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General introduction
Chapter 1

Introduction

Our unique set of experiences throughout life shape us into who we are, but there are also inherent differences between people in their response to similar experiences. Major advances in molecular genetics in the past decades have enabled biomedical researchers to investigate how genetic variation moderates the effects of the environment on behaviour and the brain. Information on such gene-environment interactions (GxE) provides clues to the biological pathways involved in a disease and may aid in its prevention or treatment. In this thesis I describe my doctoral research on the role of specific genes in the relation between environmental adversity and attention-deficit/hyperactivity disorder (ADHD), and how these GxEs influence severity of ADHD symptoms and related behaviours as well as brain structure and function. This first chapter contains a brief overview of the characteristics of ADHD, findings on its neurobiology, genetic and environmental risk factors, and the rationale behind the GxE approach. This overview is followed by an outline of the contents of the thesis.

Attention-deficit/hyperactivity disorder

ADHD is a neurodevelopmental disorder characterized by age-inappropriate levels of inattention, hyperactivity, and impulsivity. According to the Diagnostic and Statistical Manual for Mental Disorders, Fifth Edition (DSM-5), a diagnosis of ADHD requires that its symptoms interfere with daily functioning, that these are present in multiple settings, and an onset before age twelve (American Psychiatric Association, 2013). It is a prevalent disorder, with 5-7% of school-aged children meeting these criteria worldwide (Polanczyk et al., 2007). ADHD has a large economic impact due to treatment costs and missed income (Le et al., 2014), and is often negatively associated with educational attainment (Loe & Feldman, 2007) and quality of social relations (Wehmeier et al., 2010).

Individuals with ADHD vary widely in clinical profile and course of the disorder. The DSM-5 recognizes three presentations: predominantly inattentive, predominantly hyperactive/impulsive, and combined presentation, the last one being the most common. An additional distinction is being made between mild, moderate, and severe ADHD, based on the number of symptoms and impairment (American Psychiatric, 2013). Individuals may further differ in persistence of ADHD-related deficits; many still meet the criteria for a full diagnosis in adulthood, others show partial remission but continue to be functionally impaired, and a minority show full remission (Biederman et al., 2011).

Further complicating the clinical picture is the fact that the majority of individuals with ADHD also fulfil the diagnostic criteria of one or more other disorders (Jensen et al., 1997). ADHD often co-occurs with externalizing disorders such as oppositional deviant disorder (ODD) and conduct disorder (CD), as well as internalizing disorders such as major depressive disorder (MDD) and anxiety disorders. The presence of co-occurring disorders is correlated with ADHD severity and course, and has important implications for choice of treatment and quality of life (Jensen et al., 2001).
ADHD is associated with multiple cognitive and emotional deficits (Faraone et al., 2015). Many individuals with ADHD have poor working memory performance (Martinussen et al., 2005), an impaired ability to withhold inappropriate responses, known as response inhibition (Lipszyc & Schachar, 2010), as well as deficits in vigilance and planning (Willcutt et al., 2005). These are components of executive functioning, which facilitates goal-directed behaviour and has long been considered to be central to the disorder (Barkley, 1997). Individuals with ADHD also frequently show deficits in reward-related processes, choosing small immediate rewards over larger delayed ones (Sonuga-Barke & Fairchild, 2012). They may also suffer from impaired timing, motor control, and motivation (Coghill et al., 2014, Rhodes et al., 2005). Lastly, it is increasingly recognized that emotion dysregulation is an important component of ADHD (Shaw et al., 2014), and a strong determinant of quality of life (Barkley & Fischer, 2010). None of these deficits are specific to the disorder, nor are they present in all individuals with ADHD (Sjöwall et al., 2013, Sonuga-Barke et al., 2008).

**Neuroimaging**

Neuroimaging studies have reported a range of differences in brain structure and function between groups of individuals with ADHD and healthy comparison subjects. These studies have reported grey matter volume reductions in parts of the frontal cortex and the striatum, as well as many additional structural differences throughout the brain, including regions in the cerebellum, and the temporal and parietal lobe (Nakao et al., 2011, Valera et al., 2007). Longitudinal studies have indicated that ADHD is associated with delayed brain development, and that remittance is coupled to normalization of brain structure (Shaw et al., 2007). Task-based fMRI studies have further established widespread differences in brain activity; those with ADHD, as a group, generally show lower activation of frontal and parietal regions in networks underlying executive function and attention, and higher activity of brain regions in task-irrelevant networks (Cortese et al., 2012). This is in line with findings from network connectivity studies suggesting that ADHD relates to a failure to deactivate the default mode network when required, which may cause irrelevant thoughts to interfere with the task at hand (Sonuga-Barke & Castellanos, 2007). Combined, the neuroimaging literature indicates that ADHD in both children and adults involves lowered communication of the frontal cortex with striatal, parietal, temporal, cerebellar, and limbic brain regions (Cubillo et al., 2012). The association with multiple neural networks mirrors the range of impairments observed in individuals with ADHD (Castellanos & Proal, 2012). This widespread pattern may further reflect averaging over individuals who vary in clinical presentation, and the contribution of many different genetic and environmental factors to ADHD.
BACKGROUND 1: MEASURES OF BRAIN STRUCTURE AND FUNCTION

Neuroimaging has provided a tremendous boost to neuroscience, by enabling a non-invasive study of the brain in health and disease. In the upcoming chapters, we describe the research we conducted utilizing measures of brain structure, activity, and functional network connectivity. Structural magnetic resonance imaging (sMRI) scans are used predominantly to study aspects of brain grey matter, containing neuronal cell bodies and synapses, and white matter, consisting mostly of the myelinated axons that connect brain areas. Functional MRI (fMRI) takes advantage of changes in the magnetic properties of blood passing through the brain as an indicator of the relative activity of a region over time. The blood-oxygen level dependent (BOLD) signal is usually recorded while subjects perform a cognitive task, and then compared to a baseline recording to isolate the task-associated activity. FMRI data may also be used to study brain functional connectivity by calculating the coherence of activation patterns over time between regions. This may be done with task-based fMRI data, as well as with recordings while individuals are not engaged in any specific task, known as resting-state fMRI (rs-fMRI). Studies into functional connectivity have identified several brain networks, collections of regions that are consistently co-activated (Fox et al., 2005). The activation of these networks depends on the subjects’ current state of mind, with for instance activity in the executive function network being most prominent when performing a working memory task, and the default mode network becoming more active while mind wandering during resting conditions (Raichle et al., 2001).
Risk factors

The large heterogeneity in clinical presentation observed in ADHD relates to its complex, multifactorial aetiology. While there are some reports of rare genetic variants strongly predicting ADHD (Elia et al., 2010), the disorder is thought to result in most cases from the combined effect of a large set of common genetic and environmental factors with small individual effects (Banaschewski et al., 2010, Faraone et al., 2015). This dovetails with the continuous distribution of ADHD-related traits in the general population, whereby individuals diagnosed with ADHD are at the extreme end (Larsson et al., 2012). In other words, we all carry some genetic variants and are exposed to some degree to environmental factors that contribute to ADHD. The unique combination of our genetic make-up and exposure to our environment determines how our brain develops and, consequently, which specific traits or deficits we have.

Genetics

ADHD has a strong genetic component, with an estimated heritability above 70% (Faraone et al., 2005, Nicolas & Burt, 2010). Research into the genetics of ADHD has been undertaken through both hypothesis-free whole-genome investigations and hypothesis-driven candidate gene analysis. Genome-wide linkage scans, recording how transmission of small parts of the genome from parent to child relates to the inheritance of ADHD, and genome-wide association studies (GWAS), simultaneously testing large numbers of single nucleotide polymorphisms (SNPs) spread out over the genome, have produced few results so far (Neale et al., 2010, Zhou et al., 2008). Research into the role of copy number variation (CNV) throughout the genome does suggest that rare variants are involved that influence the same neurodevelopmental pathways implicated in candidate gene studies (Elia et al., 2010, Poelmans et al., 2011). In general, the pattern of findings supports the idea that, in the majority of cases, many polymorphisms with small effects contribute to ADHD, and that different genetic factors may cause the same disorder. Large-scale sequencing studies and collaborations within the context of the Psychiatric GWAS Consortium are now underway, providing the sample sizes that are necessary to discover small, or strong but rare, effects across the genome (Franke et al., 2009).

Candidate gene studies of ADHD have mostly focused on genetic variation thought to influence dopaminergic neurotransmission (Swanson et al., 2007), motivated by its central role in the cognitive functions that are impaired in ADHD and the behaviour of animal models with altered dopamine signalling (Van der Kooij & Glennon, 2007). In addition, the ADHD stimulant medication methylphenidate inhibits the dopamine transporter, slowing down removal of dopamine from the synaptic cleft in the striatum (Volkow et al., 2002). Therefore, genetic variation in the dopamine transporter (DAT1) has been a primary target of interest for ADHD genetics studies. DAT1 contains a variable number tandem repeat (VNTR) polymorphism that exists predominantly out of 9 or 10 repeats, with the 10 repeat variant being associated with higher levels of dopamine transporter (Faraone et al., 2014). Findings on its relation with ADHD have been inconsistent, with a meta-
analysis reporting significant heterogeneity between studies and a small increase in ADHD risk for 10 repeat carriers (Gizer et al., 2009). Some reports have indicated that a haplotype of this VNTR with another in intron 8 may link stronger to ADHD (Asherson et al., 2007), and that the effect of DAT1 may differ between childhood and adult ADHD (Franke et al., 2010). The dopamine receptor D4 (DRD4), mainly expressed in the frontal lobe, has also been extensively investigated. It contains a VNTR in exon 3 of which the 7 repeat (7R) variant is thought to increase the risk for ADHD (Gizer et al., 2009), possibly by producing a less responsive receptor (Asghari et al., 1995). Interestingly, the 7R allele may be protective against some cognitive deficits while conveying more behavioural problems (Swanson et al., 2000), illustrating how carrying a specific risk factor may explain some of the clinical differences between individuals with ADHD.

Genetic variation related to other neurotransmitter systems has also been implicated in ADHD, in particular genes involved in regulating the monoamines noradrenaline and serotonin. These neurotransmitters are synthesized and degraded by some of the same enzymes that regulate dopamine, and they have been found to contribute to the effects of stimulant medication (Gainetdinov et al., 1999, Prince, 2008). Studies of polymorphisms in genes encoding for noradrenaline’s transporter (SLC6A2), receptors (e.g., ADRA2A), and the degrading enzyme it shares with dopamine (COMT), have on average found no support of a relation with ADHD (Gizer et al., 2009). In the serotonin neurotransmitter system, the serotonin transporter gene (SLC6A4 or 5-HTT) has shown the strongest link with ADHD. This gene contains a polymorphism in its promoter region, 5-HTTLPR, yielding a short (S-allele) and long (L-allele) variant. The S-allele is associated with lower expression, leading to less serotonin transporter available to remove serotonin from the synaptic cleft (Heils et al., 1996). While the S-allele is associated with anxiety-related traits and depression (Clarke et al., 2010, Lesch et al., 1996), the L-allele has been found to increase risk for ADHD, although with significant heterogeneity of findings (Gizer et al., 2009).

Environmental risk factors

Environmental risk factors for ADHD include prenatal exposure to harmful substances such as alcohol and tobacco, pregnancy complications, exposure to toxins such as lead, and long-term psychosocial stress (Banerjee et al., 2007). Prenatal substance exposure affects brain development during a crucial stage, leading to a range of behaviours characteristic of ADHD, including hyperactive and impulsive behaviour, and overall executive dysfunction (Huizink & Mulder, 2006). Prolonged exposure to stress, such as physical and emotional maltreatment, has also been associated with inattentive and hyperactive behaviour (Biederman et al., 2002).

The type of environmental risk factor and the developmental stage at which exposure takes place is important for the clinical profile and persistence of ADHD. There is evidence of developmentally sensitive periods, whereby exposure to a stressor will disrupt the formation of specific neuronal circuits being formed at that time much more than at any other time (Knudsen, 2004), which may contribute to the specificity of a stressor with a certain behavioural profile. For instance, the
Romanian adoptee studies have shown that early deprivation is associated with a predominantly inattentive and persistent variant of ADHD (Kennedy et al., 2016).
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Gene-environment interactions

The effect of an environmental factor will depend on how an individual perceives and processes it, which in turn may be influenced by the individual's genetic make-up. When a genetic polymorphism moderates the effect of, or response to, an environmental factor, this is known as a gene-environment interaction, or GxE. Not accounting for the presence of such interaction effects is likely to contribute to the heterogeneity of findings seen in the search for ADHD risk factors (Buitelaar, 2005); if the response to a stressor depends on whether or not someone has a specific genetic variant, analysing the effect of either factor by itself will produce more variable results because of the ignored influence of the other factor. Through GxE we can get a more accurate estimate of the importance of both the genetic and environmental risk factors involved, and identify those individuals most susceptible to stressors (Moffitt et al., 2005).

Since the first publication describing a gene influencing the association between childhood maltreatment and adult antisocial behaviour (Caspi et al., 2002), numerous other reports of GxEs playing a role in psychiatric disorders have followed. The interplay between genes and environmental factors is also thought to play a pivotal role in ADHD (Thapar et al., 2012). Brain development is under strong genetic control, but also highly responsive to environmental stimuli, facilitating learning and adaptation to the environment (Anderson & Finlay, 2013). As ADHD is characterized by altered brain development and highly heritable, the lack of consistent findings may indicate that the influence of environmental stressors on this process has yet not been sufficiently taken into account.

Some of the initial GxE studies in ADHD looked into possible interactions of DAT1 and DRD4 with prenatal exposure to alcohol or smoking. These found evidence that carrying one or both of the genetic risk variants strengthened the relation of substance exposure in utero with ADHD (Brookes et al., 2006, Kahn et al., 2003, Neuman et al., 2007), but were followed by several replication failures (Altink et al., 2008, Langley et al., 2008). There has been somewhat stronger evidence in favour of genetic moderators of the effects of chronic stress (Nigg et al., 2010). The most investigated GxE within psychiatry is the often-replicated interaction between 5-HTTLPR and psychosocial stress, with many reports indicating that individuals carrying the S-allele are more sensitive to the negative effects of long-term stress exposure, with outcome measures ranging from depression to substance abuse (Caspi et al., 2010). This same pattern has also been reported for ADHD; while the L-allele is considered a risk factor for ADHD, S-allele carriers show a stronger relation with ADHD for those exposed to stress (Muller et al., 2008, Retz et al., 2008).

The neurobiology of gene-environment interactions

GxE takes place when the effect of an environmental factor on a biological system is amplified or dampened by a specific genetic polymorphism, and vice versa (Caspi & Moffitt, 2006). Neuroimaging may be used to enhance GxE research by identifying the neural mechanisms mediating interaction effects on behaviour. Measures of brain structure and function may better capture the influence of risk factors, because
they are more directly related to the underlying biological effect than a behavioural construct as complex as a psychiatric disorder (Meyer-Lindenberg & Weinberger, 2006). Related to this, by indicating which neural pathways are involved, neuroimaging measures provide more information on the potential of genetic and environmental factors to influence specific aspects of behaviour. This may provide clues on how and why individuals with the same disorder differ, which is beneficial for targeted study into the cause and treatment of distinct presentations of ADHD.

Dopaminergic neurons appear particularly sensitive to the neurotoxic effects of prenatal exposure to smoking or alcohol (Huizink & Mulder, 2006). Such exposure is also associated with less activity in the dopamine-rich basal ganglia and frontal cortex (Derauf et al., 2009), where DAT1 and DRD4 are predominantly expressed (Ciliax et al., 1999, Gilsbach et al., 2012). Given that prenatal exposure to smoking or alcohol, as well as variation in both these genes, has been linked to hypoactive dopaminergic neurotransmission, they may strengthen each other’s effect and potentially produce GxE effects. The specific brain regions in combination with the neurocognitive profile of children prenatally exposed to either smoking or alcohol (Huizink & Mulder, 2006) suggests these genetic and environmental factors may be particularly relevant for hyperactive-impulsive behaviour (Becker et al., 2008).

Long-term exposure to psychosocial stress is associated with a range of structural and functional brain abnormalities in frontal and limbic regions contributing to emotion processing, explaining its relation to multiple psychiatric disorders characterized by emotion dysregulation (Hart & Rubia, 2012). This overlaps considerably with the literature on the effects of serotonin and of 5-HTTLPR in particular (Pergamin-Hight et al., 2012). One mechanism proposed to explain the effects of 5-HTTLPR on emotion is the lower inhibitory control of the anterior cingulate cortex and higher amygdala activity of S-allele carriers in response to a stressor (Munafo et al., 2008, Pezawas et al., 2005), possibly related to lower transcriptional activity of that variant. Animal models and brain tissue from abused suicide victims have indicated that stress exposure influences the degree of 5-HTTLPR methylation, with higher stress levels leading to lower serotonin transporter availability (Meaney, 2010), providing a mechanism how stress and 5-HTTLPR may strengthen each other’s effect.

A stressful stimulus leads to the release of a cascade of glucocorticoids, excitatory amino acids and neurotrophic factors in the human brain through the primary stress response pathway, the hypothalamic pituitary adrenal (HPA) axis (McEwen et al., 2015). This is a highly intricate system with numerous feedback loops, reflecting its pivotal role in survival (Joels & Baram, 2009). Part of the interaction between 5-HTTLPR and stress exposure is thought to result from the regulatory role that 5-HTT plays in HPA axis activity, as S-allele carriers have a higher increase in cortisol, the human stress hormone, when exposed to laboratory stressors (Miller et al., 2013). The glucocorticoid receptor binds with cortisol and then moves to the cell nucleus to regulate expression of a large number of genes (Buckingham, 2006), thereby contributing to long-term adaptation to a stressful environment. Variation in the gene encoding this receptor, NR3C1, has also been recently linked to ADHD-related behaviour, providing evidence for involvement of the HPA axis in the
disorder (Fortier et al., 2013). Findings that polymorphisms in \textit{NR3C1} and 5\textit{-HTTLPR} jointly influence stress reactivity (Taylor et al., 2014) further illustrate how genetic and environmental factors do not operate in isolation, and that interplay among them is potentially a very large source of inconsistent findings from studies of ADHD risk factors.

**BACKGROUND 2: THE NEUROIMAGE PROJECT**

The studies described in this thesis have used data from the NeuroIMAGE project. This was a follow-up of the International Multicentre ADHD GEnetics study, or IMAGE, which took place between 2003 and 2006. IMAGE involved researchers from centres across eight countries collecting behavioural and genetic data from over a thousand children and adolescents with ADHD, as well as one or more of their siblings (Muller et al., 2011). The Dutch participants of this study were invited to take part in NeuroIMAGE, carried out between 2009 and 2012 by research groups from Nijmegen, Amsterdam, and Groningen. Including 77 newly recruited families, the NeuroIMAGE sample consisted of 1069 participants from 484 families (Von Rhein et al., 2014).

The NeuroIMAGE project was designed to investigate the course of ADHD during the scarcely studied period of transition from childhood into young adulthood, its genetic and environmental determinants, and the cognitive and neurobiological underpinnings. To this end, the extensive data collection included gathering information on participant demographics, lifestyle and experiences, cognitive testing, and a clinical interview to assess ADHD symptoms and comorbidity. Genetic data was obtained if not already available. Further, NeuroIMAGE added magnetic resonance imaging (MRI) to the measurement set, providing data on brain structure and activity from its participants. The MRI sessions included high-resolution structural scans, a diffusion-tensor imaging (DTI) scan, and fMRI scans during a response inhibition task, a visuospatial working memory task, a monetary reward task, and during resting-state. This resulted in a rich dataset with a large sample size, particularly for neuroimaging studies. Further information on study design, the sample composition, and details of specific measurements and procedures are described in Von Rhein et al. (2014), the methods sections throughout this thesis, and on www.neuroimage.nl.
General introduction

Thesis outline

In this thesis I describe our research into some of the sources of heterogeneity that are so characteristic of reports on ADHD aetiology and its clinical profile, using data from the NeuroIMAGE project (see Background 2). The following chapters document how we investigated whether genetic variation moderates the relation of pre- and postnatal stressors with ADHD, and the association of GxEs with brain structure and function. The overarching aim of this work was to contribute to a more accurate and nuanced understanding of the neurobiology of ADHD through the analysis of moderation and mediation effects (see Figure 1).

In Chapter 2, we investigated whether we can replicate inconsistent findings that DRD4 and DAT1 moderate the relation of prenatal exposure to smoking or alcohol with ADHD severity. In addition, we analysed how these genetic and environmental factors are associated with brain activity during a response inhibition task.

Chapters 3, 4, and 5 are on another GxE that has been implicated in ADHD and related behaviours, the interaction between 5-HTTLPR and long-term exposure to stress. In Chapter 3, we analysed whether this GxE explains ADHD severity, while controlling for internalizing problems. Chapter 4 describes a follow-up study where we performed a whole-brain mediation analysis searching for grey matter volume explaining the relation between 5-HTTLPR, stress exposure and ADHD severity. This approach was repeated in chapter 5, where we looked at resting-state functional connectivity of the executive control network and the default-mode network.

Chapter 6 is about the glucocorticoid receptor gene, NR3C1, and whether it moderates the relation between stress exposure with ADHD and grey matter volume. This study also included analysis of the role of 5-HTTLPR, given that both genes are thought to influence activity of the HPA axis.

In Chapter 7, we explored an alternative approach to studying the effects of genetic and environmental factors and their interplay. We applied random forest regression, a machine learning technique, to predict ADHD severity from thousands of polymorphisms in genes associated with HPA axis activity in combination with information on stress exposure.

In Chapter 8, I diverge from the topic of the other research chapters and describe how we looked into the co-occurrence of ADHD symptoms with anxiety. We investigated whether the relation between working memory-related brain activity and ADHD severity depends on the amount of co-occurring anxiety, i.e., whether there are non-additive effects signalling this combination is different from simply the simultaneous presence of ADHD and anxiety.

In Chapter 9, I provide a summary of the findings per chapter, and extrapolate from these some general ideas about the nature of the relation between risk factors and ADHD. Finally, in Chapter 10, I couple our results to the literature on ADHD, discuss their implications, and suggest future research.
Figure 1. Moderation (top) and mediation (bottom). Moderators influence the relation between a predictor and outcome, such as a gene moderating the relation between a stressor and behaviour. Mediators, on the other hand, facilitate the relation between a predictor and outcome, such as the activity of a brain region linking the effect of a gene to behaviour.
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