern for individuals taking ADHD medication and for parents of children with ADHD. Here, it was found that higher CEM was associated with reduced hippocampus volumes in the CA1 and SR/SL/SM subregions in medicated children with ADHD, and that these effects were independent of ADHD severity, sex, and age. Despite extensive research, neuroimaging studies in ADHD have garnered contradictory and irreproducible findings. This may be partly attributed to unaccounted medication effects on brain structure and head movements during scanning. Therefore, our results suggest that the effects of CEM should be considered in future ADHD neuroimaging research. Furthermore, awareness of

the structural consequences induced by medication on the hippocampus sheds light on the pathophysiology of ADHD and may influence the decision-making process of ADHD treatment in children. Although smaller hippocampus subregional volumes were not associated with the cognitive dimensions tested in our sample, previous research has shown that smaller hippocampus volumes are associated with increased vulnerability to brain disorders later in life, memory deficits, sensitivity to trauma and anti-depressant resistance. Therefore, understanding the effects of CEM on the hippocampus may be an important factor in determining the optimal duration and dosage of treatment to avoid potential negative life outcomes, which may in turn instigate revaluation of current ADHD prescription practices. Hence, the findings of the current study carry important research and clinical implications.

IV.2 *NET* Genotype is Associated with Reduced Surface Area in Brain Regions Relevant for ADHD and Higher Externalizing Disorder Scores.

As described in Chapter III section III.2, it was found that ADHD children homozygous for the *NET* risk-allele (TT) had reduced cortical surface area in brain regions important for attention, notably in the prefrontal cortex. Moreover, smaller volumes within the significant brain regions were associated with higher externalizing disorder scores. To the best of our knowledge, this is the first imaging-genetics study demonstrating the effects of a specific polymorphism within *NET* on brain structure, and subsequently associating significant brain regions to behavioural outcomes. By demonstrating the effects of *NET* genotype on cortical surface area and externalizing behaviour, our current findings are in alignment and build upon previous findings of our team.

Furthermore, if reproduced in an independent study, current findings may point to a more severe subgroup of ADHD children that may eventually be distinguishable through genetic testing.

More specifically, we reported that children with ADHD homozygous (TT) for the rs36021 risk-allele have smaller global cortical measurements, as well as significant reductions in surface area within specific regions (OFC, vmPFC, piPL, frontal and temporal cortices) in comparison to heterozygous (AT) children with ADHD. These findings were generated from a linear model that included age, sex, and cumulative exposure to ADHD medication. Selection of rs36021 was based on a previous report linking this SNP to a more severe ADHD subtype (Thakur et al., 2012). In addition, rs36021 is in complete LD with rs3785143 and rs11568324, which have been shown to be associated with ADHD in three independent studies (Brookes et al., 2006; J. W. Kim et al., 2008; Xu et al., 2008). Through a family-based associated study, our team previously showed an association between the rs36021 T risk-allele and worsened CBCL behavioural and CPT cognitive measures particularly, higher externalizing and aggression scores (Thakur et al., 2012). Children homozygous for the risk-allele (TT) displayed the largest reduction in surface area measurements in our sample, which was significantly associated with higher disordered behavioural scores.

Mean cortical thickness and total surface area did not significantly differ between the two homozygous groups (AA, TT). In other words, heterozygous (AT) children with ADHD had the greatest cortical measurements in comparison to both homozygous groups (AA, TT). The largest magnitude of brain structure differences was observed between the groups of AT and TT children. The underlying biological mechanisms giving way to the cortical differences observed between

genotype groups are beyond the scope of this study. Nevertheless, evidence exists for an overdominance model of *NET*, where cognitive functioning is modulated according to an inverted-U-shape curve of NE and DA concentrations (Arnsten & Pliszka, 2011; Cools & D'Esposito, 2011). Given this, in addition to the proposed role of rs36021 in regulating gene expression, it may be speculated that in contrast to rs36021 heterozygous children with ADHD (AT), homozygous children have sub-optimal NE and DA synaptic concentrations mediated by genetic variations within *NET*, which may in turn translate to reductions in cortical measurements and ultimately, influence behaviour. Indeed, a 2016 study (n = 487) reported that rs36021 homozygous children (AA) had lower resiliency scores, which mediated higher externalizing behaviour (Trucco et al., 2016). Higher externalizing behaviour predicted greater substance use (i.e. cigarette, marijuana, and alcohol use) in adolescence (Pederson et al., 2018; Trucco et al., 2016). The authors concluded that their findings represent individual differences in neurobiological underpinnings for an externalizing pathway to substance use disorder, which is a prevalent comorbidity in individuals with ADHD. Further research is needed to confirm these findings before directly informing intervention programs for at-risk children with ADHD. Therefore, our current findings may provide an intermediate brain structure phenotype that fits within the framework of previous reports showing an association of externalizing behaviour and rs36021. Future research in suitable animal models is required to elucidate the biological and functional mechanisms between polymorphisms within *NET*, brain structure and behavioural outcomes.

The role of norepinephrine and its transporter in ADHD have been overshadowed by research efforts investigating dopamine function. Consequently, reports primarily focusing on NE are relatively scarce, despite NE's potentially important role in ADHD pathophysiology. One

imaging-genetics study investigated the effects of the *DAT* 10-repeat allele on brain structure in children with ADHD (n = 63). It was found that the group homozygous of the 10-repeat allele was associated with reduced cortical thickness in the lateral prefrontal cortex. The authors anticipated that the cortical reductions may be associated with deficits in executive function, more severe ADHD symptoms and response to treatment, although these measures were not assessed in that study (Fernandez-Jaen et al., 2015). There are no published reports investigating the effects of *NET* polymorphisms on brain structure, and thus our work contributes novel findings to the ADHD literature, as well as complement these findings by assessing clinical measures.

Compared to ADHD genetic studies in general, our results arise from a modest number of children and reproduction in a larger, independent cohort is warranted. Nonetheless, it should be noted that the current sample size (n = 74) is within the range of previous imaging-genetics studies in the ADHD literature. Since our sample consisted of children all diagnosed with ADHD, further research comparing cases to controls is needed to determine whether the effects of rs36021 on cortical surface area are specific to ADHD or can be extended to typically-developing children. Finally, it is possible that the observed effects of rs36021 on cortical surface area may have incurred through other functional variants within *NET*, especially since rs36021 is in LD with two other SNPs associated with ADHD. In any case, this work provided evidence that genetic variation (and possibly linked SNPs) within *NET* contribute to cortical alterations and more severe ADHD behaviour.

Taken together, we report a significant association between rs36021 *NET* genotype, cortical surface area and disruptive behaviour in children with ADHD. Children with ADHD homozygous for the T risk-allele may be more at risk of having brain structure alterations associated with NE functioning, which in turn may lead to more severe clinical outcomes. If independent replication ensues, our current findings endorse an imaging-genetics based approach to disentangle the heterogeneity of ADHD, which can help define an ADHD subtype through genotype and structural biomarkers (Wallis, 2010). As such, these findings can have important research and clinical implications, and thus can ultimately promote the development of preventative and more personalized therapeutic interventions.

IV.3. Exposure to Maternal Smoking during Pregnancy

Exposure to maternal smoking during pregnancy is a frequently associated risk factor of ADHD however, inconsistencies exist across studies. As previously mentioned, our team demonstrated that a single-nucleotide polymorphism within *NET* (rs36021) was significantly associated with ADHD diagnosis and a range of behavioural and clinical dimensions (i.e. externalizing disorder scores) (Thakur et al., 2012). Interestingly, this association became highly significant when the sample was stratified according to exposure to maternal smoking during pregnancy.

An incipient approach of merging neuroimaging and epigenetics was used to objectively determine exposure to prenatal smoking and examine downstream effects on brain structure. It

was found that exposed children with ADHD, relative to non-exposed, had significant reductions in cortical surface area in brain regions previously associated with ADHD, such as the orbitofrontal cortex. Moreover, significant reductions in cortical surface area were correlated to higher commission errors in the neuropsychological assessment, denoting a higher degree of impulsivity. Children exposed to smoking in-utero have already been associated with more severe ADHD symptomatology, disorderly behaviour and poorer cognitive performance (Motlagh et al., 2011; Thakur et al., 2013). Thus, current findings provide additional supporting evidence, and implicate specific brain regions.

IV.3.1 Epigenetic Markers as a Means of Determining Exposure to Prenatal Cigarette Smoking.

The analysis in Chapter III section III.3 identified to main findings. First, the calculation of exposure to MSDP based on maternal recall led to a significant level of false-negatives. Indeed, almost one third of the children who were characterized as non-exposed to MSDP based on maternal reports were found to be carriers of a high-sensitivity epigenetic signature of prenatal exposure to cigarette smoking associated with prenatal exposure to cigarette smoking. The major limitation amongst all previous studies investigating the relationship between ADHD and MSDP is the reliance upon retrospective reports to determine children's in utero exposure status (T. Russell et al., 2004; Shipton et al., 2009). Therefore, the use of epigenetic data to determine exposure to smoking can address the current limitations prevailing in retrospective reporting by removing the non-disclosure bias and by considering the additional sources of exposure to smoking (i.e. passive smoking). Furthermore, it is possible that non-disclosure of MSDP may not have only

occurred through inaccurate reporting of smoking behaviour during pregnancy, but also from the method the questionnaire was administrated by the interviewer. In any case, the inaccurate determination of exposure status may partially explain the inconsistent association between MSDP and ADHD in the literature. Future research should take advantage of biological tools, such as epigenetic markers, to determine exposure to various environmental risk factors.

Second, by stratifying children according to their exposure of MSDP based on modifications in the epigenome, we identified significant reductions of cortical surface in areas relevant for regulating attention and impulsivity. In contrast, no effects were found when children were grouped according to maternal recall of exposure to MSDP. Although these results are preliminary, our findings illustrate the potential of using biological tools to determine exposure to environmental factors, and further support the role of exposure to prenatal cigarette smoking in ADHD pathophysiology

No effects of exposure to MSDP, as measured by either maternal recall or epigenetic markers, were identified for cortical thickness in this study. Although the literature has provided some supporting evidence for an effect of exposure to MSDP on cortical thickness, these studies were conducted in typically-developing children and adults with ADHD, whereas our sample focused on children with ADHD. In addition, relative to cortical thickness, which is more susceptible to age-dependent changes across the lifespan, cortical surface area is a more stable marker for risk factors influencing early development. Cortical surface area is largely determined during fetal development, when gyrification is occurring, and remains relatively stable throughout

life (Eyler et al., 2011). Therefore, if risk factors intervene during fetal brain development, the effects on the growing cortex may be compounded and detectable later in life as alterations in surface area.

IV.3.2 Exposure to Prenatal Smoking is Associated with Reduced Surface Area in Brain Regions Relevant for ADHD and Lower Response-Inhibition Scores.

Decreases in surface area were observed in specific brain regions (i.e. ROFc, RMTc and RPHg) in children with ADHD who were categorized, based on epigenetic markers, in the exposed group. The OFC and temporal lobes have been robustly linked to ADHD, and thus our findings provide further support for their involvement. The temporal lobes play a role in auditory processing and consolidation of memories based on sensory perceptions. Several regions of the temporal lobes are heteromodal associative areas involved in higher-order processing such as attention, memory, and emotional regulation, which are functions negatively affected in ADHD (Kobel et al., 2010). Fernandez-Jean et al. reported that children with ADHD showed decreased cortical thickness in the right temporal pole and OFC relative to typically-developing children. The authors proposed the involvement of these regions in the pathophysiology of ADHD (Fernandez-Jaen et al., 2014). The primary role of the OFC is in executive functioning, namely decision-making, motivation and reward anticipation. Lesions within the OFC have been associated with disinhibited behaviour (impulsivity), a core hallmark of ADHD. Interestingly, higher CPT commissions-error T-scores

were significantly associated with reduced cortical surface area in our sample. The CPT requires children to press the spacekey when a letter appears on the screen, apart from the letter X. CPT commissions-errors T-score measures the child's inability to withhold a pre-potent response to X (response-inhibition), where a higher score corresponds to a higher degree of impulsivity. Therefore, children with ADHD in our sample that were exposed to prenatal smoking according to epigenetic markers, had significant reductions in brain regions involved in regulating impulsivity, which translated to poorer inhibition of responses on psychological evaluation.

A study conducted in the Saguenay Lac-St-Jean founder population observed an inverse correlation between cortical thickness in the OFC and substance use in adolescence (Fernandez-Jaen et al., 2014). The authors proposed that exposure to prenatal smoking disrupts the typical development of the OFC, thereby altering reward-responses and ultimately increasing prospective substance use (Lotfipour et al., 2009). Early prevention programs designed for at-risk children with ADHD exposed to prenatal smoking may be useful to mitigate the risk of future substance abuse, by designing and offering specific interventions to minimize smoking initiation. Therefore, these findings highlight the importance of the prenatal environment and have public health implications.

While we have used epigenetic modifications in the sole purpose of determining smoking exposure status, it is important to question whether these epigenetic modifications affect the molecular pathways of development, thus providing some mechanistic explanation for the effects observed in this study. For example, growth factor GFI1 is one of the many genes found to be significantly associated with MSDP, suggesting that differential expression in various growth

factors may explain a change in brain structures and functions, which can lead to ADHD symptoms (Joubert et al., 2012; Sengupta et al., 2017). Further research is required to explore the role of these epigenetic modifications in brain development and ADHD, as this is beyond the scope of the current study.

This work has several specific strengths. First, when using maternal recall for determining MSDP status, we relied on a sample size of 109 children, which is relatively large compared to previous neuroimaging studies on MSDP. Although this suggests that the absence of any relation between MSDP and brain structure may be statistically robust in this large sample size, this finding is mitigated by the reliance on retrospective reports to assign exposure status. The second major strength is the novelty of using epigenetic markers to determine exposure to prenatal smoking in a subgroup of patients, which yielded positive findings in alignment with the literature.

The results of this study should be interpreted in view of its limitations. The acquisition of in-depth phenotyping, including neuroimaging and whole epigenome data, requires extensive resources. As such, our sample size is modest (n = 30) and is restricted to boys, and thus our preliminary findings require replication in larger independent samples that include girls. However, it should be noted that given the novelty of a combined imaging-epigenetics approach, our sample size lacks a comparative basis and contributes new findings to the literature. Moreover, since these findings originate from a clinical sample of children with ADHD, it would be interesting to investigate the effects of prenatal smoking exposure in typically-developing children to tease apart ADHD effects. Another limitation concerns the timeline for acquiring epigenetic changes resulting

from smoking exposure in children. Although we are assessing prenatal smoking exposure in relation to cortical structure, it is possible that children with epigenetic markers of cigarette smoking have been exposed after the prenatal period and/or are currently being exposed to second-hand smoke. Since the minimal time period required to establish an epigenetic smoking signature is unknown, it is difficult to disentangle embryonic effects from passive smoking during childhood. Nonetheless, this issue only arises in the children who carried epigenetic markers and whose mothers did not report smoking during pregnancy but reported smoking in the post-natal period. Out of the nine children in this category, four children had mothers who reported being exposed to second-hand cigarette smoking throughout their pregnancy. Regular exposure to second-hand smoke during pregnancy may be one explanation for the discordance between self-reports and epigenetic data. Two out of the nine mothers reported post-natal smoking with indoor smoking restrictions. There is no additional information on the remaining three mothers. In future studies when epigenetic data is not accessible, it may be worth considering passive smoking during pregnancy to have more accurate assessments of the effects of prenatal smoking exposure in children.

In summary, a significant effect of exposure to prenatal smoking was found on cortical surface area in children carrying an epigenetic signature. However, no effects on brain structure were observed when exposure groups were based solely on maternal reports. We found that one third of children were inaccurately classified as non-exposed according to maternal recall but have highly-sensitive and replicable epigenetic markers consistent with exposure to nicotine in pre/peri-natal periods through unaccounted sources (i.e. relatives, partner, etc.). To the best of our knowledge, this is the first study using molecular biomarkers to determine smoking exposure in

children with ADHD and test for association with cortical brain structures. The discrepancy between the negative neuroimaging findings according to maternal recall and the positive neuroimaging findings according to molecular data suggest that the latter may be a more accurate approach in determining exposure status to prenatal cigarette smoking, and thus increases the likelihood to uncover effects on brain structure. These results suggest that epigenetic changes associated with prenatal smoking exposure could alter brain development in regions relevant for ADHD, and therefore may represent one of the several epigenetic pathways leading to the development of ADHD. If replicated, such findings may one day endorse the practice of epigenetic screening for markers associated with prenatal smoking exposure to help identify a subpopulation of children with more severe ADHD phenotypes, and thus in need of supplementary care and more tailored resources. This can be especially relevant for adopted individuals diagnosed with ADHD, in whom the prenatal environment may be unknown. Such an approach may also be extended to other environmental factors that leave a biological trace that can be reliably measured. Ultimately, studies such as these can contribute to the collective understanding of neurodevelopment and warrant further attention.

IV.4 Overall Limitations

The overall limitations common throughout this dissertation are presented in the following paragraphs. Children included in our study were clinically evaluated based on structured diagnostic interviews by the ADHD clinic's team psychiatrists at the DMHUI, and thereby received a confirmed diagnosis of ADHD. We collected in-depth information arising from multiple sources

(i.e. child, parents, teachers) pertaining to clinical, cognitive, behavioural, neuroimaging, genetic, environmental, and epigenetic data. This enabled a thorough phenotypic characterization for each child in our sample. Nevertheless, such comprehensive data collection per participant requires extensive resources over multiple visits, countering the capacity to recruit very large sample sizes. Relative to genetic studies in general, our findings arise from a modest number of children and reproduction in a larger, independent cohort is warranted. However, it should be noted that the sample sizes, apart from the epigenetic-imaging analysis described in Chapter V, were similar to those found in most neuroimaging studies in ADHD.

Second, important challenges exist in pediatric neuroimaging. The most concerning is head motion, as it can create spurious findings by underestimating brain structure measurements (Reuter et al., 2015; Weinberger & Radulescu, 2016). Children within our sample were all pharmacologically treated for ADHD symptoms, thereby reducing head motion during the scanning process and producing viable scans that passed quality control (i.e. except for three children with ADHD). Moreover, scanning was repeated in cases where children were visibly mobile, and thus increased the opportunity of acquiring a viable brain image. Rigorous quality control was carried out on multiple occasions throughout imaging processing to select optimal brain scans. Children also practiced on a mock scanner, which has been shown to decrease anxiety levels, increase protocol compliance and improve quality of scans (de Bie et al., 2010; Poldrack, Pare-Blagoev, & Grant, 2002). Moreover, brain imaging was carried out in a single site using the same MRI scanner and technician, thus removing the potential for multi-site error. Although several actions were put in place to minimize motion and spurious effects, it cannot be stated that the brain structure measurements we acquired are completely devoid of motion artifacts. The

development of tools to address motion effects in brain imaging are currently underway and could assist in generating measurements with higher accuracy. Reproduction in future studies, correcting for motion, would help validate our findings.

Third, the FDR is typically set at 5%, however we did not observe effects at this threshold. In the vertex-wise comparisons, we discovered significant effects of genetic and epigenetic factors on cortical surface area measurements at an FDR of 15%. The FDR approach is used as an alternative method to the Bonferroni correction by informing us on the proportion of false positives, rather than guarding against making any false positive conclusions at all. FDR-based procedures reveal all the significant tests and increase power to identify truly significant comparisons, while permitting less control over erroneous claims of significance (i.e. false positives). Therefore, out of the total significant vertices found in our analyses, 15% and 85% are expected to be false positive and true positive effects, respectively.

Fourth, the presented findings were acquired through a cross-sectional design, which may help in the recruitment of participants, as well as minimize the occurrence of missing data. However, this design approach is limited in assessing the effects on brain structure development over time. Extensive neurobiological changes can occur across the lifespan, especially between childhood and adulthood. As such, the sample was restricted to a pediatric population, thereby reducing potential confounding effects arising from different developmental stages. On the other hand, this can limit generalizability of results to adolescent and adult ADHD populations. Therefore, one must interpret findings on brain structure with the understanding that a causal

relationship cannot be ascertained, and that effects may vary with age. Similarly, in Chapters III sections III.2 and III.3, the observed effects of *NET* genotype and prenatal smoking exposure on behavioural and cognitive dimensions may change over time. Nonetheless, our *NET* data are novel and if replicated in future studies, can motivate the progression into longitudinal designs.

The focus of this dissertation is on the use of brain imaging to help bridge the gap between risk factors (genetic and epigenetic) and clinical measures in ADHD. Neuroimaging enables the investigation of various risk factors, previously associated with ADHD, on brain morphology. Meta-analyses have confirmed that genetic variants show stronger associations with brain morphology in comparison to behavioural or cognitive symptoms (Mascarell Maricic et al., 2020; Rose & Donohoe, 2013). As such, combining brain imaging with genetics holds the potential to discover sizably larger effect sizes between genotype and epigenetic markers on brain structure, relative to traditional psychiatric genetic studies (Mascarell Maricic et al., 2020). In the case of ADHD, several different etiological pathways are believed to be involved in the manifestation of symptoms. Through the incorporation of brain imaging as an intermediate phenotype, we can reduce heterogeneity and complexity of ADHD by teasing apart particular subgroups of this condition that are presumed to arise through distinct etiological pathways.

It has been suggested that structural MRI measures are a promising area for endophenotype research of complex traits, such as ADHD. More specifically, individuals with a specific endophenotype (ex. cortical surface area measurements in certain brain regions) may be predisposed to develop a more severe presentation of the condition. This, in turn, can help reveal

part of the underlying biological processes playing a role in ADHD. For instance, a sample of monozygotic twin pairs concordant for ADHD found that genetic factors determined smaller volumes within orbitofrontal cortices (van 't Ent et al., 2007). Therefore, it is possible the findings presented in this dissertation, if replicated in independent samples, point towards structural neuroimaging endophenotypes of ADHD. By first investigating cumulative exposure to ADHD medication, we were able to account for potential cofounding effects on brain morphology in our sample. We then modeled the effects of genetic and epigenetic factors on brain structure and behavioural and cognitive measures, respectively. Taken together, our research provides supporting evidence that neuroimaging tools can assist genetics studies in ADHD by uncovering effects that may have not been detectable through standard genetic approaches. Therefore, imaging-genetics can improve our understanding of ADHD pathophysiology by elucidating neurobiological pathways, presenting new testable hypotheses, and investigating effects of genetic and environmental factors, as well as their interactions, on neurobiology.

Chapter V: Conclusions & Future Aims

This thesis employed imaging-genetics to investigate the effects of cumulative exposure to medication (CEM), *NET* genotype group and exposure to prenatal smoking on brain structure in children diagnosed with ADHD. This approach can aid in understanding the biological underpinnings of genetic and epigenetic factors on brain structure. Moreover, significant structural differences in specific brain regions can be subsequently associated with cognitive and behavioural measures. As demonstrated in Chapter III section III.1, higher CEM was associated with smaller hippocampus subregional volume. In sections III.2 and III.3, we reported that *NET* genotype group and epigenetic markers associated with exposure to prenatal smoking were both associated with smaller cortical surface area measurements in regions relevant for ADHD, and these cortical alterations were significantly correlated to externalizing disorder and commission error scores, respectively. Future aims to expand upon this work are presented below.

Higher CEM was associated with smaller hippocampal CA1 volume however, no effects were uncovered between smaller CA1 volume and cognitive/behavioural measures. Whether the reduction in volume was directly caused by cumulative exposure to medication, or whether the nature of these findings represent a positive or negative effect of medication on brain structure and development, remains to be established. Future longitudinal neuroimaging studies collecting structural, functional, clinical, cognitive, and behavioural data in pharmacologically treated children with ADHD are essential for determining the direct benefits and consequences of ADHD medication on brain structure and development.

We showed that the *NET* genotype was significantly associated with brain structure measurements, where children homozygous for the risk-allele (TT) had smaller cortical surface area in attentional networks. Moreover, these smaller surface area measurements were significantly correlated to more externalizing behaviour scores. In section III.3, we dichotomized children depending on exposure status to prenatal cigarette smoking, according to either maternal self-reports or epigenetic markers. No effects were found when children were grouped according to retrospective reports from mothers. However, when exposure groups were constructed according to epigenetic data, smaller cortical surface area measurements in frontal, temporal and parahippocampal regions were uncovered in the exposed group of children with ADHD. Furthermore, smaller cortical surface area measurements within significant brain regions were positively correlated to more commission errors on the Continuous Performance Test, indicating a higher frequency of impulsive replies. Since it has been previously reported that a highly significant interaction exists between rs36021 and exposure to maternal smoking during pregnancy in ADHD, it would be interesting to assess the interactive effects of these two components on brain structure. Future research projects collecting detailed genetic, epigenetic and brain imaging data would enable an interaction analysis, and thus could shed further light on aetiological pathways in ADHD. The findings presented in this thesis are novel contributions to the understanding of ADHD pathophysiology by demonstrating the effects of medication, genetic and environmental risks factors on brain structure and behaviour.

Chapter VI: References

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Appendix

Contributions to other projects

Peer-reviewed Publication

Sengupta, S.M., **Fotopoulos, N.**, Devenyi, G.A., Fortier, M.È., Ter-Stepanian, M., Sagliker, S., Karama, S., Chakravarty, M.M., Labbe, A., Grizenko, N. and Joober, R., 2018. Dissecting genetic cross-talk between ADHD and other neurodevelopmental disorders: Evidence from behavioural, pharmacological and brain imaging investigations. *Psychiatry research*, *269*, pp.652-657.

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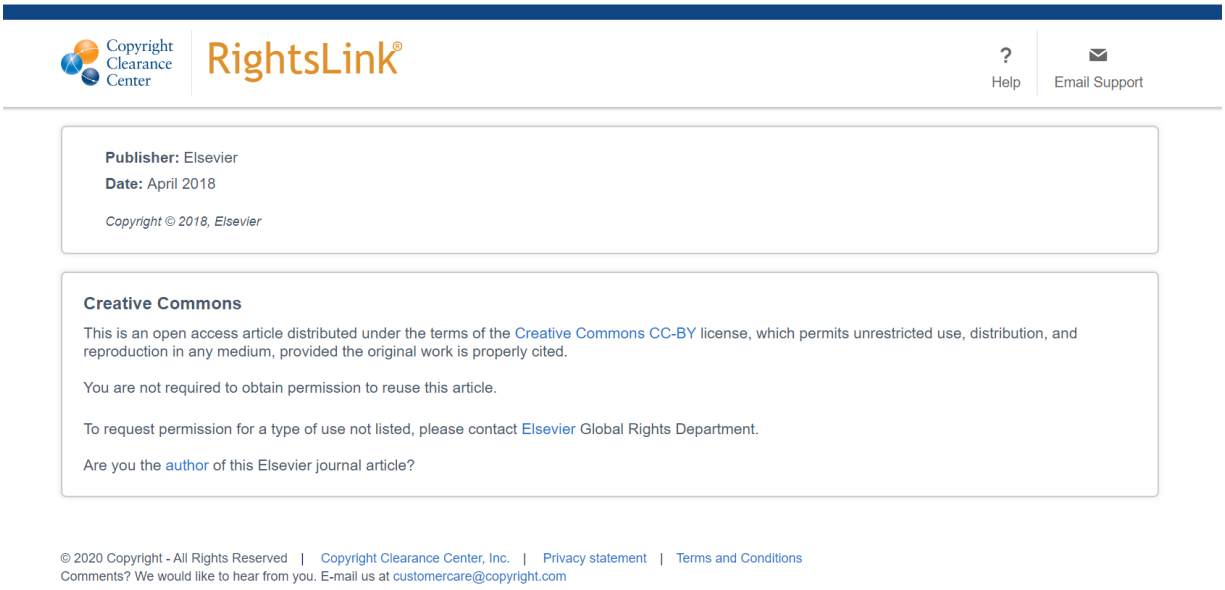

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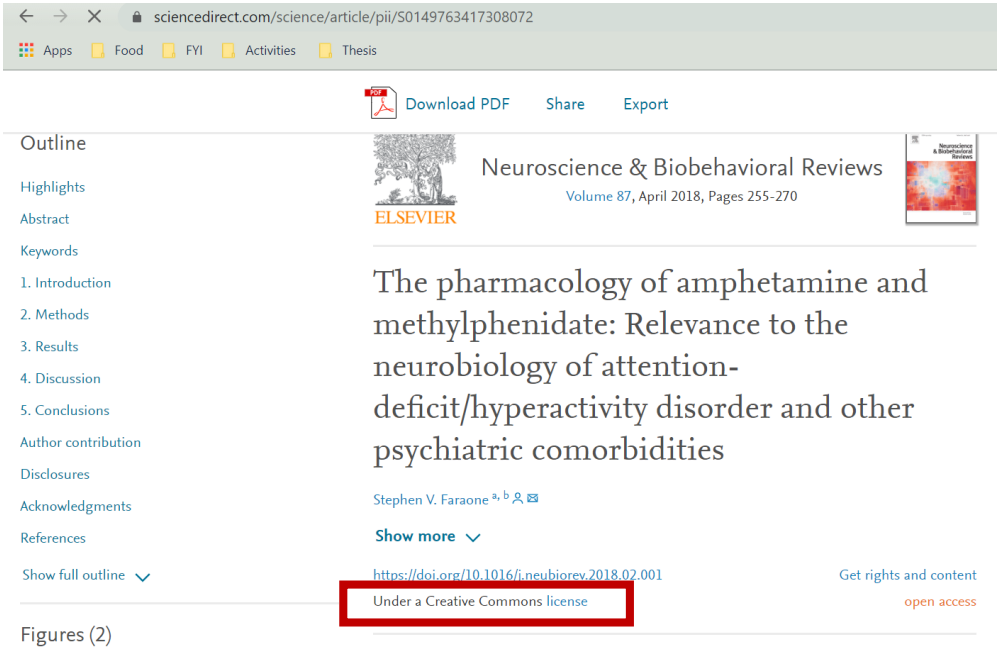

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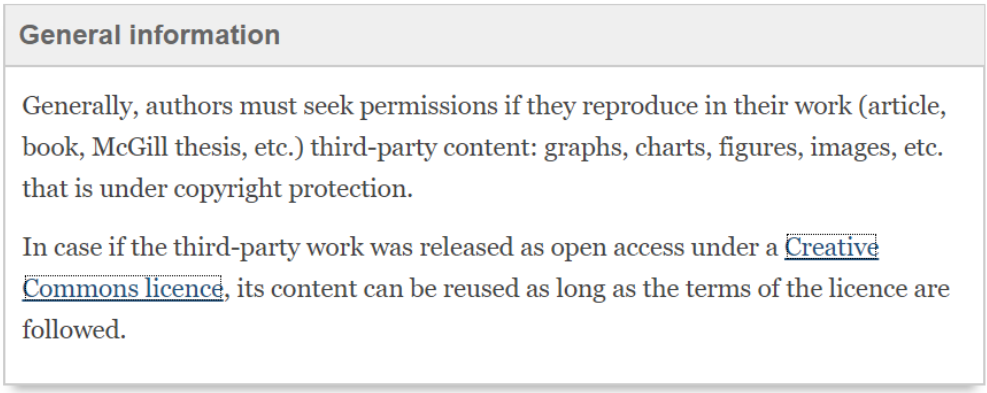

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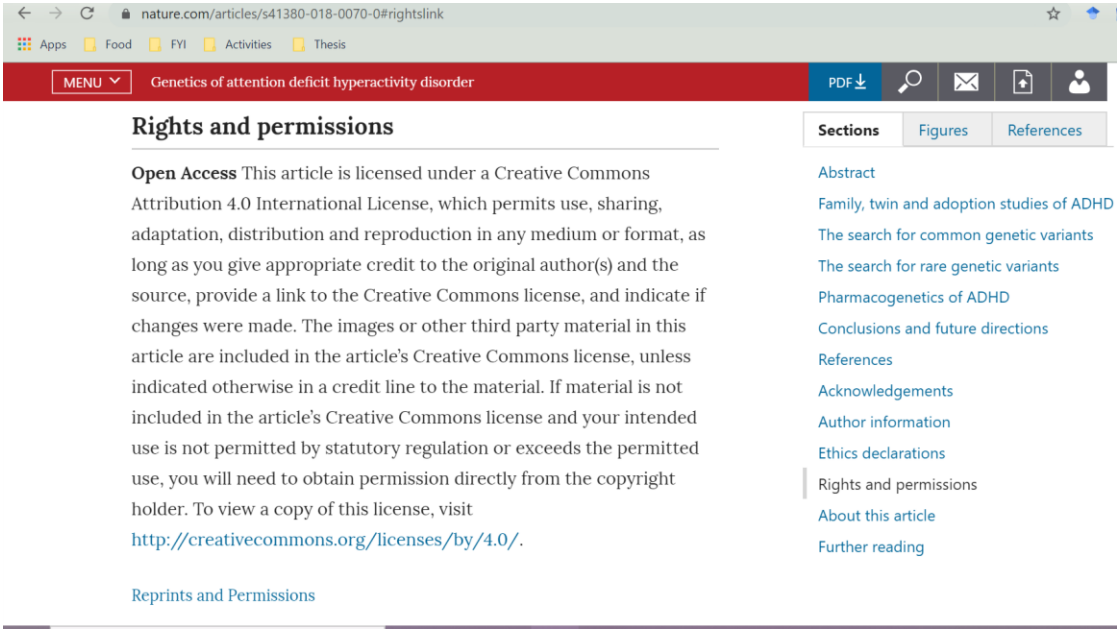

Supplemental

List of significant CpG sites associated with prenatal smoking exposure

CpG	DMP
cg14179389	mean(RUNX1)
cg19065106	Mean(BHMT2)
cg25855162	mean(CYP1A1)
cg05640346	mean(FRMD4A)
cg19796617	mean(HOXA5)
cg05549655	mean(NRP2)
cg12101586	mean(chr6)
cg13570656	mean(HOXA5)2
cg18092474	
cg22549041	
cg01359822	
cg14179389	
cg13750264	
cg21199085	
cg04180046	
cg05009104	
cg12803068	
cg19089201	
cg14157435	
cg20351668	
cg25715429	
cg00994804	
cg02869559	
cg03142697	
cg12477880	
cg26974661	
cg06758350	
cg09889857	
cg23458168	
cg01856384	
cg04198471	
cg05857999	
cg07616871	

Ethics Approval

The research protocol was approved by the Research Ethics Board of Douglas Mental Health University Institute. The study was explained to parents and children. Informed consent was provided in writing by the parents. Children gave verbal assent to participate in the MRI project.