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RESEARCH ARTICLE

Facing the Methodological Challenge in Dissecting the Genetics of ADHD: A Case for Deep Phenotyping and Heterogeneity Reduction

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Abstract

Objective: The aetiology of ADHD is complex, with genetic and environmental factors both implicated in the disorder. The most recent ADHD genome-wide association study identified 12 loci that showed significant association with the disorder. However, as highlighted by the authors, these loci "only capture a tiny fraction" of the risk for ADHD. It has been suggested that it may be important to disentangle: (1) the clinical complexity of the disorder, and (2) the complex interaction between genetic and environmental factors, in order to better dissect the aetiology of the disorder. **Method:** We have conducted a clinically-relevant Pharmaco-Behavioural Genetic study in a large group of children with ADHD (~850 families) over the last 15 years. The study includes detailed evaluation of quantitative behavioural and neuropsychological phenotypes, as well as short-term response of these phenotypes to treatment with a fixed dose of methylphenidate (0.5mg/kg in a b.i.d. dose). Specific genetic markers and environmental factors were examined for their association with these dimensions. **Results:** Here we present results that highlight the importance of examining genetic association with quantitative traits, including those constructs having relevance to Research Domain Criteria (RDoC). Further, we demonstrate that by conducting association analysis in groups of children stratified based on exposure to key environmental exposure (maternal smoking or stress during pregnancy), we are able to increase the sensitivity for finding genes involved in the disorder. **Conclusion:** These results suggest that deep phenotyping and heterogeneity reduction may be imperative in order to uncover the "missing heritability" of the disorder.

Key Words: *ADHD genetics, pharmacogenetics, environmental factors, gene-environment interplay, RDoC, cognition*

Résumé

Objectif: L'étiologie du trouble de déficit d'attention avec hyperactivité (TDAH) est complexe, puisque des facteurs tant génétiques qu'environnementaux y sont impliqués. L'étude d'association pangénomique du TDAH la plus récente a identifié 12 loci qui présentaient une association significative avec le trouble. Toutefois, comme le soulignent les auteurs, ces loci ne « représentent qu'une infime fraction » du risque de TDAH. Il est suggéré qu'il peut être important de démêler: (1) la complexité clinique du trouble et (2) l'interaction complexe entre les facteurs génétiques et environnementaux, afin de mieux décortiquer l'étiologie du trouble. **Méthode:** Nous avons mené une étude de génétique pharmaco-comportementale importante sur le plan clinique auprès d'un groupe nombreux d'enfants souffrant du TDAH (~850 familles) au cours des 15

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dernières années. L'étude comporte une évaluation détaillée des phénotypes comportementaux et neuropsychologiques quantitatifs, ainsi que la réponse à court terme de ces phénotypes au traitement par dose fixe de méthylphénidate (0,5 mg/kg dans une dose deux fois par jour). Les marqueurs génétiques spécifiques et les facteurs environnementaux ont été examinés relativement à leur association à ces dimensions. **Résultats:** Nous présentons ici les résultats qui soulignent l'importance d'examiner l'association génétique avec les traits quantitatifs, y compris ces construits qui ont rapport aux critères du domaine de recherche (RDoC). En outre, nous démontrons qu'en menant une analyse d'association dans des groupes d'enfants stratifiés selon leur exposition à une exposition environnementale principale (le tabagisme maternel ou le stress durant la grossesse), nous sommes capables d'accroître la sensibilité propice à trouver des gènes impliqués dans le trouble. **Conclusion:** Ces résultats suggèrent qu'un phénotypage profond et une réduction de l'hétérogénéité peuvent être impératifs afin de découvrir « l'héritabilité manquante » du trouble.

Mots clés: *génétique du TDAH, pharmacogénétique, facteurs environnementaux, interaction gène-environnement, RDoC, cognition*

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a complex and highly prevalent psychiatric disorder among school-aged children, occurring in 5.9-7.1% of the population (Willcutt, 2012). ADHD is heterogeneous in its clinical expression, with core symptoms of poor sustained attention, impulsiveness, hyperactivity, and accompanying cognitive deficits. Comorbidities are common in ADHD, and include- conduct disorder, oppositional defiant disorder, anxiety disorder, and learning disabilities. ADHD has adverse consequences since it is associated with grade retention, school suspensions, expulsions, difficulties with peer and family relationships in childhood (Befera & Barkley, 1985; Clark, Cheyne, Cunningham, & Siegel, 1988; Faraone et al., 1993), and academic/occupational/social impairment in adult life (Kessler et al., 2010; Sobanski, 2006). Long-term follow-up studies have concluded that the disorder is associated with significant psychiatric morbidity across the life span in men (Biederman et al., 2006) and women (Biederman et al., 2010), with increased risk for antisocial, smoking and other addictive behaviours, mood and anxiety disorders. The medications used most commonly to treat ADHD symptoms are psychostimulants (primarily methylphenidate [MPH] derivatives), estimated to be used by approximately 6% of the school-aged population in Canada (Romano et al., 2005). Significant variability in clinical response is observed, and it has been hypothesized that genetic factors may account for differences in treatment outcome (Faraone & Larsson, 2018).

ADHD is complex in its aetiology, with genetic and environmental factors implicated in the disorder (Thapar & Cooper, 2016). Family, twin, and adoption studies have concluded that the aetiology of ADHD is strongly influenced by genetic factors (Faraone & Larsson, 2018). Mean heritability has been estimated to be around 80% (Faraone & Larsson, 2018); yet the identification of "risk genes" for ADHD has been challenging, most likely due to the clinical and aetiological complexity of the disorder. It is well established that ADHD is a polygenic disorder where multiple susceptibility genes contribute to overall risk, each with a small effect (Faraone & Larsson, 2018). In addition to genetic loading, epidemiological studies have implicated environmental factors in ADHD (Thapar & Cooper, 2016). These factors include prenatal and perinatal risk factors: maternal smoking, alcohol consumption, stress, exposure to toxins particularly lead, low birth weight, and preterm birth. The common thread in these factors is that exposure occurs during critical periods of development. While there is a correlation between these environmental factors and elevated risk for ADHD, causality has yet to be confirmed using genetically-informed designs. Importantly, it has been recognized that gene-environment interplay (G-E), where the genotype of the individual modulates the sensitivity or response to the environmental risk factor, may play a pivotal role in the disorder.

Notwithstanding the challenges arising from the complexity of the disorder, much effort has been dedicated to identifying genes involved in ADHD. A large number of candidate-gene studies have been conducted, including work from our group (Choudhry et al., 2012; Choudhry et al., 2013; Choudhry et al., 2014; Fageera, Sengupta, Labbe, Grizenko, & Joober, 2018; Fortier et al., 2012; Grizenko et al., 2012; Gruber et al., 2009; Joober et al., 2007; Karama et al., 2008; Kebir, Grizenko, Sengupta, & Joober, 2009; Naumova, Grizenko, Sengupta, & Joober, 2017; Sengupta et al., 2018; Sengupta et al., 2008; Sengupta et al., 2006; Sengupta et al., 2012; Taerk et al., 2004; Thakur, Sengupta, Grizenko, Choudhry, & Joober, 2012a, 2012b; Thakur, Sengupta, Grizenko, Schmitz, et al., 2012). Most of these candidate gene studies (both work done by ourselves and others) have focused on key components of the dopamine (DA), norepinephrine (NE), and serotonin (5-HT) pathways based on evidence from animal models and pharmacological treatment of ADHD, that these neurotransmitters are involved in ADHD (Sagvolden & Sergeant, 1998). While many of the individual, candidate gene-based association

studies have been underpowered, a meta-analysis of these studies noted a significant association with common variants in a limited number of genes (Gizer, Ficks, & Waldman, 2009).

In the last ten years, Genome-Wide Association Studies (GWAS) have become the main hypothesis-free approach to investigate the genetics of complex disorders. The most recent GWAS, with 20,183 ADHD cases and 35,191 controls, identified 12 loci that showed genome-wide significant association with the disorder (Demontis et al., 2019). While these results are "*compelling,* [they] *only capture a tiny fraction of common variant risk for ADHD*" (Demontis et al., 2019). Most recently, an "omnigenic" model has been proposed to help elucidate the underlying genetics of complex traits (Boyle, Li, & Pritchard, 2017). According to this model, "*most traits can be directly affected by a modest number of genes or gene pathways with specific roles in disease etiology, as well as their direct regulators*". These "*core genes...will tend to have biologically interpretable roles in disease". However, the SNPs that contribute the bulk of the heritability* ["peripheral genes" or regulatory elements that affect the transcription of the peripheral/core genes] *tend to be spread across most of the genome*". While it is still not clear what the core genes or pathways could be for ADHD, it may be plausible to propose that genes encoding components of the DA, NE and 5-HT pathways may include, at the minimum, some of the core genes associated with ADHD.

As proposed by Ioannidis, the three major problems in genetic studies of complex disorders such as ADHD are: **(1)** false positive results, **(2)** the low *a priori* probability that a given gene is involved in a complex phenotype (low detectance), and **(3)** bias (Ioannidis, 2005). More specifically, Ioannidis has shown that a statistically significant finding is more likely to reflect a true relationship between a gene and a phenotype (positive predictive value or PPV > 50 %) when $(1- \beta)R > \alpha$, where $(1-\beta)$ is the power of the study, R is the *a priori* probability that the tested genetic variant is truly related to the phenotype and α is type I error. This relationship suggests that three major factors may contribute to the poor replicability endemic to genetic studies of psychiatric disorders including ADHD. The first problem is low power $(1-\beta)$ resulting from the small effect size of each gene. Practically, this problem has been solved by increasing sample size, through large multi-national collaborations within the framework of the Psychiatric Genetics Consortium. The second problem is the low *a priori* probability that a given gene/polymorphism is associated with a behavioural phenotype (*R* very small). An attempt to address this issue was made with the candidate gene approach, where prior knowledge of the disorder was used to select "plausible" candidate genes. However, as discussed above, this approach did not yield convincing results.

However, the other important factor that must be considered is type I error (α) (Ioannidis, 2005). Even though one can set very low α values to declare that a result is significant and thus reduce the false positive rate, this statistical criterion can be falsely reassuring if the experimental design does not reduce error in measurement of the risk and outcome variables, and does not reduce bias. Indeed, the higher the error in measurement, the less likely it is that the Odds Ratio (OR) is credible, even if it is relatively high. In contrast, a modest OR (~1.5) may have higher replicability, and may be more reliable, when the risk factor and the outcome variable are measured reliably (e.g. association between age at first breast feeding and risk for breast cancer has been consistently reported with OR ~ 1.5). This underscores one of the fundamental problems in genetic studies of complex psychiatric disorders. While the genetic risk factor can be measured with high accuracy, the outcome variables are plagued by heterogeneity at various levels (clinical heterogeneity of the disorder, differences between assessments by different observers -clinician, parents, and teachers-) and imprecise measurements. Bias can also result in highly significant results even in the absence of a true association. This is particularly true for outcomes like response to medication that can be influenced by important effects, such as response to placebo.

We have attempted to address these methodological issues using a Pharmaco-Behavioural Genetic approach where we:

(1) Increase PPV/detectance by: (a) carefully selecting quantitative phenotypes relevant to ADHD, rather than using the categorical (DSM-IV) diagnosis of ADHD as the outcome measure; and (b) conducting assessments in three different environments (clinic, home and school), by three independent observers (clinician, parents and teachers respectively).

(2) Reduce bias by assessing specific behaviours and response of these behaviours with MPH treatment using a double-blind placebo-controlled design.

(3) Minimize type I error by: (a) using an experimental design where measurement error of outcome variables (e.g. measures of behaviour and cognition), is minimized, and (b) using a family-based design (Figure 1). The two major advantages of this design, over population-based (case/control) association studies, are that it is not affected by population stratification (Nsengimana & Barrett, 2008), and that it may have increased statistical power (Haldar & Ghosh, 2011). Further, because the non-transmitted parental alleles are the control alleles, this method controls for other possible sources of bias, such as socio-economic status.

In our ongoing PBG study, a major underlying hypothesis is that the heterogeneity of ADHD needs to be reduced in order to dissect pathways leading to the disorder. While clinical

heterogeneity is reduced by examining relevant quantitative behavioural and cognitive phenotypes, we attempt to reduce etiological complexity by stratifying children based on exposure to major environmental factors associated with ADHD, while investigating the genetics of this disorder. In our studies, we do not attempt to differentiate between the two measures of G-E interplay: gene-environment interaction (GxE) and gene-environment correlation (rGE) (Rutter, 2010). Rather, the inclusion of the environmental factor is used with the view of reducing heterogeneity, and thereby to increase the sensitivity for finding genes involved in the disorder. This is because a genetic effect may be masked due to interplay with an environmental factor, which could lead to false negative results in an association study.

We have conducted an exploratory analysis with specific tag single nucleotide polymorphisms (SNPs) and functional tandem repeat polymorphisms in selected candidate genes. Candidate SNPs (Supplementary Table) were tested for association with clinical and cognitive dimensions of ADHD, therapeutic response given MPH treatment, and interaction with two environmental factors- maternal smoking and stress during pregnancy.

Methods

Subjects and evaluations

Children (between 6 and 12 years of age) diagnosed with ADHD were recruited from the Disruptive Behavior Disorders Program and the children's outpatient clinics of the Douglas Mental Health University Institute (DMHUI), a psychiatric teaching hospital in Montreal, Canada. They were referred by schools, social workers, family doctors and pediatricians. This study was approved by the Research and Ethics Board of the DMHUI. All participating children verbally agreed to participate in the study, and parents provided written consent. Exclusion criteria were an IQ less than 70, as assessed by the Wechsler Intelligence Scale for Children III/IV (WISC-III or WISC-IV), or a diagnosis of Tourette's syndrome, pervasive developmental disorder, and psychosis (including schizophrenia or bipolar disorder).

Details on the study have been published earlier (Grizenko et al., 2006; Thakur, Sengupta, et al., 2012a), and are described briefly here. During a baseline evaluation:

(a) diagnosis of ADHD and comorbid disorders (according to DSM-IV criteria) is established through a detailed psychiatric evaluation and by using the Diagnostic Interview Schedule for Children-version IV, DISC-IV (structured clinical interview of parents) (Shaffer, Fisher, Lucas, Dulcan, & Schwab-Stone, 2000);

(b) demographic data on the child and the family are collected;

(c) behaviour is assessed by the psychiatrist and clinical research staff (Clinical Global Impression for severity, CGI-severity), parents (using both Conners'-P and the Child Behavioural Checklist, CBCL) (Achenbach, Howell, Quay, & Conners, 1991; Conners & Barkley, 1985), and teachers (Conners'-T);

(d) cognitive function (IQ and executive function domains) are evaluated. Specific RDoC constructs within the cognition domain are evaluated using the following battery of neuropsychological tests: Wisconsin Card Sorting Test (WCST; measure of cognitive flexibility and set-shifting), (Heaton, Chelune, Talley, Kay, & Curtiss, 1993) Tower of London test (TOL; planning, organization, and problem-solving capacity), (Shallice, 1982) Self-Ordered Pointing Task (SOPT; visual working memory, planning and response inhibition), (Petrides & Milner, 1982) Conners' Continuous Performance Test (CPT; attention, response inhibition, and impulse control) (Conners, 1995) and Finger Windows (FW; visual-spatial working memory). (Sheslow & Adams, 1990);

(e) pre-, peri- and postnatal environmental events (including maternal smoking, alcohol use, and stress during pregnancy, and fetal hypoxia events) were assessed using the Kinney Medical and Gynaecological Questionnaire (Kinney, Yurgelun-Todd, Tohen, & Tramer, 1998) and scored using the McNeil-Sjöström scale (McNeil & K, 1995). Since information on environmental factors is collected retrospectively, and may therefore be subject to recall bias, the information provided by the mother is corroborated with medical and obstetrical records as well as an independent interview with a second individual who was present during the pregnancy (husband/maternal grandmother).

Treatment response, given treatment with placebo (PBO) and MPH, was assessed in a double-blind, placebo-controlled, within-subject (crossover) randomized control trial conducted over a two-week period, as described (trial registration number: NCT00483106) (Grizenko et al., 2006). After one week of baseline assessment (which is also a wash-out period), children receive either MPH (0.5mg/kg in a b.i.d. dose) or PBO for one week in a double-blind administration, followed by a second week of the complementary treatment. Response of behaviour (Conners'-P, Conners'-T, Clinical Global Impression [CGI]-overall improvement (Rapoport, 1985), and task-oriented behaviour measured using the Restricted Academic Situation Scale, RASS) is evaluated. In this study, MPH is used as a pharmacological probe to dynamically study the genetics of ADHD, rather than a classical trial of response to medication. In essence, the low dose of MPH was used to establish

a quasi-experimental condition to examine the mediators and moderators of treatment response.

A total of 602 children with ADHD were included in the analysis: 78.9% of subjects were male and 85.2% were of Caucasian ethnicity. The mean age was nine years (SD=1.8); 52.4% met DSM-IV criteria for the combined subtype, while 36.8% and 10.8% were diagnosed with the inattentive and hyperactive subtypes respectively. 42.3% of children in our sample had comorbid oppositional defiant disorder, 20.9% had conduct disorder, 46.2% had an anxiety disorder (including phobias), and 8.8% had a mood disorder. The mean (standard deviation) for the total CBCL, Conners'-P, and Conners'-T scores in our sample were: 68.7 (8.9), 73.3 (11.3), and 69.5 (12.5) respectively (scores higher than 65 are considered to be in the abnormal range).

Genotyping

The affected child, parents and unaffected siblings were invited to participate in the genetic component of the study. For each parent and child, DNA was extracted from a blood sample, buccal swab, or saliva sample, if the subject was only amenable to the latter. The study included 602 nuclear families having one or more children with a DSM-IV diagnosis of ADHD: 160 were complete trios with information from both parents, 195 were trios with two or more affected children, 87 were trios with information from one parent and one or more unaffected sibling, and 160 were duos including the proband and one parent.

The panel of SNPs were genotyped using Sequenom iPlex Gold Technology (Ehrich, Bocker, & van den Boom, 2005). Every plate included duplicates of two reference samples used to estimate genotyping error. Genotypes for these samples were read with 100% accuracy on each of the plates. Each of the SNPs and variable number of tandem repeats (VNTRs) successfully genotyped in the current sample are listed in the Supplementary table. Given the relatively limited sample size, variants were selected based on *a priori* probability that the tested genetic variant are truly related to the phenotype. These were selected on the basis of the following selection criteria: (1) Functional variants within genes encoding key components of the DA, NE, and 5-HT pathways, based on pharmacological evidence that these pathways are involved in ADHD. (2) Functional polymorphisms in key modulators of the stress response pathway (HPA axis), given the evidence that dysregulation of this system (Fairchild, 2010), as well as maternal stress during pregnancy, is associated with ADHD. Selected genes included *NR3C1* and *NR3C2* encoding the glucocorticoid and mineralocorticoid receptors, respectively. (3) SNPs that were reported for their significant association in other studies, with the dual objective of confirming the association in an independent sample, and examining the association with detailed phenotypes. In particular, the SNPs that showed significant association in the International Multi-centre ADHD Gene project (IMAGE) were included (Brookes et al., 2006). (4) SNPs shown to be involved in ADHD based on linkage studies (*ADGRL3*) or meta-analytic review (Gizer et al., 2009); (5) Variants that were significant in GWAS of smoking behaviours, schizophrenia spectrum disorders, bipolar and major mood disorder, obesity traits and metabolic phenotypes, intelligence, and learning disabilities. The reason for choosing these variants was to examine cross-disorder associations (Smoller et al., 2019).

Statistical Analyses

Family-based tests of association (examining transmission disequilibrium of a specific allele/haplotype from parent to affected offspring) were conducted using the FBAT statistical package (version 2.0.3) (Horvath, Xu, & Laird, 2001) (Figure 1). All the analyses were performed under the assumption of an additive model, with a null hypothesis of no linkage and no association.

FBAT was first conducted in the total sample, and subsequently in samples stratified into groups based on prenatal environmental exposure. In the latter analysis, interaction between environmental factors and genotype was examined by conducting FBAT in samples stratified into two groups: 1) first stratification based on maternal smoking during pregnancy (group 1= probands where mother did not smoke during pregnancy; group 2= probands where mother smoked during pregnancy); 2) second stratification based on maternal exposure to stressful life events during pregnancy (group 1= probands where mother experienced no or mild stress during pregnancy; group 2 = probands where mother experienced moderate or severe stress during pregnancy). Significance level was set at P=0.05.

Results

Of the genes tested for association in this exploratory analysis, tag SNPs within the following genes showed the most significant differential association with analysis stratified on either maternal smoking, or stress during pregnancy as described below: *SLC6A2* (encoding the norepinephrine transporter), *ADGRL3* (encoding Adhesion G protein-coupled receptor L3), *ANKK1* (encoding ankyrin repeat and kinase domain-containing 1), and *EGLN2* (encoding the egl-9 family hypoxia inducible factor 2, one of the top "hits" in GWAS of smoking behaviours) (Chen et al., 2011).

Stratification based on maternal smoking during pregnancy:

1. Association with tag SNP rs36021 in SLC6A2

We had reported earlier (Thakur, Sengupta, et al., 2012a), and confirm here in a larger sample, a significant association between tag SNP rs36021 and ADHD, in the group where mothers smoked during pregnancy (Tables 1, 2, 3). A significant association was observed not only with

Table 1. Association of tag SNPs in four genes with ADHD and behavioural traits, stratified based on maternal smoking/stress during pregnancy

Stratification (if any)	Total sample				Maternal smoking during pregnancy								Maternal stress during pregnancy							
Stratification (subgroups)					Yes				No				Yes Stress				No Stress			
Gene	*SLC6A2*	*EGLN2*	*ANKK1*	*ADGRL3*	*SLC6A2*	*EGLN2*	*ANKK1*	*ADGRL3*	*SLC6A2*	*EGLN2*	*ANKK1*	*ADGRL3*	*SLC6A2*	*EGLN2*	*ANKK1*	*ADGRL3*	*SLC6A2*	*EGLN2*	*ANKK1*	*ADGRL3*
Allele	***T***	***T***	***T***	***A***	***T***	***T***	***T***	***A***	***T***	***T***	***T***	***A***	***T***	***T***	***T***	***A***	***T***	***T***	***T***	***A***
Allele frequency	0.6	0.7	0.2	0.6	0.6	0.7	0.2	0.6	0.6	0.7	0.2	0.6	0.6	0.7	0.2	0.6	0.6	0.7	0.2	0.6
No of informative families	241	218	168	249	74	64	46	63	167	151	123	185	92	89	67	99	123	108	86	131
ADHD																				
• Total number DISC ADHD items																				
• Number of DISC inattention items																				
• Number of DISC hyperactivity items																				
• Number of DISC impulsivity items																				
• Number of DISC ODD items																				
• Number of DISC CD items																				
CBCL Total score																				
• CBCL Internalizing behaviour																				
• CBCL Externalizing behaviour																				
- CBCL Withdrawn																				
- CBCL Somatic complaints																				
- CBCL Anxious/depressed																				
- CBCL Social problems																				
- CBCL Thought problems																				
- CBCL Attention problems																				
- CBCL Delinquent behaviour																				
- CBCL Aggressive behaviour																				
Conners' Parents																				
Conners' Teachers																				

P values obtained from FBAT analysis are provided according to a color code, as indicated by the following scale:

	0.01-0.05
	0.001-0.009
	0.0001-0.0009
	≤0.0001

DISC = Diagnostic Interview Schedule for Children, ODD = Oppositional Defiant Disorder, CD = Conduct Disorder; CBCL = Child Behavior Checklist. The specific SNPs given for each gene are as follows: rs36021 in SLC6A2 ; rs3733829 in EGLN2; SNP rs6551665 in ADGRL3 (LPHN3); rs1800497 in ANKK1.

Table 2. Association of tag SNPs in four genes with cognitive traits

Stratification (if any)	Total				Maternal smoking during pregnancy								Maternal stress during pregnancy							
Stratification (subgroups)	Total				Yes				No				Yes Stress				No Stress			
Gene	*SLC6A2*	*EGLN2*	*ANKK1*	*ADGRL3*	*SLC6A2*	*EGLN2*	*ANKK1*	*ADGRL3*	*SLC6A2*	*EGLN2*	*ANKK1*	*ADGRL3*	*SLC6A2*	*EGLN2*	*ANKK1*	*ADGRL3*	*SLC6A2*	*EGLN2*	*ANKK1*	*ADGRL3*
Allele	*T*	*T*	*T*	*A*	*T*	*T*	*T*	*A*	*T*	*T*	*T*	*A*	*T*	*T*	*T*	*A*	*T*	*T*	*T*	*A*
WISC IQ																				
• WISC Verbal IQ																				
• WISC Performance IQ																				
WCST																				
• WCST Total errors																				
• WCST Perseverative responses																				
• WCST Perseverative errors																				
• WCST Non-perseverative errors																				
TOL																				
SOPT Total score																				
CPT																				
• Omission errors																				
• Commission errors																				
• Hit Reaction Time (RT)																				
• Hit RT standard error																				
• Variability of standard error																				
• Detectability																				
• Perseveration																				
• Hit reaction time block change																				
• Hit SE block change																				
• Hit RT ISI change																				
• Hit SE ISI change																				

Under-transmitted allele

Standard scores were used for all WCST and TOL measures, and T-scores were used for CPT measures. WISC = Wechsler Intelligence Scale, WCST = Wisconsin Card Sorting Test, TOL = Tower of London, SOPT = Self-Ordered Pointing Task, CPT = Continuous Performance Test, SE = standard error, RT = reaction time, ISI = inter-stimulus interval. The specific SNPs given for each gene are as follows: rs36021 in SLC6A2 ; rs3733829 in EGLN2; SNP rs6551665 in ADGRL3 (LPHN3); rs1800497 in ANKK1.

Table 3. Association of tag SNPs in four genes with measures of treatment response

Stratification (if any)	Total				Maternal smoking during pregnancy								Maternal stress during pregnancy							
Stratification (subgroups)					Yes				No				Yes Stress				No Stress			
Gene	*SLC6A2*	*EGLN2*	*ANKK1*	*ADGRL3*	*SLC6A2*	*EGLN2*	*ANKK1*	*ADGRL3*	*SLC6A2*	*EGLN2*	*ANKK1*	*ADGRL3*	*SLC6A2*	*EGLN2*	*ANKK1*	*ADGRL3*	*SLC6A2*	*EGLN2*	*ANKK*	*ADGRL3*
Allele	T	T	T	A	T	T	T	A	T	T	T	A	T	T	T	A	T	T	T	A
CGI - improvement PBO																				
CGI - improvement MPH																				
Conners'-P (PBO-MPH)																				
Conners'-P restless-impulsive(PBO-MPH)																				
Conners'-P emtional lability (PBO-MPH)																				
Conners'-T (PBO-MPH)																				
Conners'-T restless-impulsive (PBO-MPH)																				
Conners'-T emtional lability (PBO-MPH)																				
RASS total diff. score (PBO time2- MPH time2)																				
RASS fidgeting difference score																				
RASS vocalization difference score																				
RASS plays with objects difference score																				
RASS off seat difference score																				
RASS off task difference score																				

Conners'-P = Conners' Parents, Conners'-T = Conners' Teachers, PBO = placebo, MPH = Methylphenidate, RASS = Restricted Academic Situation Scale. P values obtained from the FBAT analysis are provided according to a color code as indicated by the scale appended after Table 1. The specific SNPs given for each gene are as follows: rs36021 in SLC6A2 ; rs3733829 in EGLN2; SNP rs6551665 in ADGRL3 (LPHN3); rs1800497 in ANKK1.

categorical DSM-IV diagnosis (i.e. the "*T"* allele was over-transmitted to the trait "ADHD", Table 1 and Figure 1) but also with several quantitative dimensions of ADHD, including: symptom measures (number of items on the DISC-IV, CBCL total and factor scores, and Conners'-P/T scores), measures of cognitive function (Table 2), and treatment response (based on the CGI-overall improvement, Conners'-P/T, as well as evaluation in the simulated academic environment-RASS; Table 3).

In this group of children with ADHD, the risk *T* allele was associated with greater severity (higher symptom scores), but also greater improvement of behavioural dimensions with MPH treatment. In terms of cognitive function, it appears that this group of children have specific deficits in sustained attention, based on the number of DISC inattention items & CBCL attention problems, and corroborated with specific CPT measures. High T-scores on Reaction Time (RT) standard error (SE) and variability of SE, as measured by the CPT, are an indication of inattention. It appears that this group of children also show deficits in vigilance (as the CPT progresses, high T-scores on hit RT block change and hit SE block change), spatial working memory (based on the SOPT), as well as updating/monitoring working memory (association with non-perseverative errors on WCST).

Interestingly, in the group of children with ADHD where mothers did not smoke during pregnancy, the results were in stark contrast. Here, a complete lack of association with rs36021 and was noted.

2. Association with tag SNP rs3733829 in EGLN2

In the group of children with ADHD where the mothers did not smoke during pregnancy, a significant association was observed between the tag SNP rs3733829 and DSM-IV diagnosis of ADHD, as well as multiple ADHD dimensions. The *T* allele was over-transmitted to children with higher symptom scores on the DISC-IV (number of ADHD/ inattention/ hyperactivity/ impulsivity/comorbid ODD and CD items), CBCL (particularly externalizing behaviour), and Conners'-P/T evaluations (Table 1). It was also associated with lower verbal IQ scores and worse performance on measures of cognitive function: SOPT and CPT (higher commission errors and deficits in reaction time variability) (Table 2). An association was also noted with treatment response (based on the Clinical Global Impression-Improvement on Medication week, Conners'-T, and specifically the dimension of fidgeting on the RASS evaluation) (Table 3). It may be noted that while an association with each of these dimensions was observed in the total sample, the statistical significance attributed to the association was higher in the non-smoking group (lack of association observed in the smoking group), and to a limited extent in the high-stress group.

Stratification based on maternal stress during pregnancy:

1. Association with tag SNP rs6551665 in ADGRL3 (LPHN3)

In the group where mothers experienced minimal stress during pregnancy, we observed a significant association with tag SNPs in *ADGRL3 (LPHN3),* with quantitative measures of behaviour (number of items on DISC, CBCL, and Conners'-P) (Table 1). As reported earlier by our group in a smaller sample (Choudhry et al., 2012), no association was observed with dimensions of cognition or treatment response, nor were any associations observed in the sample where mothers experienced moderate or extreme stress during pregnancy.

2. Association with tag SNP rs1800497 in ANKK1

With the tag SNP in *ANKK1*, a limited association was observed in the group where mothers experienced moderate or extreme stress during pregnancy (Tables 1, 2, 3). Here, a significant association was observed with symptom dimensions (particularly number of impulsivity and ODD items on the DISC) in the group where mothers experienced moderate to severe stress during pregnancy. An association was noted with improvement with MPH treatment, based on assessment by teachers (Table 3). No association was observed with cognitive dimensions (Table 2).

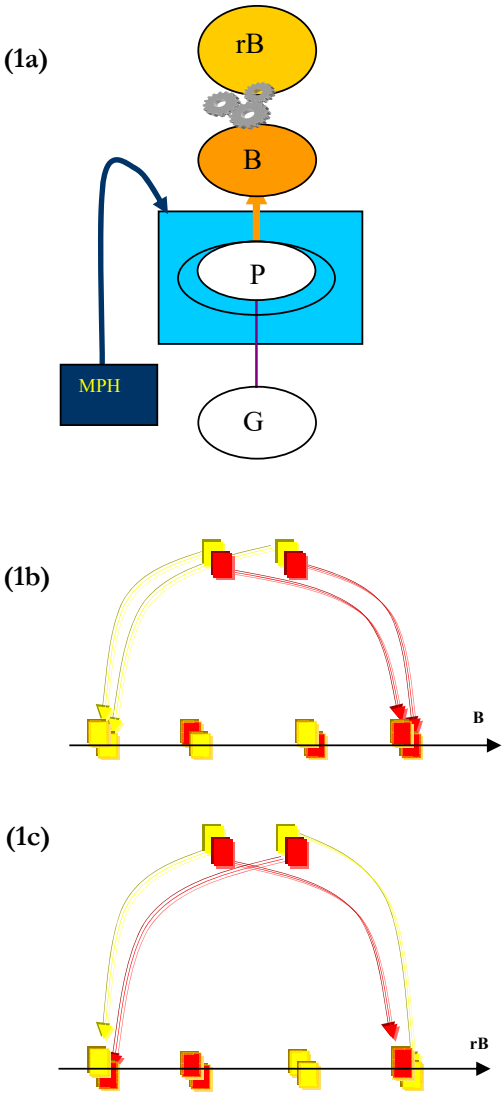
Discussion

These results: 1) provide initial insight that genetic and environmental factors combine together in distinct pathways to contribute to the etiology of the disorder, as well as the modulation of treatment response; 2) suggest that a complex network resulting from G-E interplay is involved in the distinct dimensions of ADHD. These results underscore the importance of breaking down the disorder into "component" endophenotypes, as repeatedly suggested in the extant literature (Bidwell, Willcutt, Defries, & Pennington, 2007; Castellanos & Tannock, 2002; Crosbie, Perusse, Barr, & Schachar, 2008; Doyle, Faraone, et al., 2005; Doyle, Willcutt, et al., 2005), and as promoted by the RDoC framework (Cuthbert & Insel, 2013).

Using the Pharmaco-Behavioural Genetic approach, we illustrate that it is possible to perform joint analysis of the association of a genetic locus (G) with specific behaviours (B), and the response of these behaviours to MPH treatment (rB). The main innovation of this approach is that dynamic change in behaviors, following a pharmacological challenge, is investigated in relation to a candidate gene. More specifically, four mutually exclusive hypotheses are possible when the role of G is investigated with regard to B and rB (and illustrated with the results described here):

- H_0: G is not associated either with B or rB;
- H_1: G modulates B but not rB (Figure 1);

Figure 1. Gene (G) modulates behaviour (B) but not the response of B to MPH (rB) (H1 in the manuscript)



In this illustration, G has two alleles- *G* (red) and *g* (yellow). P represents the protein encoded by G. The blue rectangle represents the brain.

Panel 1a is a depiction that G and its protein product P modulates B but does not affect rB.

Panels 1b and 1c represent family-based analysis, specifically the quantitative Transmission Disequilibrium Test (qTDT). This method tests for an excess of transmission of the risk allele (red) from heterozygous parents to affected subjects. The family-based association test (FBAT) used in our study is a generalisation of the TDT. When the test is positive, it indicates the presence of both allelic association and linkage.

Panel 1b represents over transmission of allele *G* from parents to subjects with high levels of behaviour B.

Panel 1c depicts random transmission of *G* and *g* alleles from parents to affected children in relation to drug response rB. In this illustration therefore, there is no relation between the transmission of a specific allele and rB.

- H_2: G modulates rB but not B (Figure 2A);
- H_3: G modulates both rB and B (2B).

The association with tag SNPs in *ADGRL3 (LPHN3)* in the group where mothers experienced minimal or mild stress is an illustration of H_1, where an association is observed with measures of behaviour, but not the response of behaviour to treatment with MPH. On the other hand, the associations noted with *SLC6A2*, *EGLN2* and *ANKK1* illustrate H_3, where an association is observed both with behaviour and response of behaviour to treatment.

These results also underscore the importance of minimizing the heterogeneity of the sample in order to better dissect the etiological pathways involved. One of the major problems with GWAS conducted with ADHD, is that they have "*adopted a unitary view of the disorder, comparing large heterogeneous samples of individuals with ADHD to control samples. Although this approach is pragmatic...it undoubtedly introduces noise and places an upper limit on the gene discovery*" (Hawi et al., 2015). While the latest ADHD GWAS identified 12 loci that passed the threshold for statistical significance, in fact they explain only *"a tiny fraction of common variant risk for ADHD*" (Demontis et al., 2019). We suggest that the stratification based on environmental exposure or other important factors may be necessary to shed light on what is commonly referred to as "*missing heritability*", i.e. the disorder has a strong genetic component,

Figure 2A. Gene (G) modulates response of behaviour to MPH (rB) but not the behaviour itself (B) (H2 in the manuscript).
Depiction that G and its protein product P modulates rB, but does not affect B. This modulation could be due to pharmaco-kinetic factors, that regulate drug bio-availability, or pharmaco-dynamic factors, that modulate the affinity of the receptor to the drug (illustrated by the blue crescent surrounding P).

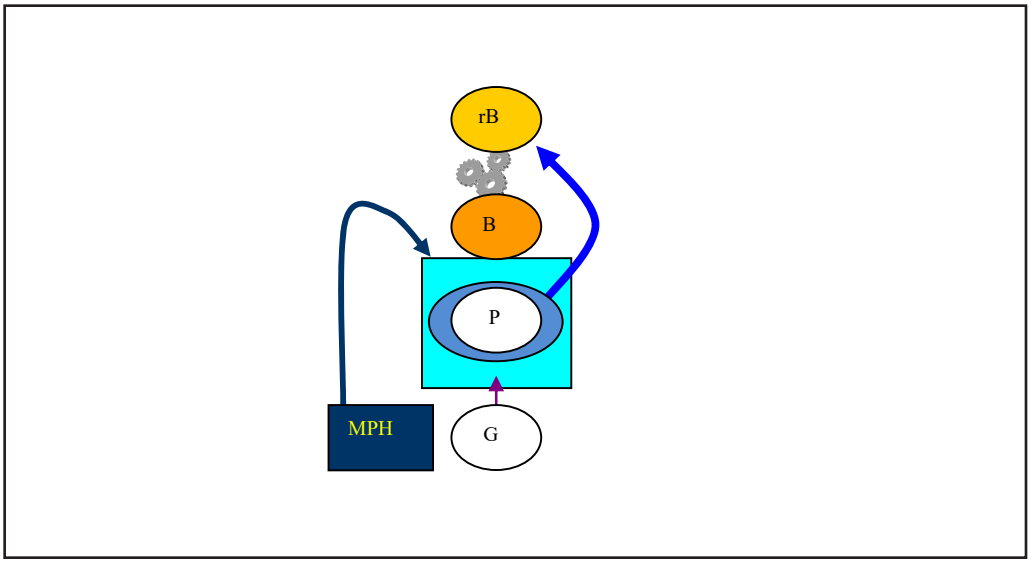
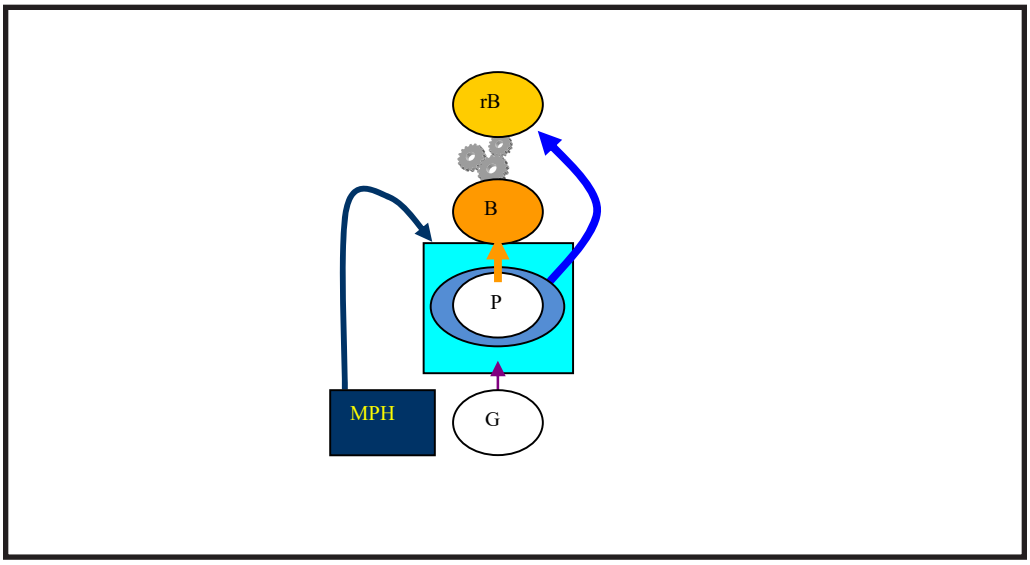


Figure 2B. Gene (G) modulates behaviour (B) and response of B to MPH (rB) (H3 in the manuscript)



but the genetic factors identified to date explain only a small portion of the variance (Eichler et al., 2010).

While these results shed some light on the complex interplay between genetic and environmental factors in the etiology of ADHD, much work is needed to disentangle these pathways. For example, the association between the variant in *SLC6A2,* which encodes the norepinephrine transporter, is intuitively interesting given the clinical efficacy of pharmacological agents that block the norepinephrine transporter, NET (both psychostimulants MPH and amphetamine, as well as atomoxetine, which is specific for NET). However it is not clear if the observed interplay with maternal smoking during pregnancy is a 'true' interaction, i.e. exposure to the toxic effects of *in utero* exposure to nicotine in those children carrying a risk allele in the gene encoding the NET, results in ADHD behaviours. An alternative possibility is that the etiology of smoking behaviour and ADHD may involve closely related pathways.

While the major limitation is the exploratory nature of the study, with no correction for multiple comparisons, the advantages are: (1) the deep phenotyping, (2) parallel investigation of environmental factors, (3) the relatively large sample examining treatment response in an unbiased, quantitative manner, and (4) analysis based on family-based association tests which are robust to population stratification. Nevertheless, replication in an independent sample is awaited before definitive conclusions can be reached.

Acknowledgements / Conflicts of Interest

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