Universität Trier

The effects of catechol-*O*-methyltransferase (*COMT*) and psychosocial risk factors on symptom severity and co-morbid Conduct Disorder in Attention-Deficit/Hyperactivity Disorder

Dissertation zur
Erlangung des Doktorgrades der Naturwissenschaften
(Dr. rer. nat.)

aus dem Fachbereich I - Psychobiologie

vorgelegt von

Haukur Örvar Pálmason

Betreuer:
Prof. Dr. Jobst Meyer
Prof. Dr. Reinhold Läßle

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Dedicated to my family

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Without a theory, the facts are silence

The sensory order

F. A. Hayek (1899-1992)

Summary

Attention-Deficit/Hyperactivity disorder (ADHD) is a common neuropsychiatric disorder characterized by inattention, motor activity and impulsiveness. Findings from genetic studies indicate that the heritability in ADHD is around 70 - 80%. The catechol-*O*-methyltransferase (*COMT*) gene plays a crucial role in the metabolism of catecholamines in the frontal cortex, which has been implicated in ADHD and Conduct Disorder (CD). It is localized in the chromosomal region 22q11.2, coding for two enzymes, soluble (S-), and membrane bound (MB-) *COMT*. A single nucleotide polymorphism (Val^{158}Met SNP) encodes the amino acids methionine (Met) or valine (Val). Carrying the Met/Met-genotype leads to a 3- to 4fold reduction of *COMT* activity compared to the Val/Val-genotype.

The aim of the present study is to assess if the *COMT* Val^{158}Met SNP is a risk factor for ADHD, ADHD symptom severity and co-morbid conduct disorder.

The main results of the study are that the *COMT* Val^{158}Met SNP is associated with ADHD, with the Met allele being over-transmitted in our sample. Secondly, that smoking during pregnancy had a significant influence on ADHD symptom severity and those with the *COMT* Met/Met genotype had the most severe ADHD symptoms in our sample. Finally, ADHD symptom severity and adverse early family circumstances during the first three years of life are positive predictors of lifetime CD in our sample.

These findings support previous results implicating *COMT* genotype in ADHD symptom severity and adverse early psychosocial surroundings as risk factors for co-morbid CD. These results reiterate the need for early intervention to prevent aggressive and maladaptive behaviour progressing into CD, reducing the overall severity of the disease burden in children with ADHD.

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Abbreviations

5-HT	Serotonin
ADHD	Attention Deficit Hyperactivity Disorder
APA	American Psychiatric Association
ANOVA	Analysis of Variance
bp	Base pair
cAMP	Cyclic adenosine monophosphate
CD	Conduct Disorder
COMT	Catechol-*O*-methyltransferase
DA	Dopamine
df	Degrees of freedom
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic acid triphosphate
DSM-IV	Diagnostic and Statistical Manual of Mental Disorder 4th edition
Gi	Guanine inhibitory
Gs	Guanine stimulation
ICD-10	International Classification of Diseases 10th edition
kb	Kilobase
LD	Linkage Disequilibrium
M	Mol
MBD	Minimal Brain Damage
mM	Millimol
nAChR	Nicotinic acetylcholine receptors
NE	Noradrenaline
ng	Nanogram
ODD	Oppositional Defiant Disorder
PCR	Polymerase chain reaction
PFC	Prefrontal cortex
pmol	Picomol
SLC6A2	Norepinephrine transporter
SLC6A3	Dopamine transporter
SLC6A4	Serotonin transporter
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for Social Sciences
TDT	Transmission Disequilibrium Test
UTR	Untranslated region
WHO	World Health Organization
µg	Microgram
µl	Microlitre

1. General Introduction

1.1 Historical introduction

Attention Deficit Hyperactivity Disorder (ADHD) is the latest diagnostic label for children with developmentally inappropriate levels of inattention, impulsivity and/or hyperactivity leading to impairments of function in everyday life (Barkley, 1998). ADHD is often thought of as a "modern" phenomenon, when in fact it has probably followed mankind from the beginning of time.

Though one can find a parallel between the behaviour of Dr. Heinrich Hoffmann's characters in "The Struwwelpeter Stories" from 1844 and the core symptoms of ADHD as defined today, however it was not, until the twentieth century, that people started to focus on this behavioral condition from a scientific point of view that the history of ADHD began to evolve (Culbertson & Krull, 1996).

In 1902, George Still published a series of lectures where he described a group of children with behavioural problems. According to Still, these children had severe problems with sustained attention, they were overactive and impulsive, defiant, resistant to discipline, excessively emotional, often aggressive and showing little inhibitory control. Still noted that in most cases, the disorder arose before eight years of age and males outnumbered females by 3:1 in his sample. Furthermore, Still noticed that among the biological relatives of these children depression, suicide, alcoholism and criminality were common. He went on to suggest that this behavioural condition was probably hereditary, but in some cases, it was the result of pre- or postnatal events (Barkley, 1998; Levy, 2001).

Since Still's publication, the nature and aetiology of ADHD has been debated among researchers. To date, many theories have been formulated in attempts to explain the nature and causes of ADHD. These theories and their effects on ADHD as a clinical disorder can be divided into periods, each influenced by the zeitgeist at the time they were formed.

1.1.1 ADHD from 1900 to the 1950s

The first period was roughly from 1900 to 1950. Here, the driving force was the connection between the behavioural symptoms of inattention, poor impulse control, hyperactivity and brain damage. In the beginning of the twentieth century, these symptoms were often mentioned in the medical literature as sequelae to encephalitis, various central nervous systems infections or head injuries (Culbertson & Krull, 1996). Following the worldwide outbreak of encephalitis (von Economo´s encephalitis) in 1917 and 1918, physicians noticed that among the children that had survived a number of them had behavioural and cognitive sequale resembling those seen in frontal lobe ablation studies in monkeys (Levy, 2001). One of the core deficits resulting from prefrontal lesions is a breakdown in the modulation of impulsive responding and regulation of goal-directed behaviour, manifested in deficits in attention, impulse control, increased restlessness and motor activity (Cohen, 1993; Fuster, 1997; Parker & Crawford, 1992).

Drawing on the similarities between behaviour in monkeys with frontal lobe lesions and hyperactive children, Levin postulated that the behaviour evident in hyperactive children could be explained by pathology in the forebrain structures (Levin, 1938). These findings fuelled theories about the connection of brain damage and the symptoms of inattention, impulsivity and hyperactivity, leading to the emergence of the concept minimal brain damage that later evolved into minimal brain dysfunction (MBD; Barkley, 1998).

A very important phase in the history of ADHD was reached in 1937 when Charles Bradley accidentally discovered the effectiveness of amphetamines in the treatment of hyperactive children. He was working at the Emma Pendleton Bradley Home for Children, and as part of diagnostic procedure the children received a pneumoencephalogram. However, this method caused the children severe headaches, and in order to ameliorate the pain, Bradley gave the children Benzedrine. The compound

was, on the other hand, far more potent than Bradley could imagine, with the children showing better self-control, increased academic performance and improved attention to tasks (Bradley, 1937).

In May 1950, M. Hartmann und L. Panizzon got the patent for a drug called Ritalin, which was purported to be useful in cases of chronic fatigue, depression or psychosis associated with depression. In 1956, Ritalin was furthermore introduced as a treatment choice for children diagnosed with MBD (Barkley, 1998).

When this era came to an end, it appeared to be widely accepted among researchers that MBD was caused by some kind of brain damage, even though the damage could not be accurately pinpointed. Furthermore, there had been progress in the treatment of MBD, and although in its infancy, the use of a Ritalin seemed promising.

1.1.2 ADHD in the 1960s

The second period was during the 1960s, and in this period the concept of MBD came under heavy criticism, whereas it was thought to be vague and over inclusive. The heterogeneity of MBD was enormous with a report from the National Institute of Neurological Disease and Blindness from 1966 counting at least 99 symptoms (Clements, 1966).

As the MBD term gradually disappeared, the concept of the Hyperactive Child Syndrome was born. In his article from 1960, Chess stresses the necessity of objective evaluation when diagnosing a child with the Hyperactive Child Syndrome. He highlighted the excessive activity as the core feature of the syndrome and separated it from the concept of MBD. Furthermore, researchers in this era stressed the relatively mild nature of ADHD symptoms and claimed that they would in most cases be resolved by puberty (Barkley, 1998). This growing emphasis on extreme activity in the Hyperactive Child Syndrome lead to the formation of the Hyperkinetic Reaction of Childhood in the Diagnostic and Statistical manual of Mental Disorders second edition (DSM-II), where it was defined in a single sentence:

> "The disorder is characterized by over activity, restlessness, distractibility, and short attention span, especially in young children; the behaviour usually diminishes by adolescence" (APA, 1968).

In this era, the focus shifted away from the brain damage theories, and the unclear concept of MBD was replaced by terms that were based on more descriptive and observable deficits in hyperactive children. However, the MBD term played a significant role in the development of ADHD, and it's legacy lies in that it lead the way for theories focusing on faulty neurological mechanisms when trying to explain the nature of Hyperkinetic Reaction of Childhood. The main features of Hyperkinetic Reaction of Childhood were excessive motor behaviour and prospects were relatively good since the children were thought to outgrow the symptoms by puberty (Barkley, 1998).

1.1.3 ADHD in the 1970s

The third phase was during the seventies, where interest in ADHD and research on the topic grew steadily. Early in the 1970, the definition of Hyperkinetic Reaction of Childhood expanded to include symptoms such as distractibility, short attention span, impulsivity, low frustration tolerance and aggressiveness (Marwitt & Stenner, 1972).

One of the most influential theory about ADHD in this decade was Douglas's model of attention and impulse control (Douglas, 1972, 1983).

In her theory, Douglas argued that the difficulties experienced by children diagnosed with Hyperkinetic Reaction of Childhood were unlikely to arise from hyperactivity alone. She maintained that poor impulse control and deficits in sustained attention played a pivotal role in conjunction with the hyperactivity, resulting in the behavioural difficulties seen in this syndrome.

According to Douglas, deficits in four areas could shed light on the symptoms of Hyperkinetic Reaction of Childhood. First, deficits in organization, investment and

maintenance of attention and effort; second, poor ability to inhibit impulsive responses; third, poor modulation of arousal levels to meet situational demands, and finally an unusually strong tendency to seek instantaneous reinforcement (Douglas, 1983). Douglas's theory had great impact on researchers for the next decade and was in all probability a major factor why the disorder was renamed Attention-Deficit Disorder in the DSM-III (APA, 1980).

Another turning point in the history of ADHD was the adoption of parent and teachers rating scales for the assessment of hyperactivity. These scales, developed by Conners, moved diagnosis and assessment of hyperactivity from a clinical impression alone to a more structured and quantitative assessment procedure (Barkley, 1998).

In this decade, researchers also started to consider the possibility that the impulsiveness and hyperactivity in ADHD would not disappear in adolescence and argued that some of these children would experience difficulties into adulthood because of these symptoms (Barkley, 1998).

A vital brick was added in the ADHD puzzle by Pontius in the seventies when she suggested that impulsive and hyperactive behaviour evident in some adults might arise from caudate and frontal lobe dysfunction, leading to variable attention, impulsivity and distractibility. Her suggestions were later confirmed by researchers using neuroimaging techniques demonstrating reduced size in caudate-prefrontal network in children with ADHD (Castellanos et al., 1996; Filipek et al., 1997)

1.1.4 ADHD in the 1980s

The fourth period began with a reconceptualization of Hyperkinetic Reaction of Childhood to Attention-Deficit Disorder (ADD), with the publication of DSM-III (APA, 1980). The DSM-III criteria set forth specific lists of symptoms, with five focusing on inattention, six on impulsivity and five on hyperactivity. The criteria incorporated a cut-off score for each of the symptom lists, guidelines for duration of symptoms and age of

onset and more importantly the exclusion of other childhood psychiatric conditions. The DSM-III criteria were significant for their emphasis on impulsivity and inattention as defining features. Furthermore, it created subtypes of ADD based on the presence or absence of hyperactivity (ADD+H, ADD-H). These subtypes of ADD were controversial at the time whereas little research existed prior to their formulation (Barkley, 1998). However, there were indications from studies that children with ADD+H differed from those with ADD-H, with ADD-H children being less aggressive, more day dreamy and experiencing more learning difficulties (Goodyear & Hynd, 1992).

In 1987, the American Psychiatric Association revised the diagnostic criteria for ADD yet again with the publication of DSM-III-R, where ADD was renamed Attention-Deficit Hyperactivity Disorder (ADHD). The diagnostic criteria changed considerably, instead of the three separate symptom lists of hyperactivity, impulsivity and inattention there was now only one list of 14 symptoms and one cut-off score that was reached when eight symptoms were present. These criteria were derived from a large field trial determining their specificity, discriminating power and sensitivity to differentiate children with ADHD from those with other psychiatric disorders (APA, 1987). The DSM-III-R no longer considered Attention-Deficit Disorder without hyperactivity to be a specific subtype, and it was downgraded to an unclearly defined category called Undifferentiated ADD. Furthermore, because of the considerable overlap or co morbidity in clinically referred children, ADHD was now classified with Oppositional Defiant Disorder and Conduct Disorder in a category called Disruptive Behaviour Disorders (Culbertson & Krull, 1996).

By the end of the 1980s, ADHD was considered to have a strong biological or hereditary predisposition. The diagnostic process had been improved considerably by the introduction of standardised questionnaires. A more important notion, and what put ADHD into a new context, was that the symptoms were thought to persist into adulthood.

1.1.5 Current diagnostic criteria and clinical characteristics

The diagnostic guidelines used when diagnosing ADHD are either the Diagnostic and Statistical Manual of Mental Disorders 4th revision (APA, 1994) or the ICD 10th revision, (WHO, 1993). The criteria in DSM-IV and ICD-10 are very similar, using the same 18 questions focusing on the cardinal symptoms of ADHD.

The diagnostic guidelines also contain specific requirements for determining when the symptoms are indicative of ADHD. The behavior must appear before age 7 and continue for at least 6 months. Above all, the behavior must create a real handicap in at least two areas of the person's life such as at home, on the playground, in the classroom or in other social settings. Finally, the symptoms can't be explained by other mental and psychiatric disorders such as anxiety disorder, mood disorder, dissociative or personality disorder. Furthermore, the behavior may not occur in the presence of pervasive developmental disorder, schizophrenia or other psychotic disorder.

In the DSM-IV, ADHD is subdivided into three primary subtypes, predominantly inattentive, predominantly hyperactive-impulsive and combined type. The criteria include 18 symptoms, nine focusing on inattentive behaviour and nine on hyperactive-impulsive behaviour. A child meets the criteria for the inattentive type when six of nine inattention symptoms are present. The criterion for hyperactive-impulsive type is met when six of nine hyperactive-impulsivity symptoms are present. With the diagnosis of a combined type then the child has at least six of nine symptoms from both the inattention and hyperactivity-impulsivity scale criteria (APA, 1994).

The ICD-10 criteria are more restrictive than the DSM-IV because they need a greater degree of symptom expression. For the diagnosis of Hyperkinetic Syndrome, a child has to meet six of nine symptoms of the inattentive part, three of five from the hyperactive part, and one of four from the impulsive part (WHO, 1993).

1.1.6 Prevalence

The exact prevalence of ADHD is hard to measure, and in past decades investigators from all regions of the world have made substantial efforts to define the prevalence of the disorder. Several literature reviews have reported highly variable rates worldwide, ranging from 1% to as high as nearly 20% among school-age children (Faraone et al., 2005). This variability in prevalence is poorly understood, but could possible be traced to methodological differences among the studies, such as the use of different diagnostic system (DSM-IV or ICD-10), application of associated criteria (degree of impairment, situational versus pervasive required for diagnoses), degree of agreement required between informants and the population being studied (Dulcan, 1997). When looking at the population being studied, factors such as male gender, young age, family dysfunction, urban living and low socioeconomic status might influence the prevalence (Szatmari, 1992). The gender ratio in ADHD also varies considerably across studies. In clinical samples, the male vs. female ratio is on average 6:1, whereas the ratio in non referred samples is 3:1 (Szatmari et al., 1989). The higher rate of males in the clinical samples might be due to referral bias, whereas males are more likely to show aggressive or antisocial behavior and thus more likely to be referred to a psychiatric center (Barkley, 1998). Follow up studies involving ADHD children suggest that 60-70% of the cases have incomplete or full syndrome in adult life (Murphy & Barkley, 1996).

The consensus of expert opinion seems to be around 3 – 5% of children have ADHD (APA, 1994). These figures are supported by a recent research using a meta analysis of 102 studies comprising 171.756 subjects, where the world-wide pooled prevalence was reported to be 5.29% (Polanczyk et al., 2007).

1.1.7 ADHD and co-morbidity

ADHD conveys a significant risk for other co-morbid psychiatric disorders, with the most common of these coexisting disorders being oppositional defiant disorder (ODD)

and conduct disorder (CD), which are diagnosed in 40 to 60% of children with ADHD (Willcutt, 1999). ODD and CD are defined as externalizing disorders characterized by aggressive and antisocial behaviours. ODD is most often seen in children under 10 years of age and is apparent by a defiant, disobedient or provocative behaviour and by the absence of more severe dissocial or aggressive acts seen in CD. Conduct disorder is a severe disorder comprising serious aggressive and antisocial behaviour, such as fighting, bullying, theft and fire setting, defined by "repetitive and persistent pattern of behaviour in which the basic rights of others or major age-appropriate societal norms or rules are violated" (APA, 1994).

Anxiety disorders are present in around 25% of the cases (Biederman et al., 1991). Anxiety can be characterized by changes in mood (anxiety, panic or tension), cognition (worrying and planning about the feared thing) and physical symptoms, such as sweating, shallow or rapid breathing and dry mouth (APA, 1994). Anxiety can also be evident in behavioural symptoms, such as agitation, rituals, attention seeking or tantrums (Pollack et al., 1996). Mood disorders are evident in about 20 to 30% of children with ADHD (Biederman et al., 1992). In mood disorders, the fundamental disturbance is a change in mood or affect, being either depression or bipolar (changing from depression to mania). Major depression in children can be evident from a sad or irritable mood or a persistent loss of interest. Associated features of childhood depression are somatic complaints, negativism, withdrawal, school refusal, school difficulties, aggression or antisocial behaviour (Spencer, 2006). The symptoms seen in manic episodes can range from decreased sleep, over talkativeness, racing thoughts or poor judgement to extreme irritability or explosive mood (APA, 1994).

Learning disorders are also frequently seen in ADHD, they are diagnosed when there is a "significant discrepancy" between one's intelligence and academic achievement. The term "Learning disorder" incorporates various deficits, such as dyscalculia, dyslexia or

dysgraphia, affecting the individual's ability to receive, retrieve, process, analyze or store information (Pennington, 1991). The prevalence of learning disorders in ADHD varies highly depending on the definition used for this “significant discrepancy” (Barkley, 1998). Semrud-Clikeman and colleagues reported dyscalculia in 55% of their ADHD children and dyslexia in 38% (Semrud-Clikeman et al., 1992), whereas Barkley (1998) reported that 28% had math difficulties, 26% had spelling difficulties, and 21% reading difficulties in his sample of children with ADHD.

2. ADHD aetiology

Despite its frequency and numerous studies over the last decades, the aetiology of ADHD is not fully understood, but it is most likely caused by a complex interaction of neurological, biological and environmental factors.

2.1 ADHD and anatomy

The search for a possible site of pathology in ADHD is a complex and difficult task, and various brain regions have been implicated the aetiology of ADHD, such as the frontal lobes, basal ganglia, cerebellum and the corpus callosum.

2.1.1 The frontal lobes

The frontal lobe comprises all the brain tissue in front of the central sulcus, making up about one third of all the neocortex and is comprised of three general areas: the motor, premotor and prefrontal cortices (Kolb & Whishaw, 1996). These areas are implicated in an enormous range of behaviour spanning from motor control to social behaviour (Parker & Crawford, 1992). The motor cortex is involved in the control and execution of individual movements (Nolte, 1993). The premotor area can be subdivided into three main areas. The premotor cortex that is involved in learning of novel motor sequences, the supplementary area that handles previously learned or routinized movements (Jenkins et al., 1994). According to Passingham, the premotor cortex chooses behaviour in response to external cues and the supplementary cortex makes a greater contribution when no external cues are available (Passingham, 1993). The frontal eye field is also a part of the premotor area. It coordinates and maintains eye and head movements and gaze shifts, and thus orienting and attentional reactions to external stimuli (Gottlieb et al., 1994).

The prefrontal region includes the dorsolateral prefrontal cortex, inferior prefrontal cortex and the medial prefrontal cortex (Kolb & Whishaw, 1996). These regions have

vast interconnections with nearly every area of the brain. The dorsolateral prefrontal cortex plays a part in working memory, planning, organizing, initiating, monitoring, evaluating and modifying our behaviour (Hale & Fiorello, 2004).

The inferior prefrontal cortex is involved in initiating and maintaining performance, inhibiting irrelevant responses, as well as behavioural and emotional regulation (Elliott et al., 1999). A part of the medial frontal cortex, the anterior cingulate, is involved in self-monitoring performance, novelty response, shifting cognitive set, inhibiting automatic responses and complex decision-making (Posner, 1994).

Neuroimaging studies focusing on the cerebral cortex in ADHD patients indicate that the total cerebral volume of ADHD individuals is smaller than in controls. Castellanos and colleagues reported that the total volume was 4.7% less than in controls, and Mostofsky and coworkers found an 8.3% reduction in cerebral volume in ADHD children (Castellanos et al., 1996; Mostofsky et al., 2002).

Researchers have described reductions in prefrontal volume, predominantly in the right hemisphere, in ADHD children (Castellanos et al., 1996; Filipek et al., 1997).

Results from functional imaging studies have indicated less function in frontal areas in ADHD individuals (Hynd et al., 1993; Zametkin et al., 1990). Results from Lou and colleagues showed that there was less regional cerebral blood flow in the frontal areas of ADHD children (Lou et al., 1984, 1989).

Results from positron emission tomography (PET) studies have indicated reduced metabolism in the frontal lobes in adult ADHD individuals (Ernst et al., 1998; Zametkin et al., 1990). Rubia et al using functional magnetic resonance imaging (fMRI), reported a decreased activity in the right medial frontal cortex in ADHD individuals (Rubia, 1999).

2.1.2 The Basal ganglia

The basal ganglia (BG) have often been implicated in the pathology of ADHD and have long been suspected to play a critical role in the disorder. They consist of a group of

sub cortical nuclei; among them are the caudate nucleus, putamen, globus pallidus, subthalamic nucleus and substantia nigra. Some include the amygdala and claustrum in their definition of the basal ganglia (Nolte, 1993). The basal ganglia have extensive connections to the cerebral cortex and are involved in a variety of processes including motor, cognitive and mnemonic functions. Among the most important projections to the basal ganglia are those coming from the prefrontal areas. The connectivity between the basal ganglia and prefrontal areas appears to be essential in the regulation of voluntary motor behaviour, enabling the basal ganglia to regulate specific cortical areas by stimulating or inhibiting them via several cortical-subcortical circuits (Alexander, 1986; Coté & Crutcher, 1991).

These circuits are topographically organised, with specific cortical areas projecting to different parts of the striatum, which therefore have specific behavioural functions (Crossman & Neary, 1995; Nolte, 1993). The best described of these circuits are the oculomotor circuit, which connects the frontal eye fields and the central region of the caudate nucleus; and the motor circuit, which arises mainly in the supplementary motor cortex and projects to the putamen. These two circuits are dedicated to motor functions. The dorsolateral prefrontal circuit connects the head of the caudate nucleus and Brodmann's areas 9 and 10. This circuit is involved in motor planning and executive behaviour, such as goal-directed behaviour, planning, flexibility and inhibition of inappropriate responses. The orbitofrontal circuit originates in the inferolateral and orbital prefrontal cortices and projects to the ventromedial caudate nucleus. This circuit is associated with socially appropriate behaviour and personality. The circuit between the anterior cingulate gyrus and the ventral striatum is called the anterior cingulate circuit, mediating motivated behaviour, see Figure 1.

Figure 1: Cortical-subcortical circuits

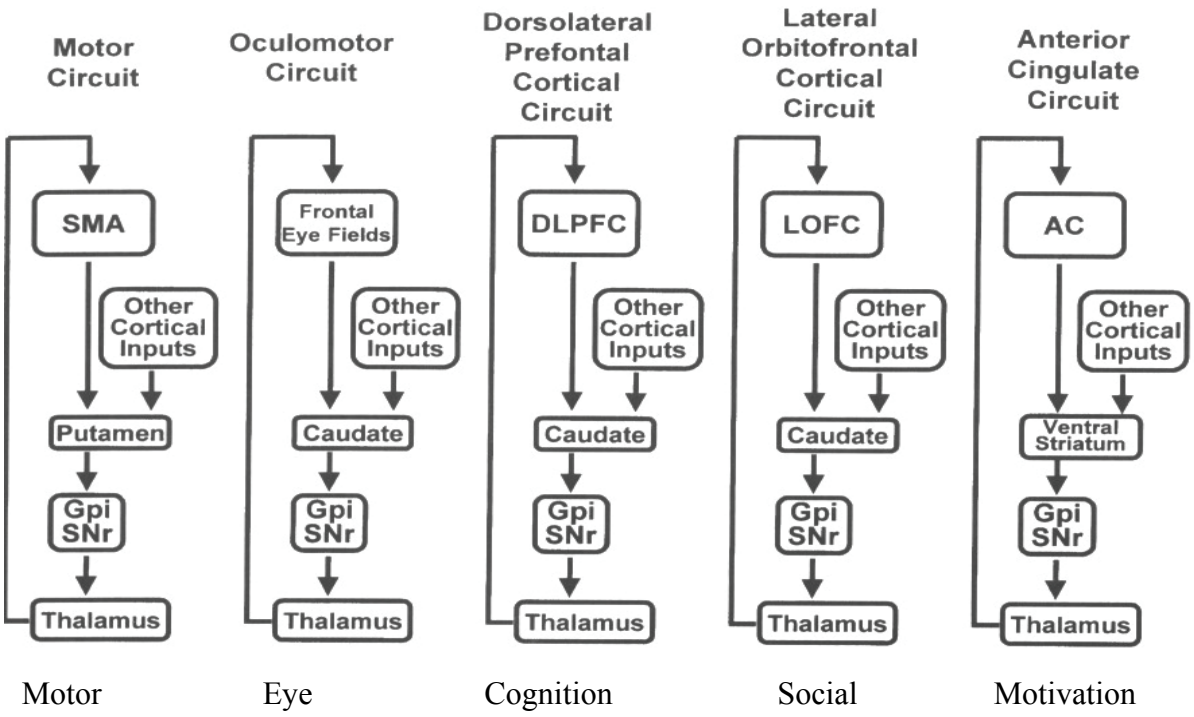


Figure taken from Bradshaw, 2001

In addition to these above-mentioned circuits, the individual nuclei of the basal ganglia furthermore participate in several subsidiary circuits, which serve to modify transmission through the basal ganglia-thalamo-cortical pathways (Alexander et al., 1986; Mega et al., 1994; Singer, 1997).

Three circuits are crucial in modulating the output from these cortical-subcortical circuits, the nigrostriatal pathway, and the direct and indirect loop. The nature of this modulation depends upon the neurotransmitters within these loops, such as γ-aminobutyric acid (GABA), glutamate, dopamine (DA) and acetylcholine (Rauch, 1996).

The nigrostriatal pathway consists of dopaminergic axons from the substantia nigra pars compacta (SNpc) to the striatum. Efferents from the SNpc terminate on DA_1 receptors in the direct loop facilitating them and on DA_2 receptors in the indirect loop inhibiting them. One role of the nigrostriatal pathway is to activate the direct loop and inhibit the indirect loop (Cooper et al., 1996).

Figure 2: Connectivity within the basal ganglia and cortex

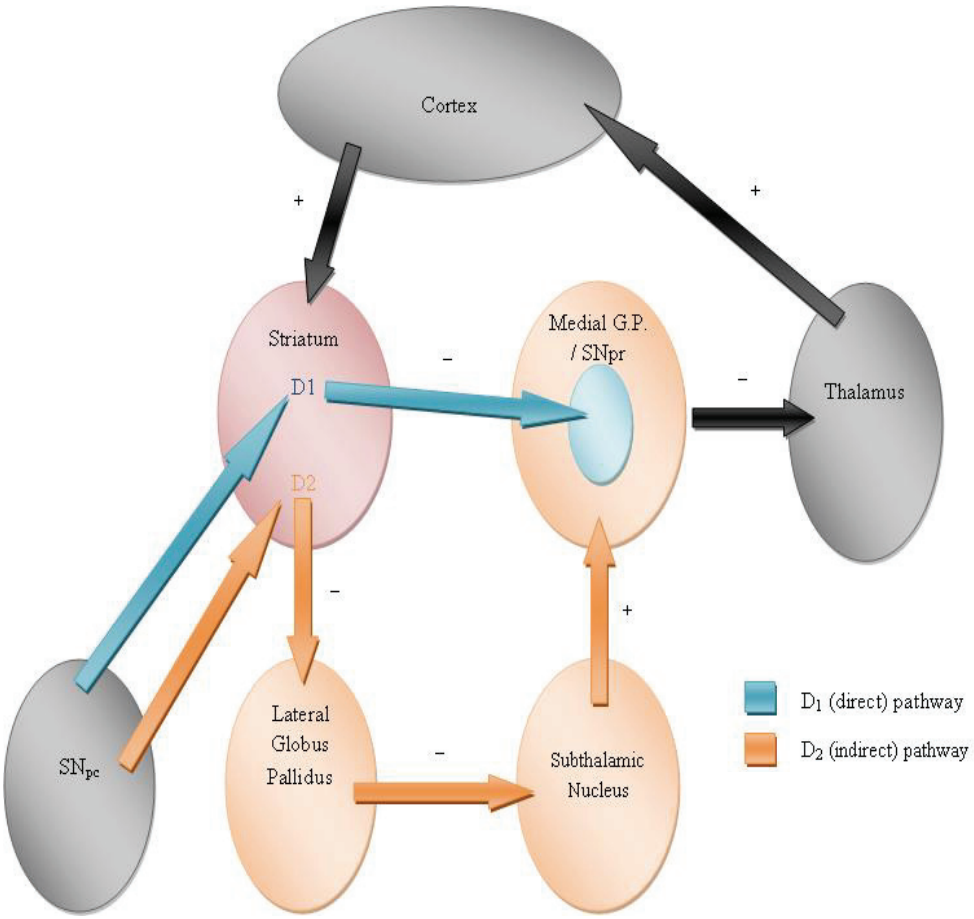


Figure taken from: http://brainybehavior.com

Findings from neuroimaging studies have been conflicting concerning specific nuclei of the basal ganglia and their role in the pathology of ADHD. To date, researchers have not reported any abnormalities or significant differences in the putamen of ADHD individuals (Aylward et al., 1996; Castellanos et al., 1996; Castellanos & Tannock, 2002). Results from magnetic resonance imaging (MRI) studies focusing on the caudate nucleus have been contradictory, with findings from Castellanos pointing to reduction in the right caudate (Castellanos, 2001), whereas other studies have pointed to reported a left sided reduction (Filipek et al., 1997). Results from Aylward and colleagues indicate that boys with ADHD have significantly smaller left globus pallidus volume and total globus pallidus volume than normal controls (Aylward et al., 1996). Whereas Castellanos et al (1996) reported smaller right globus pallidus in boys with ADHD.

2.1.3 The Cerebellum

Over the last decade, the cerebellum has received increased attention among ADHD researchers. This focus on the cerebellum is fuelled by recent findings suggesting that, in addition to its crucial role in motor function, the cerebellum is essential to the neural circuitry sub serving cognition and emotion (Katz & Steinmetz, 2002; Schmahmann & Scherman, 1998). The cerebellum is tightly interconnected with the cerebral cortex via the cortico-ponto-cerebellar system (Middleton & Strick, 2000), and has been found to play a role in attention, cognitive flexibility, verbal memory and working memory (Katz & Steinmetz, 2002).

In a study comparing 55 ADHD boys with controls, Castellanos and coworkers reported that the ADHD group had smaller cerebellum compared to controls (Castellanos et al., 1996). Mostofsky et al and Berquin and coworkers reported that the cerebellar vermis as a whole, and particularly the posterior-inferior lobules (VIII-X) were smaller in an ADHD sample compared to controls (Berquin et al., 1998; Mostofsky et al., 1998). In a study from 2001, Castellanos and colleagues reported on smaller posterior-inferior cerebellar vermis (lobules VIII-X) in a sample of 50 ADHD girls (Castellanos et al., 2001).

The exact role of the vermis is not clear. It sends efferents to the ventral tegmental area and the locus coeruleus (Snider & Sinder, 1977), and could therefore have a modulatory influence on the dopamine and norepinephrine systems and the prefrontal-subcortical circuits (Castellanos et al., 2001; Nigg, 2006).

2.1.4 The Corpus callosum

The corpus callosum is a thick bundle of fibers that connects the two cerebral hemispheres. It plays a role in interhemispheric communications and efficient transfer of information, which are vital for complex motor and cognitive functions (Banich, 1998).

Results from neuroanatomic studies focusing on the corpus callosum indicate that it is

smaller in children with ADHD compared to controls. However, findings do not agree to which part of the corpus callosum is smaller in ADHD children. Whereas, results from studies have reported that the anterior region (Baumgardner et al., 1996; Hynd et al., 1991) and the posterior region is smaller in ADHD individuals compared to controls (Semrud-Clikeman et al., 1994).

Our understanding of the pathophysiology in ADHD is still in its formative years, and results from neuroimaging studies have been contradictory when trying to pinpoint the cerebral areas implicated in the disorder. However, there is converging evidence indicating that alterations in the prefrontal cortex and its connections to the basal ganglia and cerebellum play a pivotal role in the etiology of ADHD. When looking at the findings from neuroimaging studies to date, one must bear in mind the methodological shortcomings of these studies, such as small sample size and high heterogeneity in sample characteristics, such as gender, age, inclusion of ADHD subtypes, co morbidity status and use of medication (Biederman & Faraone, 2005; Krain & Castellanos 2006; Solanto, 2002).

3. Neurotransmitters and ADHD

Researches to date indicate that a neurobiological basis is one of the mechanisms underlying the symptoms of ADHD. Converging evidence indicates that genes underlying various aspects of the monoamine[1] neurotransmitter pathway playing a major role in the aetiology and pathophysiology of ADHD (Solanto, 1998, 2002).

3.1 The dopamine system

The dopamine (DA) neurotransmitter system is probably the most extensively studied among the monoamine systems and it is known to have modulatory effects on motor regulation, attention and arousal. The main dopamine production sites in the brain are the substantia nigra (SN) and ventral tegmental area (VTA). The projection from the substantia nigra to the striatum[2] is called the "nigrostriatal pathway". The axons of the VTA neurons travel forward through the median forebrain bundle and then spread out to innervate the prefrontal cortex and ventral striatum, the "mesocortical" and "meso-limbic" dopamine pathways, respectively (Cooper et al., 1996).

Figure 3: The dopamine system

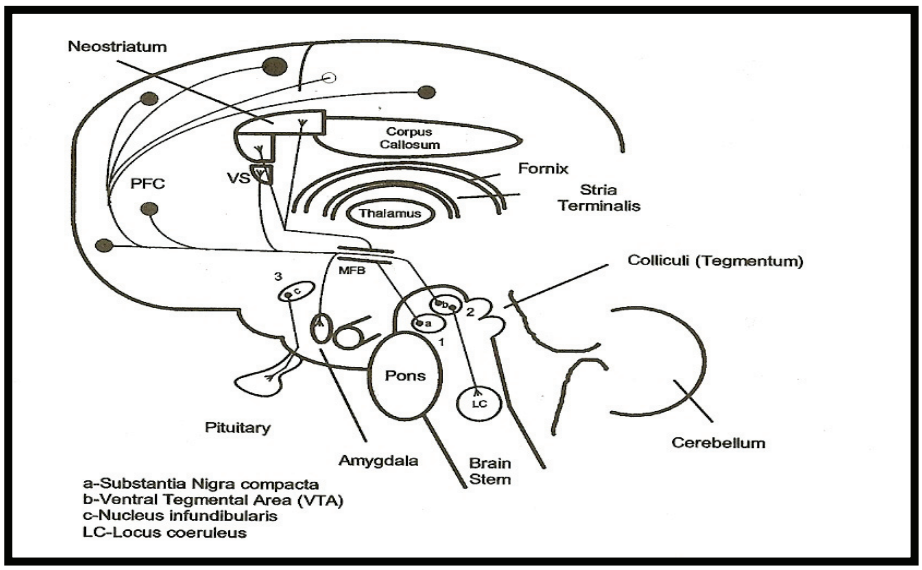


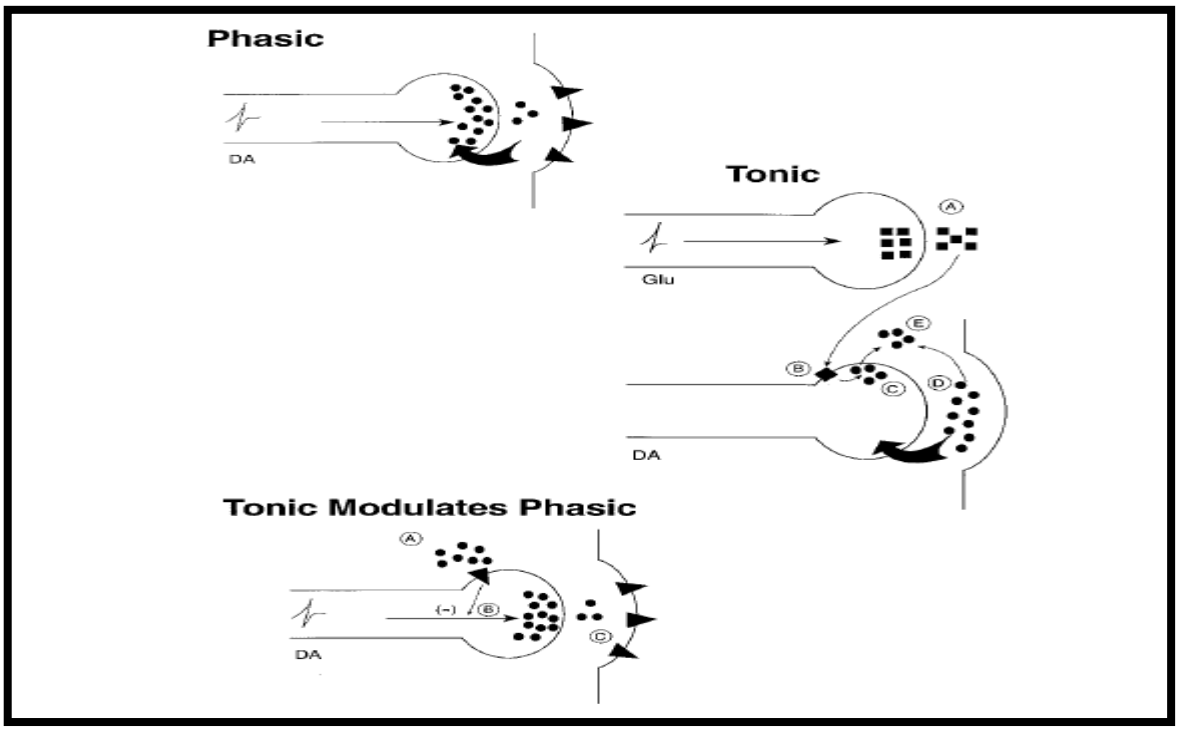
Figure taken from Pliszka, 2003

[1] Monoamines – dopamine, norepinephrine and serotonin
[2] Striatal/striatum – refers to the combination of caudate nucleus and putamen

DA neurotransmission is different in the basal ganglia compared to the frontal cortex. In the basal ganglia, the transmission is called synaptic with the dopamine transporter (SLC6A3) limiting the diffusion of DA away from the synapses and auto receptors influencing the release of DA.

The amount of DA released is controlled by so-called phasic and tonic mechanism (see Figure 4). The phasic release occurs in response to an action potential in the DA neuron. After release, most of the phasic DA is rapidly transported back into the neuron by the SLC6A3. The tonic DA, on the other hand, consists of the DA left in the synaptic cleft by the SLC6A3 and DA released through other processes. Prominent among them are glutamatergic afferents arising in the prefrontal cortex acting on heteroreceptors on the DA nerve terminal (Grace, 1991, 2001; Moore et al., 1999). There are also smaller hippocampal and amygdala glutamatergic afferents that act on these heteroreceptors (Blaha et al., 1997; Floresco, 1998). The synaptic concentration of tonic DA in the basal ganglia is too low to stimulate postsynaptic DA receptors. It is, on the other hand, sufficient to stimulate the more sensitive auto receptors leading to down-regulation of the phasic released DA (Grace, 2001, 2002). Therefore, the higher the DA tonic level, the lower the phasic release of DA and vice versa.

Figure 4: The tonic/phasic model of DA system regulation.



Top: Phasic DA is defined as the release of DA into the synaptic cleft as a consequence of action potential discharge in the DA neuron. The action potential causes release of large amounts of DA (circles), resulting in intrasynaptic DA concentrations in the millimolar range where it can stimulate postsynaptic receptors (triangles). The DA is then rapidly removed from the synaptic cleft by the DA transporter (large arrow) before it can escape into the extra synaptic space.

Centre: Tonic DA is defined as the DA that is present in the extra synaptic space. Tonic DA is proposed to be derived from two sources. Glutamate released from corticoaccumbens terminals (A; squares) diffuses to the DA terminal, where it can stimulate presynaptic glutamate receptors (B). This causes a release of DA from the synaptic terminal (C). In addition, a small portion of tonic DA is also likely derived by overflow from the synaptic cleft because of sustained activity in the DA terminal (D). The level of extra synaptic DA (E) derived from these sources is maintained at low nanomolar concentrations by numerous regulatory processes.

Bottom: The tonic DA levels in the extra synaptic space (A) are too low in concentration to stimulate the DA receptors located in the synaptic cleft, but are sufficient to activate DA autoreceptors located on the DA terminal that regulate synthesis and release of transmitter (B). Consequently, there is an inhibition of phasic, spike-dependent DA release into the synaptic cleft (C).

Circles=DA; triangles=DA receptors; squares=glutamate/glutamate receptors

Figure and text taken from Grace, 2000

In the frontal cortex, the DA transmission is called volume transmission. There are substantially fewer SLC6A3 in the frontal cortex and no auto receptors regulating the release of DA, so it diffuses away from the synapses. In the frontal cortex, the inactivation of DA is dependent on diffusion, metabolism by catechol-*O*-methyltransferase (*COMT*) and reaching norepinephrine (NE) nerve terminals where NE transporters transport DA into NE neurons (Stahl, 2000).

When released into the synaptic cleft, DA is received by a set of different DA receptors, inducing a cascade of postsynaptic events (Devinsky, 1983). These receptors can be divided into two families, D1 and D2.

The "D1 family" consists of the D1 and D5 receptors. Both of these receptors are linked to G proteins that stimulate adenylate cyclase activity. The D1 receptor is mainly found in the cerebral cortex, striatum, nucleus accumbens and the olfactory tubercle. The D5 receptor is most densely distributed in the hippocampus, but has also been found in other rostral brain regions, including cerebral cortex, striatum and the lateral thalamus.

The members of the "D2 family", the D2, D3 and D4 receptors, are linked to G proteins that inhibit adenylate cyclase activity. The D2 receptor has been mostly found in the striatum, the nucleus accumbens and olfactory tubercle. The D2 receptor can be divided into postsynaptic- and presynaptic receptors (auto receptors that are positioned on the soma, dendrites or nerve terminals of the cell). The auto receptors are 5 to 10 times more sensitive to DA than postsynaptic receptors, and can inhibit dopamine synthesis and release. Stimulation of D2 auto receptors in the somadendritic regions leads to slower firing rate of dopamine neurones, while stimulation of D2 auto receptors on the nerve terminals inhibits dopaminergic synthesis and release (Comings, 1990; Cooper et al., 1996). The D3 receptor has been found in the nucleus accumbens and olfactory tubercle. The D4 receptor is highly expressed in the frontal cortex, amygdala, hippocampus and hypothalamus. It is however, expressed at low levels in the basal ganglia (Cooper et al., 1996; Pliszka, 2003).

The DA receptors can exhibit adaptive changes following chronic exposure to dopamine antagonists or agonists. Prolonged exposure to antagonists increases the number of dopamine binding sites, leading to super sensitivity or up regulation of the DA receptors. Likewise, administrations of dopamine agonists can decrease the number of dopamine binding sites, resulting in sub sensitivity or down-regulation of the DA receptors (Missale et al., 1998).

Several lines of research indicate that the dopamine transporter gene (*SLC6A3*) is implicated in ADHD. First, stimulant medication is known to inhibit the function of the SLC6A3 and thereby increasing the levels of dopamine in the synaptic cleft (Solanto, 1998). *SLC6A3* knockout mice, which lack the gene that encodes for the dopamine transporter, show behaviour that is analogous to ADHD (Gainetdinov et al., 1999). In the *SLC6A3* knockout mice model, released DA is cleared at a slow rate from the synaptic cleft giving rise to a five-fold elevation of extracellular tonic DA or a hyperdopaminergic state in the striatum (Jones et al., 1999). Furthermore, this increase in tonic extracellular DA is accompanied by a hypodopaminergic function whereas the phasic release of DA is reduced (Gainetdinov et al., 1999). Studies have also implicated dopamine receptors, such as the dopamine receptor DRD4 in the aetiology of ADHD. The DRD4 was initially associated with the personality trait of novelty seeking (R. Epstein et al., 1996), which is thought to resemble the high levels of excitability and impulsivity often seen in ADHD (Faraone et al., 1999).

3.2 The norepinephrine system

The main norepinephrine (NE) pathway originates in the locus ceruleus (LC) and gives rise to extensive projections throughout most of the CNS, including the cortex, thalamus, hippocampus, midbrain, cerebellum and spinal cord. NE neurons also project from the LC to serotonin neurons in the dorsal raphe nucleus, thus having an influence on the output of the serotonin system (Pliszka, 2003). The LC, furthermore, displays

significant regional specificity in its projections, with areas such as the parietal cortex, superior colliculus and pulvinar nucleus receiving dense innervation (Aston-Jones, 1995; Morrison & Foote, 1986). These areas are all closely associated with attentional processing, and NE is believed to be instrumental in controlling responses to novel stimuli, and filtering out distracting information (Friedman et al., 1999), in addition to playing a vital role in sleep-wake cycle regulation, affective and cognitive functions (Aston-Jones, 1995; Cooper et al., 1996).

Figure 5: The norepinephrine system

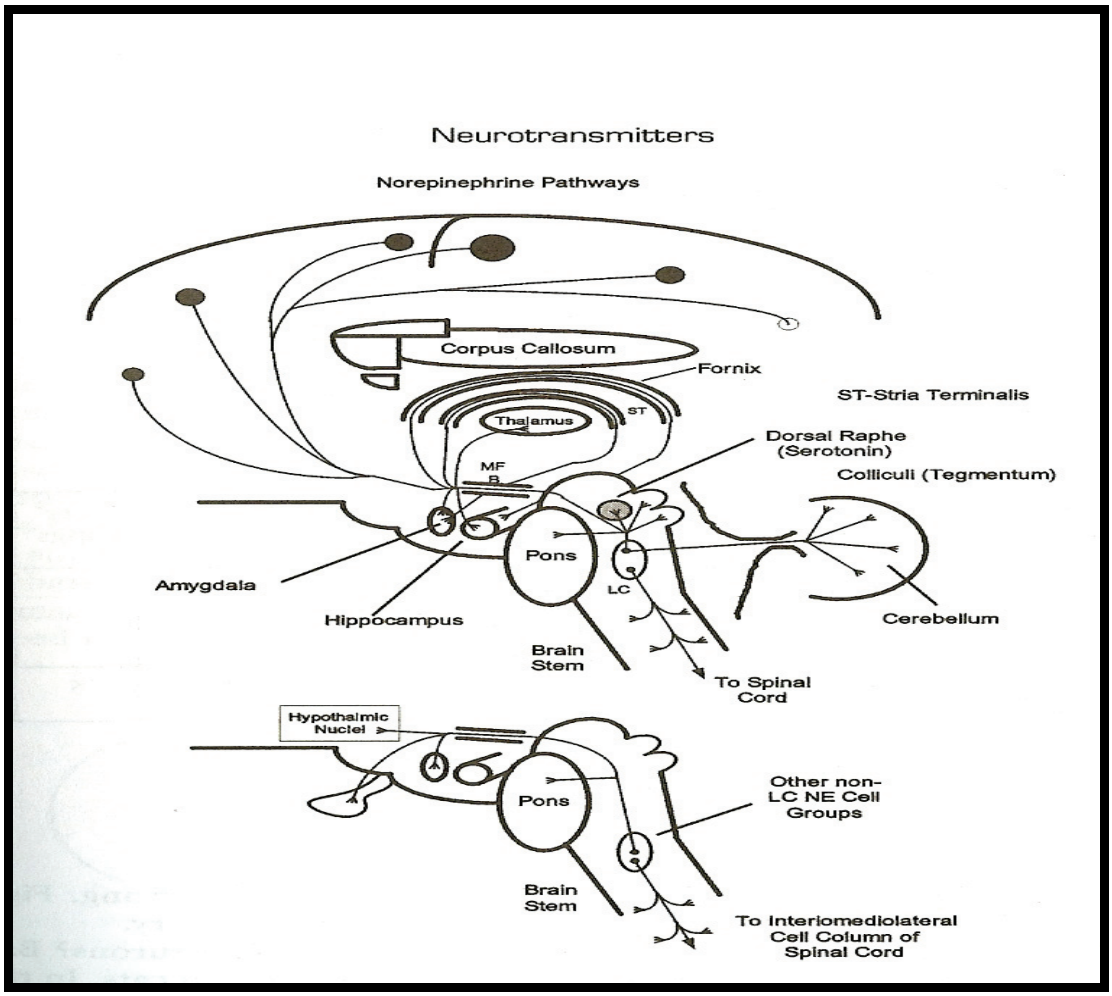


Figure taken from Pliszka, 2003

NE released into the synaptic cleft acts on NE receptors, which are all metabotropic and can be divided into three major subtypes, alpha-1, alpha-2 and beta receptors. The Alpha-1 and beta receptors are mainly postsynaptic receptors, whereas the alpha-2 is situated both pre- and postsynaptically. The beta receptors are linked to the Gs/cAMP

second messenger system, the alpha-1 receptors are coupled to the phosphoinositol system and alpha-2 receptors to the Gi/cAMP system (Cooper et al., 1996; Pliszka, 2003). The amount of NE released into the synaptic cleft is modulated via phasic and tonic mechanisms, similar to the one described in the dopamine system.

Dysregulation within the NE system might contribute to the erratic attention seen in ADHD, with too high or low levels of NE released into the synaptic cleft leading to impaired information processing and attention capacity (Aston-Jones, 1995). In line with this, researchers have begun to examine the potential role of the norepinephrine transporter gene (*SLC6A2*) in the aetiology of ADHD. SLC6A2 is responsible for the reuptake of norepinephrine from the synaptic cleft (Cooper et al., 1996). Findings from pharmacological studies show that, in addition to inhibit the function of the dopamine transporter, stimulant medication also inhibit the SLC6A2 leading to reduction in ADHD symptoms by regulating the available level of catecholamines[3] in the synaptic cleft (Solanto, 1998). Tricyclic anti-depressant medications, which block the reuptake of NE, also lead to significant improvement in ADHD symptomatology (Spencer & Biderman, 2002).

There is a need for more research on the role of NE in ADHD, but given the influence that the NE system has on modulation of higher cortical functions, such as attention, it appears reasonable to propose that faulty NE neurotransmission might contribute to the symptoms seen in ADHD.

[3] Catecholamines – dopamine and norepinephrine

3.3 The Serotonin system

Serotonin (5-hydroxytryptamine, 5-HT) has sometimes been labelled the controlling neurotransmitter in the brain and it is known to modulate numerous behavioural and physiological systems (Comings, 1990).

The main 5-HT pathways originate in the raphe nucleus, which can be divided into three main nuclei. The dorsal raphe nucleus projects through the median forebrain bundle innervating the entire cerebral cortex and striatum (Cooper et al., 1996). Furthermore, the dorsal raphe nucleus influences the output of the DA system whereas it innervates DA neurons in the SNc and the VTA (Pliszka, 2003). The median raphe also sends projections through the median forebrain bundle; the projections then separate and proceed through the stria terminalis and the fornix to reach the amygdala, hippocampus, hypothalamus and the entire cerebral cortex. The median raphe nucleus furthermore innervates the superior colliculi and cerebellum. Finally, the raphe magnus/pallidus projects downward (caudally) to the spinal cord, where it modulates sensory input (Nolte, 1993; Pliszka, 2003).

Figure 6: The serotonin system

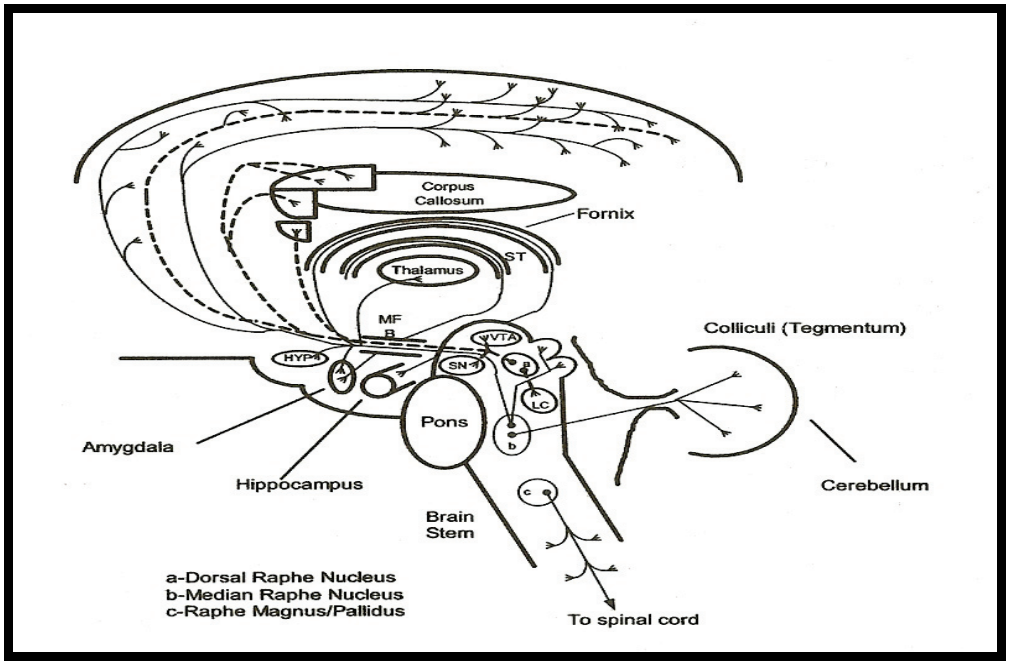


Figure taken from Pliszka, 2003

5-HT produces its effects through a variety of pre- and post synaptic receptors distributed widely throughout the central and peripheral nervous system (CNS/PNS) and coupled with an efficient reuptake system, this array of receptors provides vast signalling capabilities (Hoyer et al., 2002). To date at least 14 different 5-HT receptor subtypes have been identified. These receptors can be divided into two types, G-coupled and ligand-gated, that can be further subdivided into seven subfamilies based on pharmacological and structural characteristic (Hoyer et al., 2002; King et al., 2003).

The 5HT1, 5HT2, 5HT4, 5HT5, 5HT6, and 5HT7 receptors constitute the G-coupled type. The 5HT1 family contains the 5HT1A, 5-HT1B, 5HT1D, 5HT1E and 5HT1F receptors that all inhibit adenylyl cyclase. The 5HT2 family contains the HT2A, HT2B and HT2C receptors that all increase the hydrolysis of inositol phosphates (Aghajanian & Sanders-Bush, 2002; Kohen et al., 1996). In the 5HT5 family, there are two receptors, the 5-HT5A that inhibits adenylyl cyclase and 5HT5B. However, a functional 5HT5B has not been found in humans where the coding gene sequence is interrupted by stop codons (Grailhe et al., 2001). The 5HT4, 5HT6 and 5HT7 receptors all activate adenylyl cyclase (Hoyer et al., 2002). There is only one receptor that belongs to the ligand-gated group, and that is the 5HT3 receptor (Shih et al., 2000).

Lesions in 5-HT pathways or dysregulation in its production can compromise the brain's ability to inhibit behaviour, resulting in hyperactivity, impulsivity, depression, aggression or anxiety (Jacobs & Fornal, 2000; Pliszka, 2003).

Evidence from both human and animal studies indicates that alteration in the 5-HT system might be linked with hyperactive, impulsive and aggressive behaviour present in ADHD (Waldman & Gizer, 2006). The serotonin transporter (SLC6A4) has received increased attention among researchers, with studies reporting an association of the *SLC6A4* gene to ADHD (Curran et al., 2005; Manor et al., 2001). The SLC6A4 plays a major role in the synaptic regulation of 5-HT by transporting it from the synaptic cleft

back into the presynaptic neuron (Lesch et al., 1994). Pharmacological studies indicate that serotonin selective reuptake inhibitors (SSRIs), that act by increasing available 5-HT in the synaptic cleft, are effective in reducing ADHD symptoms (Solanto, 1998). Further evidence of the association between 5-HT and ADHD comes from the findings of Gainetdinov and colleagues (1999). They reported that stimulants decrease hyperactive behaviour in dopamine transporter knock-out mice by increasing 5-HT neurotransmission.

In summary, findings to date indicate that a neurobiological basis is one of the main mechanisms underlying the symptoms of ADHD, where faulty neurotransmission within the monoamine systems plays a pivotal role.

The effectiveness of stimulants in the treatment of ADHD and the fact that they not only influence the levels of dopamine, but noradrenaline and serotonin as well, support the notion that other neurotransmitters than DA play a vital role in the pathology of ADHD. Thus, balancing the interactions between these systems could be one of the crucial factors in the neurochemical basis of ADHD.

4. Genetics and ADHD

There is converging evidence indicating that genetics play a decisive role in ADHD, with studies implicating that the heritability in ADHD is around 80%, where many genes of small effect contribute to the disease susceptibility (Biederman & Faraone, 2005). Family studies show that first-degree relatives of ADHD individuals have a higher risk for having the disorder than relatives of controls, with around 30 to 35% of siblings of ADHD patients also fulfilling the ADHD criteria. This implies that the relative risk for ADHD is 6 to 8 times higher in first degree relatives of ADHD compared to the base rate in the population (Barkley, 1998).

Results from twin studies focusing on ADHD show that the concordance rate is 58% to 82% among MZ twins, which is significantly higher compared to 31% to 38% in same-sex DZ twins (Faraone et al., 2005).

Findings from adoptions studies provide further evidence that genes play a pivotal role in ADHD, with adoptive relatives of ADHD children being less likely than biological relatives to have the disorder or associated syndromes (Sprich et al., 2000).

Given the strong evidence implicating genes in the aetiology of ADHD there has been a steady increase during the last decade in research focusing on the genetic factors in ADHD.

The basis of research is to test a hypothesis that can be validated by other researchers, with independent replication being the alpha and omega of accepting a hypothesis. However, findings from genetic studies have been hard to replicate and a major problem hampering genetic studies is that the diagnostic criteria used for psychiatric and behavioural phenotypes are often biologically arbitrary and as such not biologically meaningful (Strachan & Read, 2004). The backbone of each genetic study is the correct categorization of individuals into groups according to similar characteristics, enhancing the possibility of detecting a gene that is involved in determining these characteristics.

The diagnosis of ADHD is no exception from this problem, with the DSM-IV classification system based only on phenomenology and completely ignoring aetiology, with no attempt to define phenotypes of the disorder with genetic bases (Barr et al., 2001).

The two main approaches used in genetic studies are association and linkage studies. These methods use genetic markers[4] to test for the association and/or linkage between a disorder and particular genes or genomic regions. The basis of both linkage and association studies is that the gene responsible for the phenotype and the marker used are located physically close on the chromosome, making it less likely that they will be separated by recombination during meiosis, so that both the marker and phenotype will co-segregate (Barr et al., 2001).

Association studies are dependent on that the chosen marker and the gene responsible for the phenotype remain together in the population over many generations and are not separated by recombination during meiosis. The finding of an association depends either on that the marker is causing the disorder or it is in linkage disequilibrium[5] (LD) with the gene that causes the disorder (Strachan & Read, 2004).

In its simplest form, association studies compare a group of cases with a group of controls; a given marker is considered associated with the disorder if it appears at a significantly higher frequency among affected individuals. Association is, however, not a genetic phenomenon, but simply a statistical statement about the co-occurrence of markers or phenotypes that can have many possible causes that are not all genetic. Stratification could be one reason, where the population in question might contain distinct subsets, and both the marker and the disorder in question are particularly frequent in one subset. Another explanation for the observed association might simply be that it is a false

[4] Genetic marker - any polymorphic Mendelian character that can be used to follow a chromosomal segment through a pedigree

[5] Linkage disequilibrium – a statistical association between particular alleles at separate but linked loci, normally the result of a particular ancestral haplotype being common in the population studied

positive association (alpha-error), resulting from a large numbers of markers used in the study without adequate statistical control (Strachan & Read, 2004).

By developing family-based association methods, researchers have been able to evade these pitfalls when conducting association studies. The most frequently used method is the Transmission Disequilibrium Test (TDT), where the marker that heterozygous parents transmit to their affected children is compared to the non-transmitted marker, using the non-transmitted marker as control. If there is an association between the disorder and the marker in question, the high risk marker should be disproportionately transmitted to the affected children. Variants of the TDT are also available such as the extended TDT (ETDT), used when only one parent is available (Waldman & Gizer, 2006). Yet another form is the sib-TDT, which looks at the difference in marker frequency between affected and unaffected siblings and is often used when there are no parents available (Spielman & Ewens, 1998).

Because LD requires that the marker be physically close to the disease-causing gene, researchers must either use a very dense map of markers or an a priori hypothesis (specific candidate) about the assumed function of the gene in a neurological system that is associated with a disorder (Barr et al., 2001). Most association studies in ADHD are driven by findings related to the effectiveness of stimulant medication in treating the disorder, indicating that the dopaminergic biochemical pathway plays a critical role in its aetiology. As a result, researchers have scrutinized the dopamine system looking for genes that might increase the susceptibility for the disorder. The fact that stimulants also influence the levels of noradrenaline and serotonin has put the focus on these systems as well and supports the notion that other neurotransmitters than DA play a vital role in the pathology of ADHD (Solanto, 1998, 2002). Variation in precursor, metabolite, receptor and transporter genes in these neurotransmitter systems can tilt the precarious balance that exists between these systems and increase the risk for developing ADHD (Waldman & Gizer, 2006).

Table 1: Candidate genes in ADHD

Gene location	Candidate gene	Positive findings	Trends	Negative findings
	Dopamine system			
5p15	Dopamine transporter (*SLC6A3*)	10	6	14
5q34-35	Dopamine receptor D1 (*DRD1*)	NR	3	2
11q22-23	Dopamine receptor D2 (*DRD2*)	2	1	3
3q13	Dopamine receptor D3 (*DRD3*)	NR	2	6
11p15	Dopamine receptor D4 (*DRD4*)	18	5	16
4p15	Dopamine receptor D5 (*DRD5*)	8	5	7
7p12	Dopa decarboxylase (*DDC*)	1	2	NR
11p15	Tyrosine hydroxylase (*TH*)	1	NR	4
	Serotonin system			
17q11	Serotonin transporter (*SLC6A4*)	7	2	4
6q13	Serotonin receptor 1B (*HTR1B*)	2	NR	NR
13q14-21	Serotonin receptor 2A (H*TR2A*)	3	2	3
Xq24	Serotonin receptor 2C (*HTR2C*)	NR	NR	1
	Noradrenaline system			
16q12	Noradrenaline transporter (*SLC6A2*)	NR	NR	6
8p11	Alpha 1C adrenergic receptor (*ADRA1C*)	NR	NR	1
10q24-26	Alpha 2A adrenergic receptor (*ADRA2A*)	2	NR	1
4p16	Alpha 2C adrenergic receptor (*ADRA2C*)	1	NR	1
Xp11	Monoamine oxidase A (*MAOA*)	3	1	1
Xp11	Monoamine oxidase B (*MAOB)*	NR	NR	1
9q34	Dopamine-beta-hydroxylase (*DBH*)	4	1	7
22q11	Catecholamine-O-methyltransferase (*COMT*)	3	1	1
	Other genes			
Xq11-12	Androgen receptor (*AR*)	NR	NR	2
15q14	Nicotinic acetylcholine receptor alpha 4 subunit (*CHRNA4*)	1	2	1
6q14-15	Nicotinic acetylcholine receptor alpha 7 subunit (*CHRNA7*)	NR	NR	3
16p13	Glutamate receptor, ionotropic, N-methyl D-aspartate 2A (*GRIN2A*)	1	2	4
10q11	Protein kinase G, cGMP-dependent, type I (*PRKG1*)	NR	NR	1
20p12-11	Synaptosomal-associated protein of 25 kDa (*SNAP25*)	5	2	7

Table adapted from (Bobb et al., 2006)

Positive findings $p < 0.05$

Trends $0.05 < p \leq 0.15$

Negative findings $p > 0.15$

NR – not reported

In linkage analysis, the correlation of a phenotype and alleles/markers is examined within families. Linkage can be defined as a genetic relationship that describes the tendency of a phenotype and markers to co-segregate in a pedigree because their determinants lie close together on a particular chromosomal region (Strachan & Read, 2004). In classical linkage studies, researchers use large, multigenerational family pedigrees, whereas in contemporary linkage studies multiple families (affected child, mother and father) or affected sib pairs are used (Craddock & Owen, 1996). Linkage studies can be divided into parametric and non-parametric studies. Parametric studies are based on precise models detailing gene frequency, mode of inheritance and penetrance of each genotype, whereas nonparametric studies are model-free and look for alleles or chromosomal regions that are shared by affected individuals (Waldman & Gizer, 2006).

When using linkage analysis it is important to distinguish between chromosomal sections that are identical by descent (IBD) from those that are identical by state (IBS). Alleles that are IBD are demonstrably copies of the same ancestral allele; whereas IBS alleles are exactly alike but their common ancestry is not demonstrable (Strachan & Read, 2004).

Affected sib pairs (ASP) analysis is a common nonparametric method that can be used to estimate the proportion of alleles shared IBD among siblings that have a particular disorder. The rationale behind ASP is that, if one randomly picks a chromosomal segment, siblings are expected to share 0, 1 or 2 parental haplotypes with the frequency of ¼, ½ or ¼, respectively. If both siblings have a genetic disorder, they are likely to share whichever chromosomal region that carries the disease locus (Strachan & Read, 2004; Waldman & Gizer, 2006).

Whole genome scans are usually the initial approach when doing linkage studies. Here researchers scan the whole genome with markers in search of regions that might harbour genes contributing to the aetiology of the disorder (Faraone & Asherson, 2005).

Those genomic segments showing no linkage between the chosen markers and the disorder can subsequently be excluded from further analysis. Those areas indicative of linkage can be scrutinized further by a new set of tightly grouped markers within the implicated region, thus narrowing the area that is linked to the disorder (Waldman & Gizer, 2006).

Table 2: Results from genome scans in ADHD

Genome studies	LOD score[6]	Chromosomal loci
Fisher et al., 2002	>1	2q22, 5p12, 4p15, 7p17, 10q26, 12p13, 12q24,
Bakker et al., 2003	>1	13q3 and **Xp22**
Arcos-Burgos et al., 2004	>1	3q13, 6q26, and 10cen
Hebebrand et al., 2006	>1	5q33
Asherson et al., 2008	>1	7q11, 8p23, 10q21, 12q24 and **Xp22**
		2q31, 11q12 and Xq27
Fisher et al., 2002	>1.5	10q26, 12q23 and **16p13**
Bakker et al., 2003	>1.5	4q16 and 13q33
Arcos-Burgos et al., 2004	>1.5	**17p11**
Asherson et al., 200	>1.5	2p24, 14q32, 16q12 and 21q22
Bakker et al., 2003	>2	9q33
Asherson et al., 200	>2	9q22
Ogdie et al., 2003	>2.5	**17p11**
Arcos-Burgos et al., 2004	>2.5	4q13 and 11q22
Hebebrand et al., 2006	>2.5	5p15
Smalley et al., 2002	>4	**16q13**

[6] LOD score - In genetics, a statistical estimate of whether two loci (the sites of genes) are likely to lie near each other on a chromosome and are therefore likely to be inherited together as a package. LOD stands for logarithm of the odds (to the base 10). A LOD score of three or more is generally taken to indicate that two gene loci are close to each other on the chromosome.

Despite the difficulties when it comes to replicating findings, molecular genetic studies during the last decades have been a major source of knowledge and improved our understanding of ADHD. However, the aetiology of ADHD is still beyond our grasp leaving us with a complex disorder where both gene-environment and gene-gene interactions play a significant role in its manifestation.

5. Environmental factors in ADHD

A wide range of environmental factors have been associated with eventual symptoms and development of ADHD, with studies estimating that environmental factors can account for around 20 to 30% of the variance in ADHD symptoms (Faraone & Asherson, 2005). These environmental risk factors can be divided into three main groups. The first group consists of pre- and perinatal events, such as prematurity, low birth weight, complications during pregnancy and birth, and finally mother's use of tobacco, alcohol or drugs during pregnancy. The second group includes family and parental factors, such as deprivation of normal parenting during infancy, childhood physical maltreatment, childhood neglect, inconsistent parenting, family conflict and violence, parental divorce and early institutional upbringing. The last group comprises acquired neurobiological risk factors such as head injury, substance abuse and toxic exposure (Kunsti & Asherson, 2004).

There is much heterogeneity in the way individuals respond to these environmental risk factors, and the key question in psychopathology is how does an environmental factor influence the nervous system to generate the symptoms seen in psychiatric diseases (Caspi & Moffitt, 2006).

The environmental factor most persistently linked to ADHD is prenatal maternal smoking (Linnet et al., 2003). Results from Milberger and coworkers indicate a 2.7-fold increased risk (Milberger et al., 1998) and findings from Weissman et al and Mick and colleagues reported a 2-fold increased risk for ADHD associated with prenatal maternal smoking (Mick et al., 2002; Weissman et al., 1999). Despite that studies have repeatedly indicated that smoking during pregnancy can adversely affect the developing fetus; the underlying biological processes are not fully understood (Neuman et al., 2007). Cigarettes include hundreds of different compounds, with the major psychoactive substance in tobacco being nicotine. Animal researches have clearly demonstrated the neurotoxic

effects of nicotine on the developing fetus (Olds, 1997). Prenatal exposure to nicotine may result in deleterious hypoxic, vascular and placental effects, leading to dysregulation in neurodevelopment inducing a higher risk for behavioural and psychiatric problems (Ernst et al., 2001).

With advances in neonatology, more children with low birth weigh (<2500 g) and very low birth weight (<1500 g) are surviving. Low birth weight (LBW) is a well-known risk factor for cognitive impairment and behavioural problems such as aggressiveness, depression and anxiety (Wiles et al., 2005). LBW is also thought to increase the risk for developing ADHD (Breslau, 1995; McCormick et al., 1996; Mick et al., 2002). Results from a meta-analysis of 16 case-control studies provide a further support for the notion that low birth weight increases the risk for ADHD significantly (Bhutta et al., 2002). Some studies have indicated that pregnancy and delivery complications raise the risk for ADHD. Birth complication can cause perinatal oxygen deficiency leading to alterations within the basal ganglia, which are very vulnerable to hypoxic-ischemic insults. These basal ganglia alterations may then lead to the emergence of ADHD symptoms (Biederman & Faraone, 2005).

Psychosocial factors such as, family dysfunction, marital distress, chronic conflict, low social class and exposure to maternal psychopathology, are also thought to play a role in the aetiology of ADHD (Banerjee et al., 2007; Langley, 2007).

Among the neurobiological risk factors often linked to ADHD is exposure to toxins such as lead. The neurotoxic effects of low levels of lead exposure on the developing brain are extensive, and can among others interfere with synapse formation (Bellinger, 1994). Several studies have shown that lead contamination can result in behavioural sequelae similar to the behaviour exhibited by ADHD children. However, most of the children with ADHD do not show signs of lead contamination, and even at relatively high levels of lead, minority of children are rated as being hyperactive (Needleman, 1982).

Furthermore, some of the studies focusing on the effects of lead on ADHD are plagued by methodological problems. The most prominent of them being that researchers did not use clinical criteria for the diagnoses of ADHD, but simply used behaviour ratings comprising only a small number of items of hyperactivity or inattention (Barkley, 1998).

6. Neuropsychology and ADHD

There is a consensus in the field of neuropsychology that neurocognitive impairments, particularly in the domain of executive functions, are common in ADHD. However, there is little agreement about the precise nature of these dysfunctions or their specificity to ADHD (Lawrence et al., 2004), with impairments ranging from the most basic levels of behaviour management to more subtle impairments affecting memory, organization and planning abilities (Brown, 2002).

The term executive functions (EF) refers to higher order cognitive processes that lay the foundation for self regulation and goal-directed behaviour, comprising processes such as working memory, set shifting, response inhibition, planning, organization, fluency and certain aspects of attention (Loring, 1999).

Pennington and Ozonoff, reviewed the literature on EF and reported that 15 of 18 studies found a significant difference between ADHD and controls on one or more measures, where 40 of the 60 EF measures used revealed a significantly poorer performance in the ADHD group (Pennington & Ozonoff, 1996). The most consistently impaired domain in the ADHD group was inhibition. Among the most frequently used measures of inhibition in EF studies are tasks, such as the Stop Task and continuous performance tasks (Pennington, 2002). The continuous performance test (CPT) is a simple task requiring the child to observe a screen, over an extended period of time (15 min), while individual letters or numbers are projected onto it at rapid pace (usually one per second). The child is asked to press a button each time the target stimulus, i.e. the letter X appears after the letter A (Nigg, 2005).

A meta-analytic review of CPT research indicates a poorer performance as measured by omission and commission error rates in ADHD children compared to controls (Losier et al., 1996). Errors of commission are responses that occur when no response is required, and are assumed to reflect impulsivity. Whereas errors of omission, that is the absence of

a response to a target, are indicative of inattention (J. Epstein et al., 2002).

In the Stop Task, the subject is taught a particular response and then later is told to inhibit the very same response on a subset of trials. This paradigm allows for the computation of a stop signal reaction time (SSRT), or the time it takes to inhibit a response. Oosterlaan and Sergeant performed a meta-analysis of studies using the Stop Task and found that ADHD children had a consistent deficit on this task, supporting the notion that children with ADHD have an impaired response inhibition (Oosterlaan & Sergeant, 1998).

Another aspect of EF that is of interest in ADHD is working memory (WM). Working memory can be defined as a limited capacity memory system providing temporary storage that allows task relevant information to be maintained briefly when solving a complex cognitive task (Loring, 1999).

Among the most influential models of WM is Baddeley's multicomponent model, which consists of two subsidiary systems. The first system includes the phonological loop and visuo-spatial sketchpad and the second system includes the central executive. The phonological loop is a temporary storage for acoustic and speech-based information, whereas the visuo-spatial sketchpad is a similar system for visual information. The phonological loop and visuo-spatial sketchpad are passive slave systems for the central executive, which is responsible for encoding, storing and retrieving of information from long-term memory (Baddeley, 1986).

Marusiak and colleagues reported that children with ADHD performed significantly worse on a working memory factor on the Stanford-Binet V intelligence test when compared to controls, but performed similar on all other factors of the test. When the working memory factor was divided into verbal and non-verbal, then the ADHD children performed significantly worse on the non-verbal working memory factor (Marusiak & Janzen, 2005).

A recent meta-analysis of working memory found a strong effect sizes (Cohen's *d* = 1.06) for tests requiring manipulation of spatial working memory compared to a moderate effect size (Cohen's *d* = 0.43) for tests requiring manipulation of verbal working memory (Martinussen et al., 2005).

WM functions are thought to be highly dependent on frontostriatal brain regions (Lewis et al., 2004) and data also suggest that different neural structures are activated depending on the modality of the central executive task, with spatial tasks more dependent on the right hemisphere and verbal tasks on the left hemisphere (for review see; Fletcher & Henson, 2001). These results thus gain support from neuroimaging studies reporting reductions in prefrontal volume, predominantly in the right hemisphere (Castellanos et al., 1996; Filipek et al., 1997) and decreased activity in the right medial frontal cortex in ADHD individuals (Rubia, 1999).

6.1 Neuropsychological models of ADHD

Various models have been put forward in order to explain the mechanism underlying the EF impairments in ADHD. Among them are Barkley's hybrid model and Sonuga-Barke's dual-pathway model.

Barkley reviewed several models of executive functions and argued for their combination into a hybrid model when trying to explain the executive dysfunctions seen in ADHD (Barkley, 1997, 1998). According to this model, behavioural inhibition is essential to the proficient performance of executive functions that control the motor system in the initiation and performance of goal-directed, future-oriented behaviour.

Barkley divides behavioural inhibition into three inter-related processes. The first one inhibits the initial prepotent response to an event. Here, prepotent response is defined as a response for which immediate reinforcement (positive or negative) is available or has been previously associated with an event. The second one stops an ongoing response or response pattern, thereby permitting a delay in the decision to respond or continue

responding. The last one is some kind of interference control, protecting this period of delay and the self-directed responses that occur within it from disruption by competing events and responses.

The executive functions in the model are divided into four categories, each with several subcategories. These four executive functions are non-verbal working memory, which can be defined as the capacity to maintain internally represented information in mind to control a subsequent response. Verbal working memory can be defined as the internalisation of speech. Allowing the individual to covertly describe, label and verbally contemplate the nature of the event or situation prior to response. The self-regulation of affect, motivation and arousal arises as a consequence of the privatisation of emotion/motivation following an event. Finally, reconstitution allows the individual to assemble multiple potential responses for the solution of a problem or the realisation of a future goal. These executive functions can shift behaviour from control by the immediate environment to control by internally represented forms of information by their influence over the last component of the model, motor control (Barkley, 1997, 1998).

Recently, there has been an increase in theories focusing on other aspects than just executive dysfunctions in ADHD. Prominent among them is the dual-pathway model put forward by Sonuga-Barke. In this model, Sonuga-Barke distinguishes between motivational and executive processing pathways in an attempt to explain the symptoms in ADHD (Sonuga-Barke, 2002, 2005).

The executive pathway is typified by dysregulations of thought and action, with the core deficit in response inhibition, which is the ability to inhibit an inappropriate prepotent or ongoing response in favor of a more appropriate alternative. Response inhibition is regarded as a prerequisite for cognitive flexibility, self-control and emotional regulation.

The motivational pathway or the delay aversion hypothesis in Sonuga-Barke´s dual

pathway model represents a radical departure from the prevailing neuropsychological explanation of ADHD. In the motivational pathway, ADHD behaviours are considered to be the product of an underlying motivational style, characterized by delay aversion, rather than resulting from a dysfunctional inhibitory control. The delay aversion hypothesis suggests that the reward processes are suboptimal in ADHD and consequently predicts that when faced with a choice between immediacy and delay ADHD children will choose immediacy. Delay aversion can thus be evident as a negative emotional reaction to delay where the child attempts to avoid or escape delay by acting on the environment to make it more interesting, resulting in impulsive, inattentive and hyperactive behaviour. This notion is supported by findings that ADHD children display hypersensitivity to delay and find it difficult to work efficiently over extended periods of time, such as in school settings or when doing their homework (Kunsti et al., 2001; Sonuga-Barke et al., 1996).

In the delay aversion hypothesis cognitive deficits, evident in working memory problems and planning difficulties arise as secondary effects of delay aversion associated with patterns of reduced task engagement

The dual-pathway model is an attempt to give a theoretical account of the interactions between cortical and sub cortical circuits implicated in the regulation of motivation and executive processes in ADHD. At a neurobiological level, there is growing evidence indicating that the dorsolateral prefrontal circuit, reviewed previously, plays a vital role in the optimal functioning of inhibitory control and executive functions within the executive pathway, whereas the anterior cingulate and the orbitofrontal circuits play a significant part within the motivational pathway (Sonuga-Barke, 2002, 2005).

Findings from neuropsychological studies to date indicate that ADHD individuals have difficulties with some aspects of EF, mainly visual working memory and response inhibition. However, there is considerable heterogeneity among ADHD patients, with performance ranging from normal to severe impairments. This indicates that EF deficits

do not contribute to ADHD in all cases and factors such as family history of the disorder, co-morbidity and symptom dimensions being potentially associated with differential performance on EF measures (Doyle, 2006; Nigg, 2005).

Despite discrepancies in findings from EF tests, they provide valuable information regarding the strengths and difficulties of children with ADHD. Furthermore, tests assessing EF can aid researchers when it comes to narrowing down the ADHD phenotype by using so-called endophenotypes, which are constructs trying to bridge the gap between genes and behaviour.

7. ADHD and endophenotypes

ADHD is one of the most intensively studied disorders in child and adolescent psychiatry and despite being armed with powerful research tools we have had little success in definitely identifying genes or gene regions contributing to the development of ADHD (Castellanos , Glaser, & Gerhard, 2006). The current psychiatric classification systems are based on observable symptoms that ignore the underlying genetic or biological pathophysiology of the disorder and are furthermore oblivious to the complex cascade of events between the genetic underpinnings of a disorder and the eventual manifestation of observable symptoms. We have had some interesting and exciting findings but the field is marred with the problem of replicating and thereby confirming these findings. These contradictory findings and problems with replicating findings have lead to the conclusion that the current symptom based classification systems do not facilitate mapping between susceptibility genes and behavioural outcomes (Cornblatt & Malhotra, 2001). The search for an appropriate way to define psychiatric phenotypes in order to enhance the power of genetic studies is of crucial importance for understanding the genetic basis of disorders with complex inheritance (Skuse, 2001).

Instead of using observable behavioural phenotypes, a more suitable construct for genetic analyses might be an intermediary measure of neuropsychiatric functioning that is involved in the pathway between genotype and the outcome of interest.

In 1973, Gottesman and Shields described the concept endophenotypes as “internal phenotypes discoverable by a biochemical test or microscopic examination” (Gottesman & Shield, 1973). Whereas Skuse (2001) refers to endophenotypes as latent traits that carry genetic loading and which are related indirectly to the behavioral symptoms as defined in DSM-IV or ICD-10. The main idea behind the use of endophenotypes is to fill the gap between observable symptoms and genes, helping researchers to answer questions about etiological model (Castellanos & Tannock, 2002).

Several researchers have put forth what should characterise a practical endophenotype for genetic analyses. Most of these definitions have in common that the endophenotype should be reliably measurable, both over time and by different observers and should be continuously quantifiable. It should be heritable and found in non-affected family members at a higher rate than in the general population. It should predict the disorder probabilistically and have its base in neuroscience, enabling it to be more closely linked to the underlying genetic factors than the behavioural phenotypes described in current classification systems (Castellanos & Tannock, 2002; Gottesman & Gould, 2003; Skuse, 2001).

This new way of looking at complex disorders is gaining momentum, and researchers have come up with various definitions of cognitive endophenotypes in ADHD, among them is the shortened delay gradient endophenotype (Castellanos & Tannock, 2002).

Castellanos and Tannock proposed a causal developmental model for ADHD with shortened delay gradient as the candidate endophenotype and delay aversion as the primary behavioural manifestation. Delay aversion, or the inability to wait, is prominent among children with ADHD; it is manifested as a tendency to select an immediate reward over a larger reward for which the subject has to wait for. Shortened delay gradient is evident in children with ADHD, and can be explained as a fast decline in the effectiveness of reinforcement as the delay between behaviour and reward increases (Sagvolden et al., 2005).

As can be seen, then Castellanos and Tannock are trying to move away from the extensive ADHD behavioral phenotype described in current classifications systems, narrowing the phenotype and focusing on the easily measurable concept of delay aversion. The model has its biological basis within the reward system situated in the basal ganglia, where dopamine neurotransmission plays a major role. The shortened delay gradient could be the result of faulty neurotransmission where an overly active dopamine

transporter, possibly caused by a polymorphism in the dopamine transporter gene, rapidly removes dopamine from the synaptic cleft. Another possible explanation could be structural abnormalities within the basal ganglia or the cerebellar vermis, but both these areas are rich in dopamine transporters (Berquin et al., 1998; Mostofsky et al., 2002).

The lack of biological basis for the classification of psychiatric disorders has led, in part, to the lack of success in genetic studies of psychiatric disorders. The use of endophenotypes, based on neurophysiological, biochemical, neuroanatomical, cognitive or neuropsychological measures, appears to be a rational step moving us away from the behavioural classification of complex disorder. Endophenotypes can help us bridge the gap between behaviour and genes, bringing us a step closer to the biological causes and improving our understanding of the genetic mechanism of complex disorders.

8. Treatment in ADHD

As to date there is no "cure" for ADHD, therefore the goal of treatment is not to eliminate the underlying cause of the disorder, but to provide a way to manage the symptoms in an effective manner. Fundamental among the treatments available is the education of the family and school staff about the nature of the disorder and its management (Barkley & Murphy, 2006). Treatment of children with ADHD often requires a comprehensive approach involving medical, educational and behavioural interventions. The severity and type of ADHD along with the presence of co-morbid condition are among the deciding factors which interventions are necessary (Shelton & Barkley, 1993).

ADHD causes a lot of strife on the family life, whereas parent training provides technique to reduce parenting stress and improve social behaviour among children with ADHD (Chronis et al., 2006). Here, the focus has been on providing the families education about the disorder, teaching parents problem solving skill and ways to encourage pro social behaviour in their children through behavioural modification techniques (Barkely, 1998).

Classroom management of ADHD is often challenging, with the symptoms causing disruption for both the child's individual learning and for peers in classroom setting. In academic setting, the most commonly used interventions are task and instructional modification, homework assistance, peer tutoring, computer-assisted instruction and strategy training (Culbertson & Krull, 1996). Another important non-medical approach used in treating children with ADHD is known as behaviour therapy or behaviour management. Behaviour therapy is based on several simple and sensible notions about what leads children to behave in socially appropriate ways. One reason is that children generally want to please their parents and feel good about themselves when their parents are proud of them. A second reason that children behave appropriately is to obtain

positive consequences, i.e. privilege or rewards, for doing so. Finally, children will behave appropriately to avoid the negative consequences that follow inappropriate behaviour.

Therefore, the goal of behaviour therapy is to increase the frequency of desirable behaviour by increasing the child's interest in pleasing parents and by providing positive consequences when the child behaves. Inappropriate behaviour is reduced by consistently providing negative consequences when such behaviour occurs (Martin & Pear, 1992).

The effectiveness of psycho-stimulants in the treatment of ADHD-like symptoms has been known for nearly 70 years or since Charles Bradley subscribed Benzedrine to children at the Emma Pendleton Bradley Home for Children (Bradley, 1937). In 1995, it was estimated that around 2.8% of children in the USA between the age of 5 and 18 years old were prescribed stimulants, by far the most common treatment for ADHD (Goldman et al., 1998).

Empirical data consistently demonstrate the efficacy of the stimulants in improving behavioural, academic and social functioning in about 70 to 80% of ADHD children, depending on the presence of co-morbid psychiatric and/or developmental disorders (Barkley, 1998).

The most common of these psycho-stimulant compounds are methylphenidate (i.e., Ritalin®) and dextroamphetamine (i.e., Dexedrine®). The precise mechanism of how these compounds work is still poorly understood, but they are mainly thought to block the dopamine transporter (SLC6A3), which is responsible for removing dopamine from the synaptic cleft.

Grace proposed a tonic-phasic model (see chapter 3.1 - The dopamine system) to explain the function of stimulants in ADHD. In a response to an action potential, DA is released in the synaptic cleft (phasic level), where it is quickly removed by the SLC6A3. On the other hand, the tonic level of DA is thought to be mediated by stimulation of the

presynaptic heteroreceptors on DA terminal by corticostriatal glutamatergic projections. Tonic DA exerts a suppressive influence on sub cortical DA systems, and the response of ADHD children to stimulants may be achieved by altering the tonic/phasic relationship to achieve more optimal regulatory levels. According to Grace, the hyperactivity and impulsivity seen in ADHD results from low tonic DA activity within the ventral striatum/nucleus accumbens, leading to abnormally high phasic DA responses (Grace, 2000, 2001).

The side effects of these psycho-stimulants are usually short term and rare, and include reduced appetite, insomnia, edginess and gastrointestinal upset. However, the effects of long-term use of psycho-stimulants are not very well documented. Psycho-stimulants have an effect on both heart rate and blood pressure, and some have expressed concerns about the effects that chronic stimulant medication might have on the developing cardiovascular system in children (Dupaul et al., 1998).

Despite overwhelming evidence for the efficacy of stimulants, it should be noted that as many as 20 to 30% of children tried on stimulants may display no response to the medications or may display worsening in behaviour in response to medication (Barkley, 1998). Contraindications for psycho-stimulant medication include children under the age of four, those with a personal or family history of tics, or thought disorder, children with severe co-morbid behavioural problems and children with internalizing symptoms (Spencer et al., 1998). For most cases, one of the greatest benefits of stimulant therapy seems to be the theoretical possibility of maximizing the effects of concurrently applied psychosocial and educational treatments [MTA Study] (Group, 1999).

For those children that do not respond favourably to stimulant medication there are various types of non-stimulant medications available, including tricyclic antidepressants (TCAs), atomoxetine and alpha-2 agonists (Faraone et al., 2006).

Tricyclic antidepressants that include compounds such as desipramine (i.e., Norpramin®) and imipramine (i.e., Tofranil®), have been used when individuals can not tolerate stimulant treatment or the children have co occurring internalizing symptoms, such as anxiety or depression (Barkley, 1998).

Atomoxetine (Strattera®) is a non-stimulant that blocks the norepinephrine transporter, which is believed to attenuate ADHD symptoms by increasing norepinephrine in the synaptic cleft. Among common side effects following Strattera® use are nausea, tiredness and reduced appetite (Michelson et al., 2002). There have also been reports of serious side effects such as increased suicidal risk and liver damage among those taking Strattera®, but these side effects are very rare.

Other compounds influencing the noradrenergic system have also been helpful in reducing the symptoms of ADHD. Alpha-2 agonists, such as clonidine (Catapres®) and guanfacine (Tenex®), have been beneficial when stimulants and tricyclics may be contra-indicated, because of side effects, tics or in very aggressive children (Stahl, 2000).

Through countless research, valuable knowledge has accumulated providing us with both a better understanding of the pathology in ADHD and leading to improvements in treatment options. There are several treatment options available for children with ADHD, with the most common form for treatment being stimulants. However, they are no panacea for treating the behavioural and attentional difficulties associated with ADHD, or should they be the sole form of therapy for individuals with the disorder.

As can be seen from the above, ADHD has been a source of discussion and debate for a long time, and despite numerous studies over the last decades the aetiology of ADHD still remains elusive. This elusiveness can be partly explained by the nature of ADHD, where it is a highly heterogeneous disorder, compounded by a significant risk for other psychiatric disorders, obscuring the boundaries from one disorder to the next.

Part II.

9. Aim of the study

There is converging evidence indicating that neurobiological factors are among the main mechanisms underlying the symptoms of ADHD, where many genes of small effect contribute to the disease susceptibility (Comings, 1990; Fisher et al., 2002).

Findings from family-, twin- and adoption- studies indicate that the heritability in ADHD is around 70 - 80%, with environmental risk factors accounting for nearly 20 to 30% of the variance in ADHD symptoms (Faraone & Asherson, 2005). Those environmental factors most persistently linked to ADHD are prenatal maternal smoking and low birth weight, with studies indicating higher rates of ADHD diagnoses and symptoms in those children exposed to nicotine during pregnancy (Linnet et al., 2003) or born with low birth weight (Bhutta et al., 2002). Psychosocial factors such as marital distress, chronic conflict, low social class and childhood maltreatment are also thought to play a role in the etiology of ADHD (Banerjee et al., 2007; Biederman & Faraone, 2005). Furthermore, childhood maltreatment is considered a risk factor for CD/ODD and the earlier children experience maltreatment, the more likely they are to develop CD/ODD (Caspi et al., 2002; Keiley et al., 2001). In addition to this, animal studies show that early life maltreatment stress can alter monoaminergic neurotransmitter systems and influence aggressive behaviour (Bennett et al., 2002; Bremner, 2003).

With regard to genetic risk factors, the efficacy of stimulants in the treatment of ADHD supports the notion that imbalance in the catecholamine system may play a pivotal role in the pathophysiology of the disorder (Grace, 2001). The catechol-*O*-methyltransferase (*COMT*) gene plays a crucial role in the metabolism of catecholamines in the prefrontal cortex (Grossman, Emanuel, & Budarf, 1992), which has been implicated in ADHD (Biederman et al., 2004) and the genesis of CD/ODD (Raine, 2002). The *COMT* gene is localized on chromosomal region 22q11.2 and it codes for two

proteins, soluble (S-COMT) and membrane bound (MB-COMT; Grossman et al., 1992). There are two promoters, P1 and P2 that control transcription of two different mRNAs (Tenhunen et al., 1994) A longer mRNA from the P2 promoter encodes mainly the MB-COMT, and a shorter mRNA from the P1 promoter encodes the S-COMT. A coding non-synonymous G/A single nucleotide polymorphism (Val^{158}Met SNP, rs4680) in codon 158 of the MB-COMT encodes the amino acids methionine (Met) or valine (Val). The Met/Met-genotype leads to a 3- to 4fold reduction of *COMT* activity compared to the Val/Val-genotype and those heterozygous having intermediate activity (Lachman et al., 1996). The *COMT* Val^{158}Met SNP has been extensively researched for its association with complex mental disorders such as ADHD, schizophrenia and conduct disorder. According to a recent meta-analysis, there does not appear to be a significant association between the *COMT* Val^{158}Met SNP and ADHD (Cheuk & Wong, 2006). Recent findings, on the other hand, indicate that 22q11.2 deletion syndrome (22q11.2 DS), which is caused by a hemizygous micro-deletion in the long arm of chromosome 22, is associated with increased risk of developing ADHD (Antshel et al., 2006; Niklasson et al., 2008). Studies also suggest that the Met allele, encoding for less active COMT enzyme with resulting increase in dopamine in the frontal lobe (Carlson et al., 1997), is significantly more prevalent in 22q11.2DS patients with ADHD compared to those without ADHD (Gothelf et al., 2007; Michaelovsky et al., 2007). Furthermore, Ettinger et al and Reuter et al found that individuals without a history of psychiatric disorders carrying the *COMT* Met/Met genotype had higher scores on an ADHD self-report scale (ASRS) compared to those with the Val/Val and Met/Val genotypes (Ettinger et al., 2006; Reuter et al., 2006).

The covariation between ADHD and aggression/CD is thought to be partly accounted for by a common genetic factor (Nadder et al., 2002), with the *COMT* Val^{158}Met SNP suspected of influencing the expression of aggression in ADHD (Thapar et al., 2005). Catecholamines are thought to lower the threshold for an aggressive response to

environmental stimuli and if aggressive behaviour is enhanced by catecholaminergic activity, then lower activity of *COMT* should indirectly enhance aggression (Bilder et al., 2004). Evidence from animal studies supports this notion, where increased levels of dopamine were associated with aggressive behaviour in *COMT* knock-out mice (Gogos, 1998).

Despite conflicting results, *COMT* can be regarded as one of the candidate genes for the regulation of aggression, with evidence available to date largely supporting the hypothesis that low COMT activity indirectly enhances aggression (Volavka et al., 2004).

As *COMT* was associated with ADHD as well as CD symptoms in previous studies, the aim of the this study is to assess if the *COMT* Val^{158}Met SNP is a risk factor for ADHD as well as for severity of ADHD symptoms and co-morbid CD in children with ADHD.

First, it is hypothesized, that the *COMT* Val^{158}Met SNP is associated with ADHD in this family based study of 166 children with ADHD and their parents. Secondly, it is predicted that ADHD children with the *COMT* Met/Met genotype will have more severe ADHD symptoms and are more likely to have co-morbid CD than those children with either Val/Met or Val/Val genotype. Finally, as interaction of *COMT* genotypes with birth weight has been reported, we aimed to assess the same interaction as well as further genotype*environment interactions factors (smoking, alcohol during pregnancy, SES and early familial risk factors) for association with ADHD symptom severity and co-morbid CD and ODD.

10. Material and Methods

10.1 Sample

The orginal sample consisted of 663 individuals, composed of children diagnosed with ADHD according to DSM-IV (APA, 1994), their parents and siblings. For the present study, prenatal history, psychosocial and environmental risk factors were assessed in 166 clinically referred, unrelated children with ADHD aged between 6 and 13 years and their parents. Children with a birth weight < 2000 g, born preterm before pregnancy week 32, with any severe pre-, peri- or postnatal biological or medical risk factors (including severe chronic medical condition of mother, illegal drug abuse during pregnancy), autism-spectrum disorder, psychotic symptoms, history of epilepsy, mental retardation (IQ < 70), a known genetic syndrome or any other severe medical condition were excluded from the study.

Participants were recruited from the Department of Child and Adolescent Psychiatry at the Mutterhaus in Trier and the Department of Child and Adolescent Psychiatry at the Saarland University Hospital. Further assessment took place either at the Department of Neuro behavioral Genetics at the Institute of Psychobiology at the University of Trier or at the Saarland University Hospital. Informed consent for participation and publication was obtained by the parents. The study protocol was approved by the local Ethical Committee (*Ethikkommission der Ärztekammer des Saarlandes*).

10.2 Methods

A number of diagnostic instruments were administered when gathering data from the participants. Furthermore, polymerase chain reaction, restriction endonuclease digestion and gel electrophoresis were performed with DNA from the participantes to identify their genotypes from the $Val^{158}Met$ single nucleotide polymorphism ($Val^{158}Met$ SNP, rs4680).

10.2.1 Diagnostic Instruments

- Diagnostisches Interview bei psychischen Störungen bei Kindern und Jugendlichen (Kinder-DIPS) - answered by parents
- Hyperkinetic Syndrome check list (DCL-HKS) - answered by parents
- Conners rating scale – answered by parents and teachers
- Strength and Difficulties Questionnaire (SDQ) - answered by parents and teachers
- Childhood Behaviour Checklist (CBCL) - answered by parents
- Teacher Rating Format (TRF) - answered by teachers
- Kaufman Assessment Battery for Children (K-ABC)

10.2.2 The Kinder-DIPS

The Kinder-DIPS (Unnewehr et al., 1995) is a structured diagnostic interview designed to assess current and past episodes of psychopathology in children and adolescents according to ICD-10 and DSM-IV criteria. Probes and objective criteria are provided to rate individual symptoms. The main psychiatric disturbances assessed with the Kinder-DIPS are:

1. Behavioural disturbances
    a. Attention Deficit Hyperactivity Disorder
    b. Oppositional Defiant Disorder
    c. Conduct Disorder

2. Enuresis/Encopresis
    a. Functional Enuresis
    b. Functional Encopresis

3. Affective disturbances
    a. Severe Depressive episode (SDS)
    b. Dysthymic Syndrome (with and without SDS)

4. Anxiety disturbances
    a. Separation Anxiety
    b. Panic syndrome without Agoraphobia
    c. Panic syndrome with Agoraphobia
    d. Agoraphobia without Panic Disorder
    e. Specific phobia
    f. Social phobia

g. Obsessive-Compulsive Disorder
h. Generalized Anxiety
i. Posttraumatic Stress Disorder

5. Eating disturbances
    a. Anorexia nervosa
    b. Bulimia nervosa

10.2.3 Hyperkinetic Syndrome check list (DCL-HKS)

A German Hyperkinetic Syndrome check list (DCL-HKS), rating each of the 18 DSM-IV derived symptom on a scale between 0-3, a score of 3 indicating most severe problems (Döpfner & Lehmkuhl, 1998). Symptom reports were obtained both prior to starting medication and when the child was on medication.

10.2.4 Conners rating scale

Parents and teachers filled in the Conners rating scale, which is a questionnaire focusing on DSM-IV derived ADHD symptoms to ensure pervasiveness of ADHD symptoms across different settings. The parents' short version contains 27 items and the teachers' short version has 28. The results are summerized in four factors, Hyperactive, Cognitive Problems/Inattention, Conduct Problems and ADHD index. The results are given in T scores with a mean of 50 and a standard deviation of 10. T-scores between 60 and 70 are a cause for concern and have interpretive value, while scores above 70 indicate significant imapairments (Conners, 1997).

10.2.5 Strength and Difficulty Questionnaire (SDQ)

The SDQ is a brief behavioural screening questionnaire for 3-16 year olds. It exists in several versions to meet the needs of researchers, clinicians and educationalists. All versions of the SDQ ask about 25 attributes, some positive and others negative. These 25 items are divided between 5 scales:

- Emotional symptoms (5 items; score ranges from 0 to10 points)
- Conduct problems (5 items, score ranges from 0 to10 points)
- Hyperactivity/inattention (5 items, score ranges from 0 to10 points)
- Peer relationship problems (5 items, score ranges from 0 to10 points)

These four scales are added together to generate a total difficulties score ranging from 0 to 40 points, where a score of 17 or more is indicative of significant problems.

- The fifth scale assesses prosocial behaviour (5 items, score ranges from 10 to 0) - here a score of 10 is indicative of good prosocial behaviour and a score of 0 is indicative of poor prosocial behaviour (Goodman, 1997).

10.2.6 Child Behaviour Checklist and Teacher Rating Format

Parents filled out the Child Behaviour Checklist for ages 4-18 (CBCL 4-18) and teachers filled out the Teacher Rating Format (TRF). The CBCL 4-18 obtains reports from parents regarding children's competencies and behavioral/emotional problems. Parents provide information for 20 competence items covering their child's activities, social relations and school performance. The CBCL 4-18 has 118 items that describe specific behavioural and emotional problems. Parents rate their child for how true each item is now or within the past 6 months using the following scale: 0 = not true (as far as you know); 1 = somewhat or sometimes true; 2 = very true or often true.

The CBCL 4-18 scoring profile provides raw scores, *T* scores (a mean of 50 and a standard deviation of 10) and percentiles for three competence scales (Activities, Social and School), Total Competence, eight cross-informant syndromes, and Internalizing, Externalizing and Total Problems. The cross-informant syndromes scored from the CBCL 4-18 are Aggressive Behavior; Anxious/Depressed; Attention Problems; Rule-Breaking Behaviour; Social Problems; Somatic Complaints; Thought Problems and Withdrawn/Depressed.

The TRF has 118 problem items, of which 93 have counterparts on the CBCL 4-18. The remaining items concern school behaviors that parents would not observe, such as difficulty following directions, disturbs other pupils, and disrupts class discipline. Teachers rate the child for how true each item is now or was within the past two months, using the same three-point response scale as for the CBCL 4-18.

The TRF is designed to obtain teachers' reports of children's academic performance, adaptive functioning and behavioral/emotional problems. Teachers rate the child's academic performance in each subject on a five-point scale ranging from 1 (far below grade level) to 5 (far above grade level). Space is also provided for reporting cognitive and achievement test scores for the child, if available. For adaptive functioning, teachers use a seven-point scale to compare the child to typical pupils for how hard he/she is working, how appropriately he/she is behaving, how much he/she is learning, and how happy he/she is.

The TRF scoring profile provides raw scores, *T* scores (T score of 50 corresponds to the mean and the standard deviation is 10), and percentiles for Academic Performance, Total Adaptive Functioning, the eight cross-informant syndrome scales as in the CBCL and Internalizing, Externalizing and Total Problems.

On both the CBCL 4-18 and the TRF, scores that are 64 and below are in the normal range. Scores that are between 65 and 69 are in the borderline clinical range and scores that are 70 and above are in the clinically significant range (Achenbach, 1991).

10.2.7 The Kaufman Assessment Battery for Children (K-ABC)

The Kaufman Assessment Battery for Children (K-ABC) is a standardized test that assesses intelligence and achievement in children aged two years, six months to 12 years, six months. Administration of the K-ABC takes between 35 and 85 minutes. The older the child, the longer the test generally takes to administer. It is comprised of four global test scores that include:

- Sequential processing scales
- Simultaneous processing scales
- Achievement scales
- Mental processing composite

There is an additional nonverbal scale that allows applicable subtests to be administered through gestures to hearing impaired, speech/language impaired, or children who do not speak English.

The test consists of 16 subtests—10 mental processing subtests and six achievement subtests. Not all subtests are administered to each age group, and only three subtests are administered to all age groups. Children from age two years, six months are given seven subtests, and the number of subtests given increase with the child's age. For any one child, a maximum of 13 subtests are administered. Children from age seven years to 12 years, six months are given 13 subtests.

The Sequential processing scale primarily measures short-term memory and consists of subtests that measure problem-solving skills where the emphasis is on following a sequence or order. The child solves tasks by arranging items in serial or sequential order including reproducing hand taps on a table, and recalling numbers that were presented. It also contains a subtest that measures a child's ability to recall objects in correct order as presented by the examiner.

The Simultaneous processing scale examines problem-solving skills that involve several processes at once. The seven subtests comprising this scale are, facial recognition, identification of objects or scenes in a partially completed picture, reproduction of a presented design by using rubber triangles, selecting a picture that completes or is similar to another picture, memory for location of pictures presented on a page and arrangement of pictures in meaningful order.

The Achievement scales measure achievement and focus on applied skills and facts

that were learned through the school or home environment. The subtests are expressive vocabulary; ability to name fictional characters, famous persons and well-known places; mathematical skills; ability to solve riddles; reading and decoding skills and reading and comprehension skills.

The Sequential and Simultaneous processing scales are combined to comprise the mental processing composite. This composite measures intelligence on the K-ABC and concentrates on the child's ability to solve unfamiliar problems simultaneously and sequentially. The simultaneous processing scales have a greater impact on the mental processing composite score than do the sequential processing scales. The mental processing composite score is considered the global estimate of a child's level of intellectual functioning (Kaufman & Kaufman, 1983).

10.2.8 Axis 5 Psychosocial interview

Psychosocial risk factors were assessed by a semi-structured, detailed interview with the parents or mother on the psychosocial axis (Axis V) of the WHO multiaxial classification system showing good reliability (Goor-Lambo, 1987; Poustka et al., 1994). This interview lists nine abnormal psychosocial situations associated with psychopathology: abnormal intrafamilial relationships; mental disorder, deviance or handicap in the child's primary support group; inadequate or distorted intrafamilial communication; abnormal qualities of upbringing; abnormal immediate environment; acute life events; societal stressors; chronic interpersonal stress associated with school/work and stressful events/situations resulting from the child's disorder.

In this study, a summary score of risk factors during the first 3 years of life was formed to assess the influence of early family risk factors. This variable focuses on abnormal intra-familiar relationship patterns, distorted communication within the family and parental separation/divorce or institutional education outside the family during the first three years of life.

10.2.9 Socio-Economic-Status (SES)

Socio-economic status (SES) of the family was allocated by occupational status of both parents.

10.2.10 Parental ADHD

Current parental ADHD was assessed by two self-report questionnaires on current (ADHS-SB; Rosler et al., 2004) and childhood ADHD symptoms (WURS-K; Retz-Junginger et al., 2003).

10.2.11 ADHD-SB

The ADHS-SB is a 22 item questionnaire focusing on current ADHD symptoms, with 18 of these items derived from the current DSM-IV and ICD-10 ADHD criteria. The remaining items focus on the age of onset, strain of symptoms and how they affect daily life, work and social function. Each item is rated as following: 0= no effect, 1=small effect, 2=medium effect and 3=severe effect upon daily life. The score is then added up and can range from 0 to 66. In this study, a cut-off score of 15 was used as an indication of ADHD problem. According to Rosler et al (2004), this cut-off score has a sensitivity of 77% and specificity of 75%.

10.2.12 Wender Utah Rating Scale - short version

Wender Utah Rating Scale- short version (WURS-K) is a 25 item list assessing childhood ADHD symptoms on a scale of 0 (no effect) to 4 (severe effect), with the score ranging from 0 to 100. In this study a cutoff score of 30 was used as an indicator of childhood ADHD. This score has a sensitivity of 85% and specificity of 76% (Retz-Junginger et al., 2003).

Parental ADHD was categorized in probably present (WURS-K > 30 points; meeting criteria in the ADHS-SB >15 points), possibly present (WURS-K > 30 but not meeting criteria in the ADHS-SB or ADHS-SB missing; meeting criteria in the ADHS-SB but not meeting criteria in the WURS-K or WURS-K missing) and definitely absent. Data from both parents were assessed together.

10.3 Equipment and chemicals for biological methods

Apparatives	Manufacturer
D-50 Digital Camera	Nikon, Düsseldorf
Electrophoresis Power Supply EPS200	Pharmacia Biotech, Freiburg
Gel rack LKB GNA 100	Pharmacia Biotech, Freiburg
Gene Quant 2 RNA/DNA Calculator	Pharmacia Biotech, Freiburg
Gel chamber Multiphor 2	Pharmacia Biotech, Freiburg
Gene Amp® PCR-System 9700	PE Applied Biosystems, Weiterstadt
Microwave	Sharp, Hamburg
Thermoshaker 5436	Eppendorf, Hamburg
Centrifuge Z 233 M	Hermle, Wehingen
UV-Illuminator N-90M	INTAS UV – Systeme, Wiesloch
Vortex Genie2	Scientific Industries, Bohemia/NY
Avanti Js	Beckman, Krefeld
Scale L420P	Sartorius Laboratory, Göttingen
Heat Block Schutron	Schnipptherm Wolf, York/UK

Reagents	Manufacturer
Agarose Roti®garose NEEO	Fa. Roth, Karlsruhe
Ammonium chloride (NH_4Cl)	Fa. Roth, Karlsruhe
Bovine Serum Albumin (BSA)	New England Biolabs, Frankfurt am Main
Bromphenolblue-Na-Salt (BPB)	Serva, Heidelberg
Ethylenediaminetetraacetic acid (EDTA)	Merck, Darmstadt
Dimethyl sulfoxide (DSMO; C_2H_6OS)	Fa. Roth, Karlsruhe
Ethidium bromide	Fa. Roth, Karlsruhe
Ethanol Rotipuran® (C_2H_6O)	Fa. Roth, Karlsruhe
Ficoll 400	Promega, Mannheim
Gene Ruler^{TM} 100bp DNA Ladder Plus	Fermentas, St. Leon-Roth
Hydrochloic Acid (HCl)	Fa.Roth,Karlsruhe
Magnesium chloride ($MgCl_2$)	Fa. Roth, Karlsruhe
*Hsp*92II	Promega
Kaliumchlorid (KCl)	Fa. Roth, Karlsruhe
Kaliumhydrogencarbonat(KHCO3)	Fa. Roth, Karlsruhe
2-Propanol (C_3H_8O)	Fa.Roth, Karlsruhe
Pronase E	Sigma-Aldrich,Deisendorf
Sodium2-EDTA	Fa. Roth, Karlsruhe
Sodium chloride (NaCl)	Sigma-Aldrich, Deisenhofen
Sodium Dodecyl Sulphate (SDS)	Fa. Roth, Karlsruhe
Taq-Polymerase Amplitaq Gold^{TM}	PE Applied Biosystems, Weiterstadt
TE-Buffer	Fa. Roth, Karlsruhe
TBE Buffer (10x) Rotiphorese®	Fa. Roth, Karlsruhe
Tris ($C_4H_{11}NO_3$)	Fa. Roth, Karlsruhe
Tween®20	Fa. Roth, Karlsruhe
Xylencyanol	Serva, Heidelberg

PCR-buffers 10x concentration:

	Tris-HCl (pH 8.3)	KCl	Tween20	BSA	$MgCl_2$
Buffer A	100 mM	500mM	0.25%	0.25 mg/ml	7.5 mM
Buffer B	100 mM	500mM	0.25%	0.25 mg/ml	10 mM
Buffer C	100 mM	500mM	0.25%	0.25 mg/ml	15 mM
Buffer D:	100 mM	500mM	0.25%	0.25 mg/ml	20 mM

Kits:

dNTPs (ATP, CTP, GTP, TTP) Fermentas, St. Leon-Roth

Primers:

Forward (# 220) 5'-ACT GTG GCT ACT CAG CTG TG-3'
Reverse (# 221) 5'-CCT TTT TCC AGG TCT GAC AA-3'

10.4 Isolation of genomic DNA

Blood samples, 1 to 9 ml, were taken from all participants by medical staff. DNA was extracted from whole blood according to the salting out procedure (Miller et al., 1988).

The first step in this method is the selective lysis of the erythrocytes with 4°C red cell-lysisbuffer (RC-lysisbuffer, 155 mM NH_4Cl,10 mM $KHCO_3$, 0.1 mM EDTA, ph 7.4) leaving the leukocytes intact. Each blood sample and RC-lysisbuffer is pipetted into a sterile 50 ml tube (mixing ratio 1:3). The samples are mixed gently by inversion and incubated for 15 minutes on ice. After incubation the tubes are centrifuged for 15 minutes at 1500 rpm at 4°C (Centrifuge, J-25, Fa. Beckmann, Krefeld). The supernatant is carefully removed, leaving behind a visible leukocytes pellet at the bottom of the tube. White cell lysisbuffer (10 mM Trish HCl pH8, 400 mM NaCl, 2 mM Na_2EDTA-buffer pH 8.2), 10% sodium dodecyl sulphate (SDS) solution and Pronase E (20 µg/ml) is added to the leukocyte pellet and vortexed thoroughly until the pellet is dissolved. The sample is then incubated and shaken at 37°C overnight (Onkyo/Gallenkamp, UK). The next step is to precipitate the proteins by adding saturated NaCl (6M) to the tube, vortex it thoroughly and centrifuge it for 20 minutes at 4000 rpm at 20°C, separating a protein

pellet at the bottom of the tube. The supernatant, which contains the DNA, is then carefully poured into a new 50 ml tube. The supernatant is centrifuged again, cleaning it from proteinaceous particles. Finally, 100% Isopropanol is pipetted to the sample, and gently mixed by inversion until the DNA strands precipitate in the solution. A sterile blunt-ended glass rod is used to "fish" the DNA strands carefully out of the alcohol. The DNA is then dried for around 30 seconds or until the alcohol is no longer visible and transferred into a sterile 1.5 ml tube containing TE-buffer (10 mM Tris-HCl pH 8, 0.1 mM EDTA).

After the DNA has dissolved in the TE-buffer, an aliquot of the sample (5 µl DNA) is diluted 1:20 with UV treated water to measure the quality and concentration of the DNA by Spectrometer (UV/VIS-Spectrometer, Fa. Pharmacia, Uppsala). Readings at 260 nm allows calculation of the concentration of nucleic acid in the sample and readings at 280 nm gives the amount of protein in the sample. For this reason, the ratio of 260/280 nm absorbance is a good general indicator of the relative purity of a solution. In this study, the absorbance ratio was usually between 1.6 and 1.9. Finally aliquots with a concentration of 20 ng DNA per µl were made and stored at 4°C, with the stock DNA being stored at minus 20°C.

10.5 Polymerase Chain Reaction (PCR)

PCR is a simple and widely used method which makes it possible to amplify specific regions of a DNA strand (DNA target) to use for further analyses. The most important reagent for a PCR is the *Taq* polymerase. This enzyme is extracted from bacteria (*Thermus aquaticus*) that live in hot springs and, unlike human polymerase which denaturates at 42°C, can tolerate temperatures of up to 110°C.

A PCR consists of three steps that are repeated in 35 to 40 cycles: denaturation, annealing and elongation/extension. The denaturation step is performed at 94°C to “melt” the DNA template. This disrupts the hydrogen bonds between complimentary bases of

DNA strands and results in single stranded DNA. During the annealing step, the temperature is lowered which allows annealing of primers to the single stranded DNA template. Primers, also called oligonucleotides, are short (18 to 35 nucleotides) synthetic DNA fragments that are complementary to the 5´and 3´ ends of the DNA region that is supposed to be amplified.

The optimal temperature for annealing of the primers is between 50 and 68°C, depending on the length and base structure of the fragment. For the elongation step, the reaction temperature is raised to 72°C because that is the optimum activity temperature for the *Taq* polymerase. The enzyme synthesizes a strand of new DNA that is complementary to the template by attaching complementary dinucleotidetriphosphates (dNTP´s) to the template in the 5´to 3´ direction. With each cycle the amplification of the DNA fragment increases exponentially. After the last cycle, the PCR is completed with a final elongation step to ensure that all single-stranded DNA is completely extended. After that the PCR-product is kept at 4°C for storage.

10.6 COMT PCR

SNP rs4680 G/A → Val/Met

The following primers were used in the PCR:

5'-ACT GTG GCT ACT CAG CTG TG-3' (forward)

5'-CCT TTT TCC AGG TCT GAC AA-3' (reverse)

ACTGTGGCTACTCAGCTGTGCGCATGGCCCGCCTGCTGTCACCAGGGGCGAGGCTCA

TCACCATCGAGATCAACCCCGACTGTGCCGCCATCACCCAGCGGATGGTGGATTTCGCTG

GCGTGAAGGACAAGGTGTGCATGCCTGACCC**GTTGTCAGACCTGGAAAAAGG rs4680 G/A**

Expected PCR-product 169 bp

The PCR was done in a 50 µl reaction mixture containing:

100 ng of genomic DNA, 200 µM of each dNTP´s, 10 pmol of each primer, 0.5 U *Taq*-polymerase, Buffer C (KCl 50 mM, Tris-HCl 10 mM, Tween 20 0.025%, BSA 0.025 mg/ml, and 1.5 mM $MgCl_2$) and 5% dimethylsulfoxide (DMSO).

To reduce measuring errors a master mix of all reagents – except the DNA templates – was prepared. All the individual amounts were multiplied by the number of DNA samples in the PCR (plus 1 to make up for losses), then mixed, vortexed and distributed between reaction tubes.

The PCR reaction had an initial temperature of 94°C (5 min), followed by 40 cycles of denaturation (95°C, 30 s), annealing (57°C, 30 s), and extension (72°C, 30 s). An extension period of 7 min at 72°C followed the final cycle. The PCR reactions were done using an ABI GeneAmp®9700 cycler.

10.7 Agarose gel electrophoresis

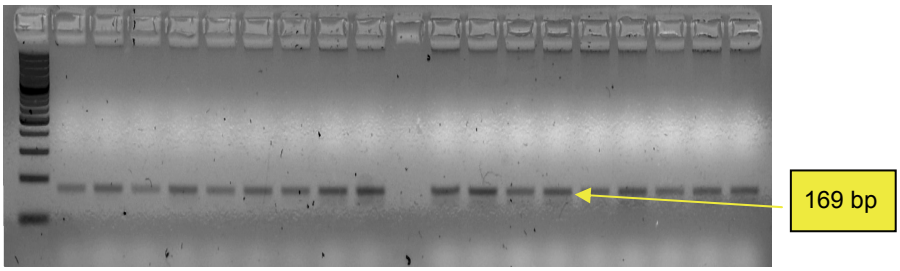
Agarose gel electrophoresis can be used in order to separate DNA fragments and to ascertain their lengths. In this method, an electrical current is applied to an agarose gel matrix through which the negatively charged DNA molecules move to the positive anode. Since shorter fragments migrate faster and further through the matrix than longer ones, different sized molecules can be detected as separate bands. Their lengths can then be estimated with a DNA size marker, which consists of DNA fragments of defined lengths that allow an estimate of the size of the target at hand. In the present study, a gel with a 1.5% agarose (Agarose Roti®agarose NEEO) concentration was used when visualizing the PCR product and a gel with 2.5% agarose concentration was used when visualizing the PCR product digested with the *Hsp*92II.

In order to produce a 2.5% concentrated gel, one combines 2.5g of agarose with each 100 ml 0.5 x TBE buffer (0.5 x Rotiphorese®TBE-Puffer, pH 8.3, 1.0mM Tris-Borat, 20mM EDTA, Fa. Roth, Karlsruhe). The mixture is heated to a boil in a microwave oven

until the agarose is completely dissolved, then approximately 1µl ethidium bromide per 100 ml is stirred into the liquid. The ethidium bromide intercalates with nucleic acid and makes it fluoresce with a pink colour under ultraviolet light. The agarosegel is poured into a plastic rack in which "combs" are inserted. That way, after cooling and solidifying, small indentations called wells or slots are left in the gel. The gel is then placed into a tank that is filled with 0.5 x TBE buffer so that the gel is completely covered in liquid and the slots are at the end at which the negative current is applied.

Before loading the slots with the samples, they are mixed with 6 x loading buffer (0.25% Bromphenolblau, 0.25% Xylencyanol, 15% Ficoll), with 1 µl loading buffer per 5 µl PCR product. Ficoll keeps the DNA fragments from floating up in the TBE buffer before they have a chance to move through the gel. Whereas, Bromphenolblau and Xylencyanol help to monitor the progress of the gel electrophoresis because they result in two visible band moving towards the anode. DNA size marker was filled in the outer slots (RangeRuler™100 bp/50 bp DNA Ladder, Fa. Fermentas, St. Leon-Roth) and the electrophoresis chamber was connected to a power source. 120 V at a maximum of 400 mA were applied for about 45 minutes for the PCR product and about 2 hours for the PCR product digested with *Hsp*92II. 10 µl of the PCR product were resolved on a 1.5 % agarose gel (ROTI®GAROSE NEEO, Roth, Karlsruhe), the DNA bands were observed under a UV-lamp and photographed with a digital camera.

Figure 7: COMT 169 bp PCR product using buffer C



A restriction endonuclease digestion was conducted in order to determine which base (adenine or guanine) occurs at the rs4680 SNP location. In this study, the PCR product was digested with *Hsp*92II, which is know to cut at a certain recognition site.

_Hsp_92II recognition site CATG^

5'... CATG↓ 3' ..

3'... GTAC↑ 5' ..

Expected sizes after digest

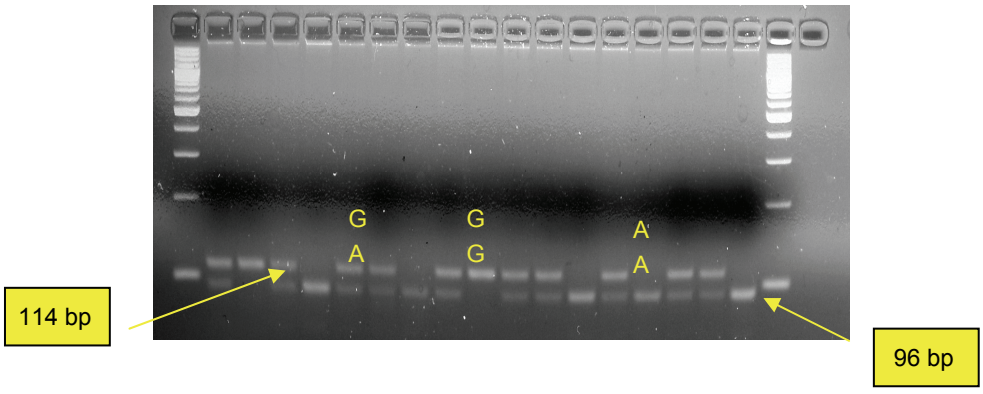
AA 96, 29, 26, 18 bp

GG 114, 29, 26 bp

GA 114, 96, 28, 26, 18 bp

A 30µl solution was made with 20 µl PCR product, 3 µl Puffer K (10mMTris-HCl,10mM $MgCl_2$,150mM KCl), 0.3 µl BSA (1:10), 6.2 µl ddH_2O and 0.5 µl *Hsp*92II (10U/µl). This solution was then digested overnight at 37°C and fragments were separated on 2.5% agarose gel (ROTI®GAROSE NEEO, Roth, Karlsruhe) and genotyped according to the length of the digested PCR product. Genotypes were called by two individuals who were blind to the clinical data.

Figure 8: COMT PCR product digested with _Hsp_92II – A allele 96 bp – G allele 114 bp



10.8 Software

Manufacturer/ web site	
Statistical Package for Social Science (SPSS)	SPSS, Chicago, USA
Human Genome Browser Gateway	http://genome.ucsc.edu/
Primer3	http://frodo.wi.mit.edu/

10.9 Statistical analyses

Statistical analyses were performed by SPSS, version 16.0 (SPSS Inc, Chicago, IL). Family based association with ADHD was assessed by UNPHASED, version 3.0.13 (Dudbridge, 2008). No adjustment for multiple testing was done for the family based association test, as only one hypothesis of the association of *COMT* Met/Met with ADHD was assessed.

Descriptive statistics were calculated by χ^2-test or analysis of variance as appropriate. The impact of biological and psychosocial factors on ADHD symptom severity was assessed by, linear regression analysis, controlling for the following possible confounding variables: sex, age, IQ, alcohol during pregnancy (yes/no), parental ADHD and SES. Independent variables of interest included early familial psychosocial risk factors, smoking during pregnancy, birth weight, *COMT* genotype, *COMT* genotype*gender, *COMT* genotype*birth weight, *COMT* genotype*SES, *COMT* genotype*Smoking during pregnancy, *COMT* genotype*early familial risk factors and *COMT* genotype*alcohol during pregnancy interaction.

The effects of biological and psychosocial factors on co-morbid lifetime CD were assessed by logistic regression analysis. Independent variables in the logistic regression model were chosen analogous to the linear regression model explaining ADHD symptom severity mentioned above. Additionally, the ADHD symptom severity score was included as a possible confounding variable increasing risk for CD.

Residuals were normally distributed.

11. Results

Our sample consisted of 166 children with a DSM-IV derived ADHD diagnoses, where 66.3% had the combined type, 22.9% the inattentive type and 10.8% the hyperactive/impulsive type. The male – female ratio was 84.3% versus 15.7%. The distribution of ADHD subtypes and the male-female ratio was similar to other clinical samples, where both males and the combined type were overrepresented. The mean age of the sample was 9.69 years (SD=1.79) and mean IQ was 100.68 (SD=11.08) and 41% of the children in the sample were receiving medication. In our sample, 39.2% of the children had a co-morbid oppositional defiant disorder and 23.5% had a co-morbid conduct disorder. Among the parents, 22.3% had a possible ADHD diagnoses whereas 7.2% had a probable ADHD diagnoses.

There was no evidence of deviation from the Hardy-Weinberg equilibrium, when looking at the distribution of genotypes (χ^2=0.0594, df=2, p=0.971).

The results from the transmission disequilibrium test (TDT) using an additive model showed that the A allele (Met) was significantly (Z=2.1; p=0.0335) over transmitted in our sample, see table 3.

Table 3: Transmission disequilibrium test for the *COMT* A and G allele

Marker	Allele	Allele Freq	Fam#	S-E(S)	Var(S)	Z	P
COMT	1 (A allele)	0.556	87	11.500	29.250	2.126	0.0335
COMT	3 (G allele)	0.444	87	-11.500	29.250	-2.126	0.0335

Analyses of variance showed that the three genotype groups differed significantly on ADHD symptom severity ($p<0.05$), where those with a Val/Val genotype had a significantly lower score than those with Met/Met and Val/Met genotype. The groups also differed on the ADHD-HI ($p<0.05$), where those with the Met/Met and Val/Met genotype had a significantly higher score than those with the Val/Val genotype. Furthermore, carriers of Val/Val showed a higher rate of the inattentive ADHD subtype as well as lower hyperactive and combined ADHD symptoms than the Met/Met and the Met/Val carriers. Whereas, no difference was observed between the three genotype groups regarding sex, age, IQ, medication use, biological or psychosocial risk factors.

Clinical and demographic information for the children stratified according to genotype are present in Table 4.

11.1 Demographic and clinical characteristics

Table 4: Demographic and clinical characteristics

	Met/Met (N=54) 32.5%	**Val/Met (N=80) 48.2%**	**Val/Val (N=32) 19.3%**	**p-values**
Male N (%)	45 (83.3%)	65 (81.3%)	30 (93.7%)	n.s.
Female N (%)	9 (16.7%)	15 (18.8%)	2 (6.3%)	
Age year mean (SD)	9.5 (1.8)	9.8 (1.8)	9.8 (1.9)	n.s.
IQ (SD)	99.9 (11.6)	100.8 (11.1)	101.6 (10.3)	n.s.
Birth weight in g (SD)	3335 (475)	3390 (484)	3379 (495)	n.s.
Total ADHD score (SD)	38.4 (7.7)	37.0 (8.8)	32.2 (9.9)	**F (2.163)=5.406, *p*=0.005**
Total Inattention (SD)	19.3 (3.8)	19.5 (4.9)	17.9 (4.2)	n.s.
Total Hyperactivity (SD)	19.1 (5.2)	17.5 (6.4)	14.3 (7.4)	**F (2.163)=6.060, *p*=0.003**
ADHD subtypes				**χ^2= 18.43, df=4, *p*=0.001**
Combined	42 (77.8%)	55 (68.8%)	13 (40.6%)	
Inattentive	8 (14.8%)	14 (17.5%)	16 (50%)	
Hyper-impulsive	4 (7.4%)	11 (13.7%)	3 (9.4%)	
Medication				n.s.
Yes	24 (44.4%)	32 (40%)	12 (37.5%)	
No	30 (55.6%)	48 (60 %)	20 (62.5%)	
Bleeding during pregnancy				n.s
Yes	5 (9.3%)	9 (11.2%	7 (21.9%)	
No	49 (90.7%)	71 (88.8%)	25 (78.1%)	
Smoking during pregnancy				n.s.
Yes	18 (33.3%)	23 (28.7%)	10 (31.2%)	
No	36 (66.7%)	57 (71.3%)	22 (68.8%)	
Alcohol during pregnancy				n.s.
Yes	9 (16.7%)	9 (11.2%)	2 (6.2%)	
No	45 (83.3%)	71 (88.8%)	30 (93.8%)	
Parental ADHD				n.s
No ADHD	40 (74.1%)	56 (70%)	21 (65.6%)	
Possible ADHD	11 (20.4%)	17 (21.2%)	9 (28.1%)	
Probable ADHD	3 (5.5%)	7 (8.8%)	2 (6.3%)	
Early family risk factors (SD)	2.0 (3.2)	2.9 (3.0)	2.6 (4.1)	n.s.
Parenting quality last 6 months	1.1 (1.8)	1.7 (1.6)	0.8 (1.3)	n.s.
Acute life events last 6 months	0.6 (1.2)	0.6 (0.9)	0.8 (1.2)	n.s.
Social economic status	2.8 (0.7)	2.7 (0.7)	3.0 (0.6)	n.s.
Family environment score (GSEFU)	79.7 (10.3)	79.1 (10.9)	77.6 (11.4)	n.s.
DSM-IV Lifetime CD				
Yes	18 (33.3%)	13 (16.2%)	8 (25%)	n.s.
No	36 (66.7%)	67 (83.8%)	24 (75%)	

N: number of subjects SD: standard deviation DF: degree of freedom NS: non significant

11.2 ADHD symptom severity and risk factors

The etiology of ADHD still eludes us, leaving us with a complex disorder shaped by the interaction between genes and environment. Findings to date indicate that neurobiological factors play a central role in the etiology of ADHD, where many genes of small effect contribute to the disease susceptibility (Fisher et al., 2002). Environmental factors are also thought to influence the development of ADHD, with smoking during pregnancy, low birth weight and adverse psychosocial factors often linked with the disorder (Banerjee et al., 2007). Among the genes implicated in the etiology of ADHD is the *COMT* gene, which plays a crucial role in the metabolism of catecholamines in the prefrontal cortex (Grossman et al., 1992), which is instrumental when it comes to the guidance of behaviour. Among the central components of prefrontal dysfunction is a breakdown in the modulation of impulsive responding and regulation of goal-directed behaviour (Parker & Crawford, 1992), often manifested in the holy trinity of ADHD, namely deficits in attention, impulse control and excessive motor activity.

The aim of this analysis is to assess whether *COMT* genotype influences ADHD symptom severity. As Met/Met genotype has been associated with ADHD symptom severity in resent studies (Ettinger et al., 2006; Reuter et al., 2006) and the Met allele was found to be significantly more prevalent in 22q11.2DS patients with ADHD compared to those without ADHD (Gothelf et al., 2007; Michaelovsky et al., 2007). We predict that children with the *COMT* Met/Met genotype will have more severe ADHD symptoms than children with either Val/Met and Val/Val genotype. Furthermore, given the potential importance of gene-environment interaction on the development of ADHD, we aimed to assess the interaction of *COMT* genotypes with environmental risk factors previously associated with ADHD.

The total ADHD symptom severity was used as the dependent variable when assessing the impact of biological and psychosocial risk factors on ADHD symptom

severity. The initial analysis revealed that the interaction terms between *COMT* genotype and environmental factors previously associated with ADHD did not have a significant effect on ADHD symptom severity in our sample.

The only variable having a significant effect on ADHD symptom severity was maternal smoking during pregnancy. Therefore, we constructed another model where all the interaction terms were left out. Subsequently, the following variables became significant, maternal smoking during pregnancy and *COMT* genotype.

The results of the linear regression analysis, without the gene*environment interaction terms, are presented in Table 5. Table 6 shows the model parameters for ADHD symptom severity risk factors.

Table 5: Risk factors for ADHD symptom severity

Dependent Variable: ADHD symptom severity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2548.296[a]	12	212.358	3.031	0.001
Intercept	970.329	1	970.329	13.851	0.000
Gender	20.206	1	20.206	0.288	0.592
Alcohol in pregnancy	5.183	1	5.183	0.074	0.786
Smoking in pregnancy	555.039	1	555.039	7.923	**0.006***
***COMT* Genotype**	877.966	2	438.983	6.266	**0.002****
Parental ADHD	165.617	2	82.809	1.182	0.309
Age	24.609	1	24.609	0.351	0.554
Birth weight	15.402	1	15.402	0.220	0.640
IQ	114.465	1	114.465	1.634	0.203
Early family risk factors	200.782	1	200.782	2.866	0.093
SES	120.367	1	120.367	1.718	0.192
Error	10578.606	151	70.057		
Total	231908.000	164			
Corrected Total	13126.902	163			

a. R Squared = 0.194 (Adjusted R Squared = 0.130)
* significant at 0.010
** significant at 0.005

Table 6: Parameter estimates for ADHD symptom severity risk factors

Dependent Variable: ADHD Symptom severity						
Parameter	B	Std. Error	T	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Intercept	30.933	9.378	3.299	0.001	12.405	49.462
Gender	1.036	1.928	0.537	0.592	-2.774	4.846
Alcohol during pregnancy	0.569	2.092	0.272	0.786	-3.565	4.703
Smoking during pregnancy	-4.274	1.519	-2.815	**0.006***	-7.275	-1.274
Met/Met Genotype vs Val/Val Genotype	6.756	1.942	3.479	**0.001****	2.919	10.592
Val/Met Genotype vs Val/Val Genotype	5.235	1.828	2.863	**0.005***	1.622	8.847
No parental ADHD vs probable ADHD	-3.305	2.640	-1.252	0.213	-8.521	1.911
Possible parental ADHD vs probable ADHD	-1.559	2.847	-0.547	0.585	-7.185	4.067
Age	-0.223	0.376	-0.593	0.554	-0.965	0.520
Birth weight	0.001	0.001	0.469	0.640	-0.002	0.003
IQ	0.079	0.062	1.278	0.203	-0.043	0.201
Early family risk factors	0.354	0.209	1.693	0.093	-0.059	0.767
SES	-1.346	1.027	-1.311	0.192	-3.374	0.683

* significant at 0.010
** significant at 0.005

11.3 Smoking and ADHD symptom severity

A large body of literature exists suggesting that exposure to nicotine in utero is associated with several adverse behavioural outcomes such as ADHD, conduct disorder and antisocial behavior (Linnet et al., 2003; Thapar et al., 2006). Studies of children whose mothers smoked during pregnancy have demonstrated neurocognitive deficits such as poor school performance and lower scores on intelligence tests (DiFranza et al., 2004). Furthermore, an almost universal observation is that maternal smoking during pregnancy is associated with low birth weight (Mick et al., 2002).

A binary variable for prenatal smoking was created, indicating those who smoked at all during their pregnancy and those who did not. In our sample smoking during pregnancy was a significant risk factor for more severe ADHD symptoms. Children of smoking mothers had a mean ADHD symptom severity score of 39.90 compared to 35.03 in the non smoking group, and are these findings in accordance with many previous findings. Those children whose mother smoked during pregnancy had both lower birth weight and IQ compared to children not exposed to nicotine in utero. The psychosocial environment was also more adverse among the children exposed to nicotine in utero, where both early familial risk factors and parental quality during the last six months were significantly inferior in that group. Finally, the smoking group had significantly more severe problems as rated by the Child Behaviour Checklist (CBCL) compared to the no smoking group (See Table 7).

Table 7: The effects of smoking on behaviour and psychosocial environment

		N	Mean	SD	Sig.
Birth weight	No smoking	115	3430	476.51	0.017*
	Smoking	51	3237	468.69	
ADHD total symptom severity	No smoking	115	35.03	8.632	
	Smoking	51	39.90	8.723	0.001**
ADHD inattentive type	No smoking	115	18.53	4.245	
	Smoking	51	20.41	4.570	0.014*
ADHD hyperactive impulsive type	No smoking	115	16.50	6.583	
	Smoking	51	19.49	5.644	0.004**
IQ	No smoking	115	102.02	10.715	
	Smoking	51	97.67	11.415	0.023*
CBCL – externalizing behaviour	No smoking	100	65.30	7.326	
	Smoking	48	69.25	7.541	0.003**
CBCL – internalizing behaviour	No smoking	100	62.44	8.820	
	Smoking	48	64.31	9.408	0.239
CBCL – scholastic and social competence	No smoking	96	48.07	9.798	
	Smoking	44	43.00	11.254	0.012*
CBCL – aggression	No smoking	100	66.51	8.915	
	Smoking	48	70.42	8.695	0.013*
CBCL – antisocial behaviour	No smoking	100	61.91	7.068	
	Smoking	48	66.35	8.687	0.003**
CBCL – Anxiety	No smoking	100	62.44	8.934	
	Smoking	47	64.64	8.808	0.164
Early family risk factors	No smoking	113	1.73	2.794	
	Smoking	51	3.45	3.997	0.007**
Parental quality last 6 months	No smoking	113	0.08	1.35	
	Smoking	51	3.45	1.94	0.036*
Acute life events last 6 months	No smoking	113	0.49	0.814	
	Smoking	51	1.04	1.47	0.014*
SES	No smoking	115	2.75	0.636	
	Smoking	51	2.58	0.631	0.109

CBCL - childhood behaviour checklist

* significant at 0.010
** significant at 0.005

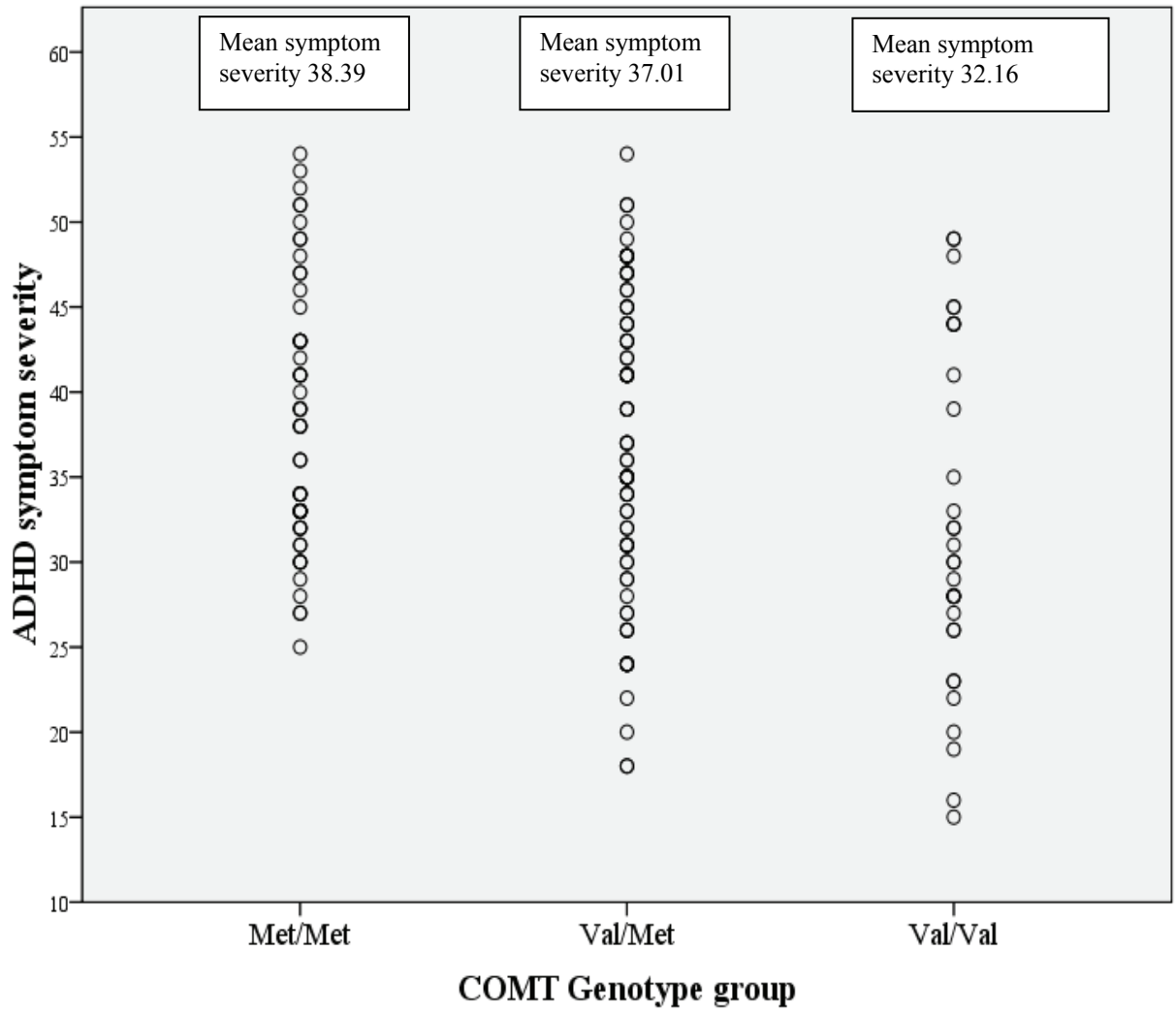
11.4 *COMT* genotype and ADHD symptom severity

The *COMT* gene plays a crucial role in the metabolism of catecholamines in the prefrontal cortex. It therefore has a considerable influence on the optimal functioning of the prefrontal cortex, which is instrumental when it comes to the guidance of behaviour (Grossman et al., 1992). Results from studies focusing on the associations between the *COMT* gene and ADHD have been contradictory. However, recent findings indicate that carriers of the *COMT* Met/Met genotype have more severe ADHD symptoms compared to carriers of the Val/Val and Met/Val genotypes (Ettinger et al., 2006; Reuter et al., 2006). Furthermore, the Met allele was found to be significantly more prevalent in 22q11.2DS patients with ADHD compared to those without ADHD (Gothelf et al., 2007; Michaelovsky et al., 2007).

As *COMT* genotype was associated with ADHD symptom severity in recent studies, we wanted to test the hypothesis that *COMT* Met/Met genotype is a risk factor for increased severity of ADHD symptoms.

Looking at ADHD symptom severity among the genotypes revealed that the Met/Met group had a mean ADHD symptom severity of 38.39 that was significantly higher compared to the symptom severity of 32.16 in the Val/Val group ($p > 0.002$). Similarly, the Val/Met group had a mean ADHD symptom severity of 37.01 that was significantly higher compared to the Val/Val group ($p > 0.014$). The difference between the Met/Met and Val/Met genotypes was not significant. Figure 9 shows the effects of genotype on symptom severity.

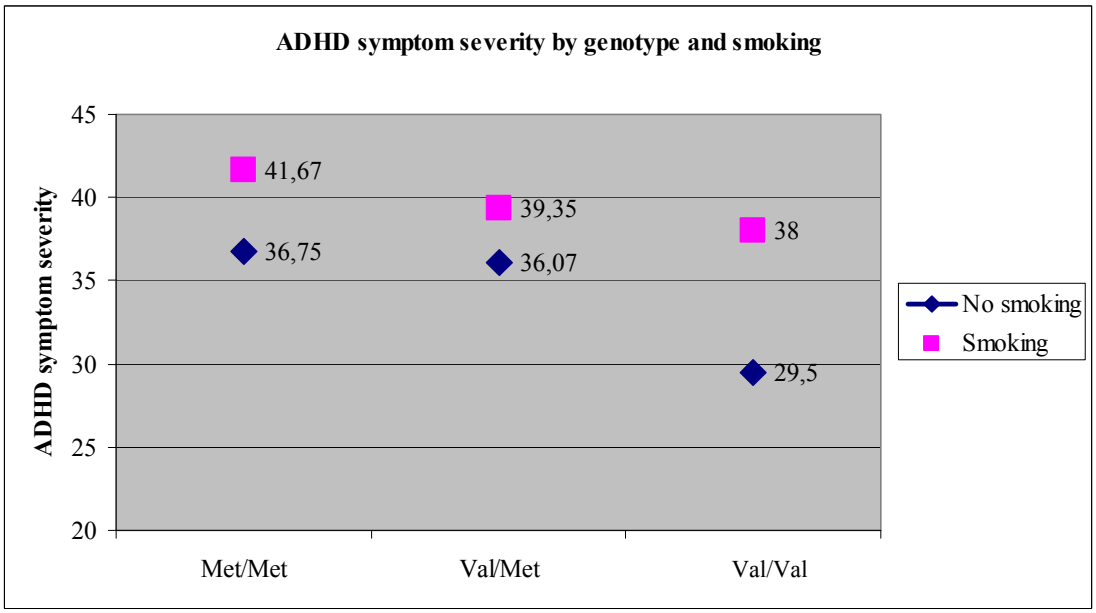
Figure 9: Genotype and ADHD symptom severity



11.5 Effects of *COMT* genotype and smoking on ADHD symptom severity

As can be seen from the results of the regression analysis both smoking and genotype have a significant effect on ADHD symptom severity in our sample. Figure 10 shows us the effect of smoking on ADHD symptom severity within each genotype group. Looking at the Met/Met genotype group there is a significant difference between children of smoking and non smoking mothers, where children of smoking mothers had a mean ADHD symptom severity score of 41.67 compared to 36.75 in the non smoking group ($p > 0.026$). In the Val/Met genotype group the symptom severity is not signficantly different among the two groups ($p = 0.133$), where those who had a mother that smoked had a mean ADHD symptom severity score of 39.35 compared to 36.07 in the non smoking group. In the Val/Val genotype group, children of smoking mothers had a significantly higher mean ADHD symptom severity score than the non smoking group ($p > 0.022$), 38.0 compared to 29.5 respectively (See Figure 10).

Figure 10: The effect of *COMT* genotype and smoking on ADHD symptom severity



11.6 Risk factors for co-morbid conduct and oppositional defiant disorders in ADHD

Among clinically referred children, co-morbidity is frequently found between ADHD and CD or ODD, with studies indicating that 54% to 67% of ADHD children will meet full diagnostic criteria for ODD and as many as 20% to 56% will be diagnosed with CD (Barkley, 1998). ODD and CD are externalizing disorders characterized by aggressive and antisocial behaviours (Bassarath, 2001).

Of these two disorders, CD is a more serious problem with children acting out aggressively and expressing anger inappropriately. Furthermore, they engage in a variety of antisocial and destructive acts, including violence towards people and animals, destruction of property, lying and stealing. Oppositional defiant disorder is a less severe disorder than CD and is characterised by a recurring pattern of negative, hostile, disobedient and defiant behaviour (APA, 1994).

Both genetic and environmental factors are thought to influence the genesis of antisocial behaviour. Despite great effort over the last years, researchers have been unsuccessful in their attempts to elucidate the specific environmental and genetic factors underlying the association between ADHD and co-morbid CD/ODD.

Given the potential importance of gene-environment interaction, whereby genes modify susceptibility to environmental factors, we wanted to identify those environmental factors that influence ADHD symptom severity and co-morbid CD and ODD, by testing for interaction as well as main effects of the *COMT* Val^{158}Met genotypes with several environmental risk factors previously associated with ADHD.

11.7 Risk factors for co-morbid conduct disorder in ADHD

The initial logistic regression model including the gene*environment interaction terms revealed that total ADHD symptom severity and early family risk factors were significant predictors of lifetime CD in our sample. None of the interaction terms included in the model had a significant effect on CD in our sample. An additional model was constructed which did not include any of the interaction terms. In this model, total ADHD symptom severity and early family risk factors were significant predictors of lifetime CD. This model classified 82.9% of the children with lifetime CD correctly. The *COMT* genotype variable showed a trend towards having a significant impact on lifetime CD in our sample, with the p value being 0.051. Among the Met/Met genotype group, there were 33% of the children that had co-morbid CD, in the Val/Met group, 16% of the children had co-morbid CD and in the Val/Val group 25% of the children had co-morbid CD. When odds of having CD was compared among the genotype groups, using the Met/Met genotype as a reference group, those with Val/Met were significantly less likely to have co-morbid CD compared to Met/Met, $p > 0.016$. Those with Val/Val genotype were less likely to have co-morbid CD compared to those with Met/Met genotype, the difference was however not significant $p = 0.654$.

The results of the logistic regression analysis, without any interaction terms, are present in Table 8.

Table 8: Risk factors for co-morbid CD in ADHD

	B	S.E.	Wald	df	Sig.	Exp(B)	95,0% C.I.for EXP(B)	
							Lower	Upper
Gender	0.881	0.785	1.258	1	0.262	2.412	0.518	11.238
Age	0.124	0.122	1.046	1	0.306	1.133	0.892	1.438
ADHD symptom severity	0.065	0.027	5.708	1	**0.017***	1.067	1.012	1.125
Alcohol in pregnancy	-0.375	0.637	0.346	1	0.556	0.687	0.197	2.396
Smoking in pregnancy	-0.662	0.477	1.922	1	0.166	0.516	0.202	1.315
Birth weight	0.000	0.000	0.204	1	0.651	1.000	0.999	1.001
COMT Genotype			5.969	2	0.051			
COMT Genotype Val/Met vs Met/Met	-1.164	0.485	5.754	1	0.016	0.312	0.121	0.808
COMT Genotype Val/Val vs Met/Met	-0.285	0.635	0.201	1	0.654	0.752	0.216	2.613
IQ	0.005	0.019	0.060	1	0.807	1.005	0.967	1.043
Parental ADHD			1.207	2	0.547			
Parental ADHD Possible parental ADHD vs. No parental ADHD	-0.825	0.768	1.154	1	0.283	0.438	0.097	1.974
Parental ADHD Probable parental ADHD vs. No parental ADHD	-0.829	0.839	0.976	1	0.323	0.437	0.084	2.260
Early familial risk factors	0.186	0.063	8.690	1	**0.003****	1.204	1.064	1.363
SES	-0.189	0.358	0.279	1	0.597	0.828	0.411	1.669
Constant	-5.843	2.899	4.063	1	0.044	0.003		

Model coefficients: χ^2 (13) = 39.7, p = 0.000
** significant at 0.005
* significant at 0.050

11.8 The effect of smoking and co-morbid CD on behavioural and psychosocial measures

Findings to date indicate that maternal smoking during pregnancy increases the risk for cognitive deficits, ADHD and behavioural problems, such as CD (Huijbregts, 2007; Wakschlag et al., 1997). Furthermore, a large body of literature exists suggesting that ADHD children with co-morbid CD are more likely to experience greater symptom severity and persistence, lower IQ and have more negative correlates such as adverse family environment than children with just ADHD (Drabick, 2006).

Though smoking during pregnancy did not have a significant influence on CD in our sample, we wanted to compare those children exposed to nicotine in utero and diagnosed with CD to children with just ADHD and not exposed to nicotine on behavioural and psychosocial measures.

When we divided the participants into groups according to ADHD and not exposed to nicotine in utero and ADHD with co-morbid CD plus exposure to nicotine in utero it was apparent that children in the latter group had significantly more behavioural problems and had more adverse psychosocial environment than those with just ADHD. Those children with co-morbid CD plus exposure to nicotine had significantly more severe ADHD symptoms on all ADHD subscales compared to children with just ADHD. When looking at the outcome of the CBCL subscales, children in the CD plus smoking group were significantly more aggressive and antisocial. Furthermore, they had significantly more internalizing symptoms, but anxiety/depressive symptoms were similar in the groups. In addition, the CD plus smoking group had less scholastic and social competence compared to the ADHD group. The children in the ADHD group had higher mean IQ compared to the CD plus smoking group, 101.77 and 96.61 respectively, but the difference was not significant.

Looking at the environmental factors, the social economical status (SES) was lower

in the CD plus smoking group compared to children with just ADHD. The early family risk factors and parental quality during the last six months were significantly poorer in the CD group and finally they also experienced significantly more acute life events during the last six months than those with just ADHD. The effect of CD and smoking on behavioural measures and psychosocial environment can be seen in Table 9.

Table 9: The effects of co-morbid CD and smoking on behavioural and psychosocial measures

Group Statistics					
		N	Mean	SD	Sig.
IQ	No smoking and no CD	97	101.48	11.58	
	CD and smoking	20	96.61	13.69	0.099
ADHD total	No smoking and no CD	97	34.41	8.56	
	CD and smoking	20	43.90	7.20	0.000**
ADHD In	No smoking and no CD	97	18.64	4.16	
	CD and smoking	20	21.60	5.10	0.006*
ADHD HI	No smoking and no CD	97	15.78	6.62	
	CD and smoking	20	22.30	4.01	0.000**
CBCL – externalizing	No smoking and no CD	86	64.57	7.04	
	CD and smoking	20	74.00	5.97	0.000**
CBCL – Social and scholastic competence	No smoking and no CD	82	48.28	9.33	
	CD and smoking	18	38.61	14.63	0.014*
CBCL – aggression	No smoking and no CD	86	65.79	8.53	
	CD and smoking	20	75.70	7.24	0.000**
CBCL - antisocial behaviour	No smoking and no CD	86	60.88	6.69	
	CD and smoking	20	71.70	6.09	0.000**
CBCL – anxiety/depression	No smoking and no CD	86	62.76	8.98	
	CD and smoking	19	66.37	8.63	0.114
CBCL – internalizing	No smoking and no CD	86	62.64	8.98	
	CD and smoking	20	67.05	8.36	0.048*
SES	No smoking and no CD	97	2.75	0.650	
	CD and smoking	20	2.35	0.790	0.016*
Early family risk factors	No smoking and no CD	95	1.53	2.64	
	CD and smoking	20	5.50	5.06	0.003**
Parental quality last 6 months	No smoking and no CD	95	0.73	1.29	
	CD and smoking	20	2.05	2.34	0.018*
Acute life events last 6 months	No smoking and no CD	95	0.42	0.793	
	CD and smoking	20	1.25	1.713	0.047*

** significant at 0.005
* significant at 0.050

11.9 Risk factors for co-morbid oppositional defiant disorder in ADHD

The initial logistic regression model including the gene*environment interaction terms revealed that total ADHD symptoms were a significant predictor of co-morbid oppositional defiant disorder in our sample. None of the gene*environment interaction terms were significant so an additional model was constructed which did not include any of the interaction terms. In this model, ADHD symptom severity, age and IQ were significant predictors of lifetime co-morbid ODD. This model classified 72.8% of the children with lifetime ODD correctly. The results of the logistic regression analysis, without any interaction terms are present in Table 10.

Table 10: Risk factors for co-morbid ODD in ADHD

	B	S.E.	Wald	df	Sig.	Exp(B)	95.0% C.I.for EXP(B)	
							Lower	Upper
Gender	0.137	0.561	0.059	1	0.808	1.146	0.382	3.443
Age	0.294	0.122	5.757	1	**0.016***	1.341	1.055	1.705
ADHD symptom severity	0.068	0.026	6.885	1	**0.009***	1.070	1.017	1.125
Alcohol in pregnancy	0.708	0.676	1.094	1	0.296	2.029	0.539	7.640
Smoking in pregnancy	0.127	0.485	0.069	1	0.793	1.136	0.439	2.939
Birth weight	0.000	0.000	0.072	1	0.789	1.000	0.999	1.001
COMT Genotype	-1.039		4.966	2	0.083		0.137	0.912
COMT Genotype Val/Met vs Met/Met	-0.293	0.483	4.631	1	0.031	0.354	0.216	2.579
COMT Genotype Val/Val vs Met/Met	-0.041	0.633	0.215	1	0.643	0.746	0.922	0.999
IQ	-2.866	0.021	4.004	1	**0.045***	0.960	0.003	0.950
Parental ADHD	-3.049		4.154	2	0.125		0.002	0.911
Parental ADHD Possible parental ADHD vs. No parental ADHD	0.098	1.436	3.983	1	0.046	0.057	0.930	1.309
Parental ADHD Probable parental ADHD vs. No parental ADHD	-0.090	1.508	4.086	1	0.043	0.047	0.460	1.814
Early family risk factors	0.093	0.087	1.270	1	0.260	1.103	0.382	3.443
SES	0.137	0.350	0.067	1	0.796	0.914	1.055	1.705
Constant	0.294	2.882	0.001	1	0.974	1.097		

Model coefficients: χ^2 (13) = 24.9, p = 0.024

* significant at 0.050

12. Discussion

Despite that ADHD is now better understood and more clearly defined than it once was, it remains a controversial entity, with children diagnosed with ADHD representing a highly heterogeneous population, displaying great diversity in their symptom severity and in the co-occurrence of other disorders, such as ODD and CD (Pennington, 2002). The etiology of ADHD is still a mystery that is moulded by the interaction between genes and environment. Findings to date indicate that neurobiological factors play a large role in the pathology of ADHD, where many genes of small effect contribute to the disease susceptibility, affecting the optimal functioning of various brain regions such as the frontal lobes, basal ganglia and cerebellum (Arnsten & Li, 2005; Fisher et al., 2002).

The *COMT* gene has been implicated in the etiology of several psychiatric disorders, including ADHD and CD (Biederman et al., 2004; Caspi et al., 2008). It plays a crucial role in the metabolism of catecholamines in the prefrontal cortex (Sesack et al., 1998), which is influential in the control of executive functions and guidance of behaviour (Arnsten & Li, 2005).

As *COMT* was associated with ADHD as well as CD in previous studies, the aim of this study is to assess if the *COMT* Val^{158}Met SNP is a risk factor for ADHD as well as for severity of ADHD symptoms and co-morbid CD in children with ADHD.

First it is hypothesized, that the *COMT* Val^{158}Met SNP is associated with ADHD in this family based study of 166 children with ADHD and their parents. Secondly, it is predicted that ADHD children with the *COMT* Met/Met genotype will have more severe ADHD symptoms and are more likely to have co-morbid CD than those children with either Val/Met or Val/Val genotype. Finally, given the potential importance of gene-environment interaction, whereby genes modify susceptibility to environmental factors, we wanted to identify those environmental factors influencing ADHD symptom severity and co-morbid CD and ODD, by testing for interaction as well as main effects of the

COMT Val^{158}Met SNP with several environmental risk factors previously associated with ADHD.

The main results of the study are that *COMT* Val^{158}Met SNP is associated with ADHD in our sample, with the Met allele being over-transmitted in our sample. Secondly, then smoking during pregnancy had significant influence on ADHD symptom severity, and those with the *COMT* Met/Met genotype had the most severe ADHD symptoms in our sample. Finally, ADHD symptom severity and adverse early family circumstances during the first three years of life are positive predictors of lifetime CD in our sample. Whereas *COMT* genotype showed a trend towards being a significant predictor of lifetime CD. When looking at those variables influencing co-morbid ODD, then ADHD symptom severity, age and IQ were positive predictors of ODD in our sample. None of the gene*environment interaction had a significant effect on ADHD symptom severity or the occurrence of co-morbid CD or ODD in our sample.

12.1 Risk factors and ADHD symptom severity

Results from genetic studies indicate that environmental factors may play a role in the individual difference in ADHD symptom severity, accounting for around 20 to 30% of the variance in ADHD symptoms (Faraone & Asherson, 2005). This variance in ADHD symptom severity may result from environmental factors, such as pre-, peri- and postnatal complications, diseases, trauma, toxins or other neurologically compromising events that may occur during the development of the nervous system before and after birth.

12.1.1 Alcohol

Alcohol is one of the environmental factors frequently linked to ADHD, and it is widely recognized as a teratogenic agent that can disturb the neurochemical and structural environment of the developing fetal brain, resulting in impaired mental functioning, including fetal alcohol effect and fetal alcohol syndrome (Aronson et al., 1997; Coles et

al., 1997). Children with fetal alcohol syndrome tend to be hyperactive, exhibit cognitive deficits and deficits in adaptive behaviour and are at increased risk for psychiatric disorders (Mick et al., 2002). This similarity in the presentation of ADHD and the ADHD-like behavioural component of fetal alcohol syndrome suggests that alcohol may also play a role in the etiology of ADHD (Coles et al., 1997).

Our findings indicate that alcohol consumption during pregnancy did not have a significant effect on ADHD symptom severity in our sample. These results reflect findings from other studies, indicating that maternal alcohol use is not a specific risk factor, neither for ADHD nor for ADHD symptom severity once other risk factors have been controlled for during analysis, especially smoking during pregnancy (Knopik et al., 2006; Kotimaa et al., 2003; Mick et al., 2002). A possible explanation for these negative results could be that the assessment of alcohol use was retrospective and thus susceptible to a recall bias, with mothers possibly reporting that they used less alcohol than they actually did. A more plausible explanation is that only 12% of mothers reported that they had consumed alcohol during pregnancy and most of them said that it had been in great moderation. This is supported by the fact that when those children exposed to alcohol were compared to those that were not exposed to alcohol during pregnancy, the two groups were virtually identical on all behavioural scales. The psychosocial environment was furthermore similar in the two groups, and there were no indications that alcohol use during pregnancy was more common among those mothers who lived in poorer psychosocial environment compared to those who lived in a better psychosocial environment.

12.1.2 Low birth weight

Low birth weight (LBW) is another environmental factor that has been implicated in ADHD; it has multiple causes and often reflects an accumulation of psychosocial and physical risk factors that are difficult to tease apart. Chomitz and colleagues reviewed the

literature and identified several factors as possible causes of LBW, such as inadequate maternal health and nutrition, cigarette use, alcohol or other substance use during pregnancy, maternal illness, domestic violence leading to fetal injury or premature labour due to severe emotional stress and possibly other significant maternal emotional stress (Chomitz, 1995).

Our results indicate that birth weight does not influence ADHD symptom severity in our sample. These results could be explained by the strict exclusion criteria implemented in the study, excluding all children with a birth weight below 2000 g, with only 6 individuals or 3.6% of the sample having a birth weight below 2500 g. Many of the studies reporting an influence of low birth weight on ADHD and ADHD symptom severity were based on highly selective groups ranging from extremely low birth weight (<1000 g) to low birth weight (< 2500 g) thus possibly limiting the conclusion to children with a birth weight that is less than 2500 g (Bhutta et al., 2002).

It is clear that psychosocial adversity and poor maternal health converge on a range of physical and emotional stressors complicating the picture in research focusing on LBW; however, results from comprehensive meta analysis and reviews indicate that the largest single and most preventable cause of LBW is maternal smoking during pregnancy (Chomitz, 1995; Kramer, 1987).

12.1.3 Smoking

The variable that had most severe effects upon ADHD symptom severity and outcome on behavioural measures was maternal smoking during pregnancy. In our sample, 51 mothers (30.7%) reported having smoked during pregnancy, compared to the reported mean of 24% in many Western countries (Huijbregts, 2007). Our results that smoking during pregnancy is associated with higher ADHD symptom severity (39.90 versus 35.03) are similar to those seen in case-control (Milberger et al., 1998) and longitudinal studies (Kotimaa et al., 2003) on children with ADHD. Those mothers that

smoked during pregnancy gave birth to significantly lighter children than mothers that did not smoke, 3237 g compared to 3430 g respectively, these results are in accordance with previous findings (Ernst et al., 2001, Mick et al., 2002).

IQ was significantly lower in children exposed to nicotine in utero compared to those not exposed (M=97.67 and 102.02, respectively), which is similar to reports from DiFranza and Weitzman (DiFranza et al., 2004; Weitzman et al., 2002). In addition, those children exposed to nicotine in uterus had significantly more behavioural problem on nearly all measures used in the study.

Finally when looking at early family risk factors, then they were significantly higher in children from smoking mothers (M=3.45) than in those who had non-smoking mothers (M=1.73). Langley reported similar findings where maternal smoking during pregnancy and environmental adversity indexed by lower social class, independently influenced the clinical presentation of ADHD (Langley, 2007).

A large body of literature exists suggesting that maternal smoking during pregnancy has adverse effects on the risk of developing ADHD and CD. In the present study, those children whose mothers smoked during pregnancy were more likely to have accompanying CD than those children not exposed to nicotine during pregnancy, though the difference was not significant. This is similar to previous studies, reporting a two- to fourfold increased risk for CD in children exposed to nicotine in utero (Ernst et al., 2001; Wakschlag et al., 1997; Weissman et al., 1999). When we looked at the combined effects of exposure to nicotine and CD on the behavioural measures it was apparent that those children with co-morbid CD and exposed to nicotine in utero had significantly more behavioural problems and lived in a more adverse psychosocial environment than those children with just ADHD and not exposed to nicotine.

The children in the CD plus smoking group had significantly more severe ADHD symptoms on all ADHD subscales compared to children with just ADHD and were more

aggressive. In addition, the CD plus smoking group had less scholastic and social competence compared to the ADHD group. Looking at the environmental factors, the social economical status (SES) was lower among those children with CD and exposed to nicotine compared to children with just ADHD. Those children with CD plus exposure to nicotine had more early family risk factors and the parental quality during the last six months was significantly poorer in the CD group. Furthermore, they also experienced significantly more acute life events during the last six months than those children with just ADHD (see Table 9).

Despite studies that have repeatedly indicated that smoking during pregnancy can adversely affect the developing fetus, the underlying biological processes are not fully understood (Neuman et al., 2007). Cigarettes include hundreds of different compounds, with the major psychoactive substance in tobacco being nicotine. Animal research have clearly demonstrated the neurotoxic effects of nicotine on the developing fetus (Olds, 1997). Nicotine is rapidly absorbed into the blood stream and reaches the fetus at concentrations equal to or even higher than those in the mother (Ankarberg et al., 2001; Dempsey & Benowitz, 2001). It interacts with nicotinic acetylcholine receptors (nAChR), that are ligand-gated, rapid-onset, and excitatory ion channels widely expressed both in the central and peripheral nervous system (Dani & Bertrand, 2006; Stahl, 2000). These receptors exist in several different subtypes that have important functional implications, with the two most abundant subtypes being the heteromeric α4β2 and homomeric α7 nAChRs (Ernst et al., 2001; Huang et al., 2007). nAChR are expressed early during embryonic development in both human and animal fetuses and are highly expressed in brain areas implicated in the aetiology of ADHD, such as the frontal cortex and cerebellum (Adams et al., 2002). The nAChR play a vital a role in neuronal pathfinding, cell proliferation, regulation and differentiation acting on various neurotransmitter systems, such as dopamine, noradrenaline, serotonin, GABA and glutamate. Therefore,

prenatal exposure to nicotine might directly influence brain development by leading to dysregulation in neurodevelopment inducing a higher risk for behavioural and psychiatric problems, such as ADHD (Ernst et al., 2001).

12.1.4 *COMT* genotype

Many theories of ADHD have postulated that the prefrontal cortex plays a primary role in the disorder (Barkley, 1997, 1998; Sonuga-Barke, 2002). The gist of theses theories is that ADHD represents a deficit in prefrontal cortex inhibition leading to deficits in executive functions, such as verbal working memory, non-verbal working memory and self-regulation of affect.

The prefrontal cortex is sensitive to its neurochemical environment and changes in catecholamine modulation can have deleterious effects on its ability to guide behaviour (Arnsten & Li, 2005). Findings from animal studies indicate that the *COMT* gene plays a key role in modulating dopamine neurotransmission in the prefrontal cortex (Gogos, 1998), being responsible for nearly 60% of dopamine degradation in this area (Karoum et al., 1995). The *COMT* Val^{158}Met polymorphism encodes the amino acids methionine (Met) or valine (Val), giving rise to a trimodal distribution of the enzyme, where homozygozity for the Met allele yields a three- to four-fold reduction in *COMT* activity relative to those homozygous for the Val allele (Weinshilboum et al., 1999). Considering the importance of *COMT* for optimal function of the frontal lobe it is a prominent candidate gene for various psychiatric disorders, such as schizophrenia, bipolar disorder, obsessive-compulsive disorder and ADHD.

Our findings indicate that *COMT* genotype has a significant effect on ADHD severity, with *COMT* enzyme activity having an inverse relation to symptom severity. Therefore, those with the Met/Met genotype and the least active enzyme had the highest ADHD symptom severity; whereas those with the Val/Val genotype and the most active enzyme had the least severe ADHD symptom severity, see Figure 11.

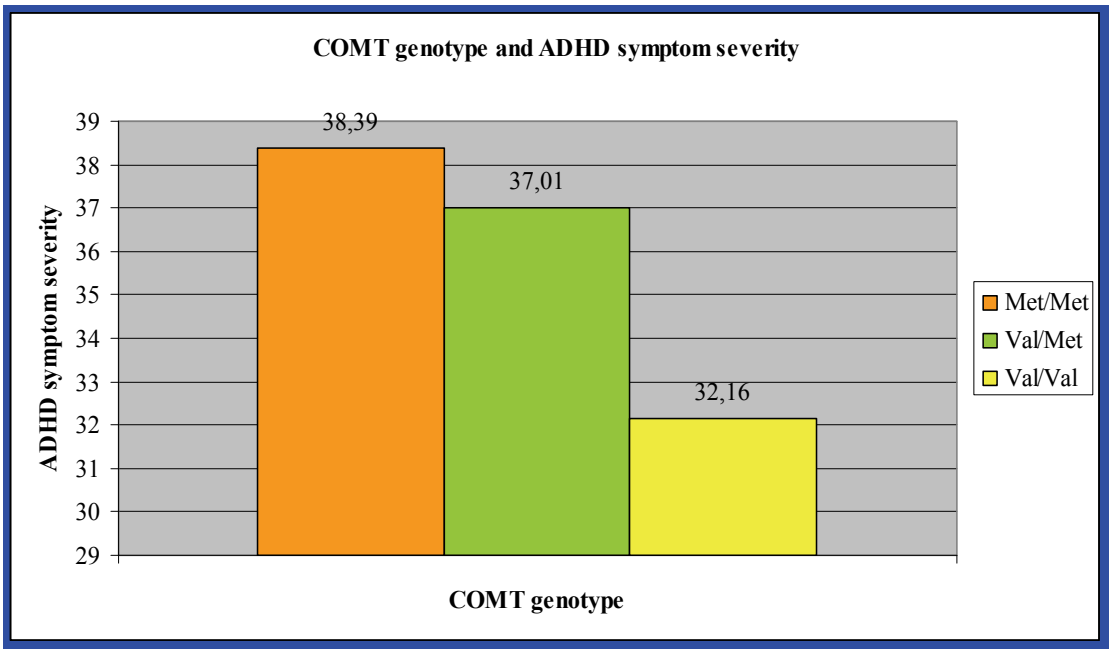


Figure 11: *COMT* genotype and ADHD symptom severity

Our results complement resent reports from different cohorts suggesting that the Met allele plays a role in ADHD. Ettinger et al (2006) and Reuter et al (2006) reported that healthy individuals homozygous for the *COMT* Met allele had the highest scores on an ADHD self report scale (ASRS) supporting the involvement of *COMT* genotype in ADHD. Studies focusing on 22q11.2 deletion syndrome (22q11.2 DS), which is caused by a microdeletion on the long arm of chromosome 22 where the *COMT* gene resides, also reported an association between the Met allele and ADHD (Gothelf et al., 2007; Michaelovsky et al., 2007). Where those children with 22q11.2 DS and co-morbid ADHD had significantly higher frequency of the Met allele compared to those 22q11.2 DS children without co-morbid ADHD.

In our sample of 166 children with ADHD, the distribution of *COMT* genotypes does not breach the Hardy-Weinberg equilibrium; and the results from the transmission disequilibrium test show that the Met allele is over-transmitted and thus associated with the disorder in our sample.

Faraone et al reviewed studies that have examined the association between the Val[158]Met polymorphism and ADHD. From the seven studies reviewed, five did not find

an association between the disorder and the polymorphism (Faraone et al., 2005). Two studies reported statistically significant associations, Eisenberg et al (1999) and Qian et al (2003). The Eisenberg group reported an over transmission of the Val allele in a sample of 48 children, diagnosed with the DSM-IV criteria. This over-transmission of the Val allele was confined to the combined and hyperactive-impulsive subtypes, but Eisenberg and colleagues did not correct for multiple testing in their sample and subsequently corrected their report to include less over-transmission of the Val allele than originally reported (Eisenberg et al., 1999). Qian and colleagues used both a case–control and family-based analyses, including 340 Han Chinese children diagnosed according to DSM-IV ADHD criteria, to examine the relation between ADHD and Val^{158}Met polymorphism (Qian et al., 2003). The family based design revealed no association or linkage between *COMT* and ADHD, when the whole sample was included. However, when the analysis was restricted to males without a co-morbid diagnoses, the results showed a significant over transmission of the Met allele among boys with ADHD. The case–control analyses reported by Qian et al. (2003) showed somewhat different results. Similar to the family-based analyses, there was no evidence for association between *COMT* and ADHD when the entire sample was included in the study. However, there was significant evidence for association between *COMT* and ADHD among girls with the Val allele being overrepresented, consistent with the original association reported by Eisenberg et al (1999).

Though our results harmonize with some previous findings, the evidence supporting an association of the *COMT* Val^{158}Met polymorphism with ADHD remains inconclusive. These inconsistent results have raised questions about effect of sample stratification, phenotype definition and the mechanisms by which this polymorphism exerts its effects.

Looking at sample stratification, first of all there is difference in the allele distribution among ethnic groups, i.e. with the genotype distribution being 3% Met/Met, 30%

Val/Met and 67% Val/Val among Chinese compared to 31% Met/Met, 47% Val/Met and 22% Val/Val among Caucasians in the UK, making comparison between studies difficult. Sample size is also a problem in some of these studies focusing on *COMT,* ranging from 48 up to 340 individuals. When looking at a phenotype with extensive genetic heterogeneity as is the case in ADHD, large samples are imperative, it is thus possible that some of the samples lacked statistical power and the results reported could be either false negative or false positives results. Another problem is that the gender ratio in most of the studies focusing on ADHD is skewed, with boys being over representative in these studies. This can make the interpretation of the results difficult, are the findings related to the *COMT* Val^{158}Met polymorphism or do they arise because of gender difference in COMT activity and hormones that can influence *COMT* expression. For example, it is known that women have a 20 to 30% lower COMT activity compared to men (Boudikova, 1990; Fahndrich, 1999). Females also have higher levels of estrogens than males and high estrogen levels can lower *COMT* expression and activity (Xie et al., 1999). Another possible reason for these inconsistencies could be that researchers did not take into account the effect of age and maturity when composing their samples, grouping together children as young as 4 and as old as 17 years. Having such a large age span in a research group can lead to false conclusion. First of all, then the dopaminergic system appears to be among the most age-sensitive neurotransmitters system (Joseph, 1990). Secondly then, COMT activity increases by about 10-fold from birth to adulthood (Guldberg & Marsden, 1975), and finally, DA levels decrease in the brain with age (Volkow, 1996). Thus, comparing a 5-year-old boy with the Met/Met genotype to a 17-year-old male with the same genotype might be questionable based on the effect that age has on the level of COMT activity. Not to mention if one would compare a 5-year-old girl to a 17-year-old female, where you have both the effect of age and the rise in estrogens that accompany sexual maturation influencing COMT activity.

The definition of phenotypes for genetic studies is a challenging endeavour and this represents a significant problem for ADHD researchers, reflected in inconsistent findings. First of all, the DSM-IV and ICD-10 ADHD criteria are based only on phenomenology, intentionally ignoring etiology, so there is no overt attempt to define ADHD phenotypes according to genetic bases.

Second, despite that the DSM-IV and ICD-10 diagnoses are based on the same 18 symptoms, and none of them are necessary or sufficient for the diagnoses, decisions rules about subtypes of the disorder and co-morbid condition differ between these diagnostic systems. For example, DSM-IV recognizes three subtypes of ADHD (combined, inattentive, hyperactive/impulsive) and encourages diagnoses of many co-morbid disorders, whereas the ICD-10 classification system only uses Hyperkinetic Disorder as a subtype and only lists one co-morbid condition, Hyperkinetic Conduct Disorder. The various combinations of inclusion criteria based on subtypes and co-morbid disorders can produce at least 12 possible phenotypes of ADHD (Barr, Swanson, & Kennedy, 2001). If researchers only use the broad DSM-IV definition of ADHD-combined, ADHD-inattentive or ADHD-hyperacitive/impulsive and do not split their samples into more homogenous groups according phenotype and co morbidity, they run the risk of masking phenotypic variation in their samples. Therefore, the primary concern for researchers should be the correct classification of individuals into homogeneous groups, in order to enhance the possibility of detecting genes that are involved in determining characteristics or traits (Barr et al., 2001). This problem of phenotype definition in ADHD research is highlighted in nearly two decades of incompatible research findings, making it painstakingly obvious that symptom based diagnostic classification systems such as the DSM-IV and ICD-10 do not facilitate mapping between susceptibility genes and behavioural outcomes (Cornblatt & Malhotra, 2001).

The search for an appropriate way to define psychiatric phenotypes is crucial for the

understanding of the genetic basis of psychiatric disorders. It has been suggested that a focus on phenotypic or latent traits, rather than broadly defined behavioural syndromes, would be a more prosperous approach and could in due course contribute to the redefinition of traditional psychiatric syndromes (Skuse, 2001). Latent genetically influenced traits, which may be related only indirectly to the classic disease symptoms defined in DSM-IV or ICD-10, are known as endophenotypes (Gottesman & Gould, 2003). The main idea with endophenotypes is to bridge the gap between behaviour and genes, with genes acting from the inside out through proteins, cells and multiple pathways exerting their influence on the behaviourally expressed phenotype, bringing us a step closer to the biological causes and improving our understanding of the genetic mechanism of complex disorders (Schulze, 2004; Skuse, 2001).

Another way to explain these discrepancies among studies is to look at the functional aspects of the *COMT* polymorphism from the perspective of the tonic-phasic model put forward by Grace (1991). In brief, this theory states that the dynamics of DA regulation is controlled by so-called phasic and tonic mechanism. The phasic mechanism releases DA into the synaptic cleft, with the tonic mechanism regulating the amplitude of the phasic release via autoreceptor position on the presynaptic neuron. Therefore, the higher the tonic level is, the lower the phasic release of DA will be and vice versa. The Val allele, associated with high-activity COMT, increases phasic and reduces tonic DA transmission subcortically and decreases DA concentrations in the PFC. The Met allele, associated with low-activity COMT, decreases phasic and increases tonic DA transmission subcortically, and increases DA in the PFC. The relationship between the *COMT* gene and PFC activity is, however, more complex than simply stating that either allele is good or bad. One way to look at the association of the *COMT* gene and optimal function of the PFC is to see it as an inverted U-shaped curve, where either too low or too high DA levels attenuate performance (Goldman-Rakic et al.,2000; Seamans et al., 2004).

Looking at it from this point of view, the precise effect of COMT activity on PFC function is likely to be dependent on where on the inverted U-shaped curve the individual in question lies in any given environmental or genetic context. The position of each individual on the U-shaped curve is governed by many factors, one of them being stress. It is know that stress can increase the extraneuronal levels of DA in the PFC, where it is suspected that steroids such as estrogens promote cortisosterone release (Caticha, 1993). This increase in corticosterone potently blocks extraneuronal catecholamine transporters leading to increased DA levels in the PFC (Gründemann et al., 1998). This augmentation in DA levels results in the activation of D5 receptor positioned on the dendritic stem reducing signal transfer in the PFC impairing its control of behaviour (Arnsten, 2001). Thus it is possible that individuals with the highly active Val/Val genotype, living in a very stressful environment with resulting increment in PFC DA levels, could have similar DA levels as those individuals that have the less effective Met/Met genotype living in an environment with "normal" stress level. Thus, it is vital that researchers take into account stress-inducing factors, such as severe martial discord, low social class, maternal mental disorder and foster placement, when looking at the effects of *COMT* upon behaviour.

Many of the studies investigating the association between ADHD and *COMT* have focused solely on the *COMT* Val^{158}Met polymorphism yielding controversial and confusing finding. Although stratification problems or poor control of environmental factors, such as stress, in the study design might explain some of the inconsistencies, a more plausible explanation might be the genetic variations in the coding sequence and/or regulatory regions contributing to the gene expression and enzymatic activity (Michaelovsky et al., 2007).

A highly advantageous method for studying the effects of genetic variations on biological functions and disease susceptibility is the construction of haplotypes, as they characterize the linkage disequilibrium (LD) structure of several markers that might show

a stronger association with illness than a single marker, such as the *COMT* Val^{158}Met polymorphism (Schaid, 2004). Recently, it was found that a risk haplotype (GAA) composed of the G allele from the SNP in the P2 promoter of the MB-COMT (rs2097603), the A allele of the *COMT* Val^{158}Met SNP (rs4680) and the A allele from the SNP in the 3' region of the gene (rs165599) was associated with ADHD in a sample of children with the 22q11.2 deletion syndrome (Michaelovsky et al., 2007).

Figure 12: Polymorphic sites on the *COMT* gene

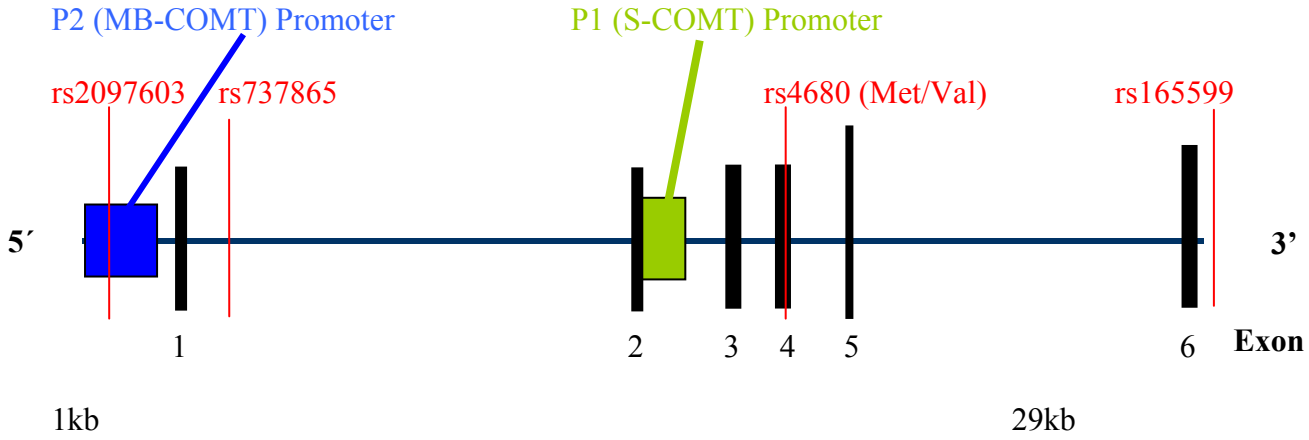


Figure adapted from (Meyer-Lindenberg et al., 2006)

Each of these polymorphisms has previously been implicated in modulation of *COMT* enzymatic activity. Having the A allele in the rs4680 SNP leads to marked reduction in *COMT* enzymatic activity (Lachman et al., 1996), the G allele in the P2 promoter SNP (rs2097603) can reduce the *COMT* enzyme activity even further (Chen, 2004) and the 3'SNP (rs165599) was shown to affect *COMT* mRNA expression in post-mortem brain tissue (Bray et al., 2003).

The effect of this risk haplotype on ADHD susceptibility could be attributed to variations in function of the individual SNPs and/or to additional effects of the entire haplotype, where the G-A-A haplotype increases the risk of ADHD, while the A-G-G haplotype reduced the risk of developing ADHD (Michaelovsky et al., 2007). However, these findings from Michaelovsky and colleagues cannot be extrapolated directly to

ADHD in the general population, since 22q11.2 deletion syndrome represents a particular and unique case. However, these findings gain support from a study looking at the functional aspects of a *COMT* haplotype composed of the same SNPs, where it was found that individuals without any psychiatric disorders and carrying the G-A-A haplotype had the poorest performance on prefrontal working memory tasks (Meyer-Lindenberg et al., 2006), supporting the notion put forward by Bilder and colleagues that phenotypic expression of the *COMT* Val^{158}Met SNP affects both a broad range of neuropsychiatric syndromes (Bilder et al., 2004).

Thus, *COMT* may contain at least three functional polymorphisms that have impact on its biologic actions and confound its clinical associations. To make things even more complicated, findings from Nackely et al suggest that a synonymous change in the coding region of the *COMT* gene (rs4818) can affect secondary mRNA structure and consequently protein quantity and enzymatic activity (Nackley et al., 2006). The potential complex interactions of functional variations in *COMT* imply that the overall functional state of the gene in an individual, presumably critical for phenotypic association, may not be easily deduced from *COMT* genotype information alone. Thus, haplotypic information is crucial if multiple functional sites, affecting transcription and protein function, are implicated within a single gene and can provide important information about disease susceptibility.

Therefore, the molecular genetic findings presented thus far should be considered preliminary and interpreted with caution, taking into account confounding factors like small sample size, heterogeneous samples and difference in phenotype definition.

The hope of genetic studies is that they may untangle genetic subtypes yielding a meaningful relation between genes and ADHD, but the success of future studies hinges on the degree to which researchers can correctly address these issues in the planning and design of future studies.

12.2. Risk factors for ADHD and co-morbid CD and ODD

Conduct problems, such as oppositionality, defiance, delinquency, verbal and physical aggression account for the majority of reported co-morbidity in ADHD. In the DSM-IV classification system these behaviour can be classified under two distinctive behaviour disorders, oppositional defiant disorder (ODD) and conduct disorder (CD). Symptoms of ODD include defiance of authority, failure to comply with adult requests, bullying, blaming others and performing acts that constitute minor violations of age-appropriate societal norms. CD describes children with aggressive and delinquent behaviours that represent major violations of age-appropriate social norms. CD symptoms can be divided into two categories aggressive and non-aggressive acts. Among the aggressive acts are fights or committing assaults, whereas non-aggressive acts include lying or stealing without confronting the victim (APA, 1994). Studies indicate that around 50% of ADHD children meet the criteria for either CD or ODD (Barkley, 1998). ODD and CD are often interrelated, therefore they are frequently discussed together in the literature. Despite the common co-occurrence of these disorders, little is known about the causes of this overlap, but studies indicate that both genetic loading and exposure to environmental risk factors predispose individuals to ADHD and co-morbid CD and ODD (Faraone et al., 2000; Thapar et al., 2001).

Finding the answer to the question why some children with ADHD engage in such behaviour is important and has both clinical and societal implications, whereas those children with co-morbid CD/ODD have more severe symptoms and serious clinical course with worse prognosis than do individuals with ADHD only (Burt, 2005). Furthermore, as the definition of these conditions includes either physical harm to others or property damage, the societal cost of CD/ODD is enormous.

This covariation between ADHD and CD/ODD is considered to be partly accounted for by a common genetic factor, and based on results from studies linking increased levels

of catecholamines with aggressive behaviour (Volavka et al., 2004), the *COMT* Val^{158}Met SNP has been suspected of playing a role in the expression of antisocial behaviour in ADHD (Thapar et al., 2005). Environmental factors are furthermore, thought play a large role in emergence of CD/ODD, with families characterized by social isolation, broken homes and weak social support are more likely to neglect or physically abuse their children, increasing the risk that the children develop aggressive behaviour (Bassarath, 2001; Caspi et al., 2002).

12.2.1 ADHD symptom severity and co-morbid CD

Co-morbidity of ADHD is one of the most actively studied topics in the field of child and adolescent psychiatry. Among clinic-referred children co-morbidity is frequently found between ADHD and CD; with co-morbid CD being related to greater ADHD symptom severity (Connor et al., 2003).

This raises questions about the potentially different etiologies of ADHD alone and ADHD with co-morbid CD, are these disorders separate entities or do those with ADHD and co-morbid CD have a more severe variant of ADHD compared to those who have just ADHD (Hurtig et al., 2007). Findings indicate that relatively pure cases of both can be found and that these disorders are likely to have different correlates and outcomes (Hinshaw, 1987; Jensen et al., 1997). Children with co-morbid CD often come from backgrounds with greater social adversity and higher prevalence of psychiatric disorders among their parents and relatives than ADHD children without CD (Biederman et al., 1996; Jensen et al., 1997). ADHD children, on the other hand, are more likely to have developmental delays and cognitive deficits compared to children with CD. While, children with both disorders often display mixture of the cognitive and attentional deficits typical of ADHD in addition to the difficulties arising from factors such as social adversity, family psychiatric problems and family conflict (Jensen et al., 1997).

Gaining a better insight and understanding of the relationship between ADHD and

CD is important and has major clinical implications. The presence of a co-morbid condition in a child has significant consequences since those children often have more serious clinical course, with poorer outcome. They have more negative parent-child relationships, show more aggression, and in adulthood, have higher rates of psychiatric in-patient admissions, than those individuals with just single disorder (Burt, 2005).

When looking at those factors influencing co-morbid CD in the present study, ADHD symptom severity was a positive predictor of lifetime CD in our sample. Those children with co-morbid CD had on average significantly higher total ADHD symptom score compared to those with just ADHD, 41 and 35 respectively. These findings are in accordance with previous findings, with Taylor and colleagues reporting that hyperactive symptoms in childhood predicted conduct problems in adolescence (Taylor et al., 1996). Furthermore, Connor et al (2003) and Kuhne et al (1997) reported that co-morbid CD was related to greater ADHD symptom severity in their samples (Connor et al., 2003; Kuhne et al., 1997).

It is imperative for clinicians to be aware of variables that are associated with increased co-morbidity in children with ADHD. The identification of these variables is important for developmental psychopathology because they are central to understanding how risk factors lead to disorders. Furthermore, the detection of these variables may stimulate further research efforts and enhance the knowledge of co-morbidity in ADHD. Identifying potential risk factors, such as ADHD symptom severity, for developing CD has significant clinical implications. Early recognition and intervention may prevent the progression from aggressive and maladaptive behaviours to CD and may reduce the overall severity of the disease burden in children with ADHD.

12.2.2 Early family risk factors and co-morbid CD

Considerable progress has been made in the genetics of ADHD and it is estimated that the heritability in ADHD is around 80%, where many genes of small effect contribute

to the disease susceptibility (Faraone et al., 2005; Fisher et al., 2002).

Furthermore, it is estimated that environmental factors can account for around 20 to 30% of the variance in ADHD symptoms, with a wide range of environmental factors associated with eventual symptoms and development of ADHD (Faraone et al., 2005). These environmental risk factors can be divided into three main groups. The first group consists of pre- and perinatal events, the second group includes family and parental factors and the last group comprises acquired neurobiological risk factors (Kunsti & Asherson, 2004). Among those environmental risk factors linked to mental disorders are, maternal stress during pregnancy, maternal substance abuse during pregnancy, birth complications, low birth weight, deprivation of normal parental care during infancy, childhood physical maltreatment, childhood neglect, premature parental loss, exposure to family conflict and violence, low social economical status, parental psychopathology and stressful life events involving loss or threat (Caspi & Moffitt, 2006).

In children with ADHD, psychosocial adversity has great impact on the course and future outcome of the disorder. Factors such as family conflict, poor quality of parent-child relationship, decreased family cohesion and parental psychopathology increase the risk for aggressive behaviour; and the development of a co-morbid condition such as conduct disorder (Caspi et al., 2002; Johnston, 2001; Thapar et al., 2006).

In the research literature, aggression has been subtyped as either proactive or reactive. Although many aggressive children show aspects of both types of aggression, some children can be classified as having predominantly proactive (well planned, instrumental and affectless) or reactive (impulsive, hostile and affective) aggression (Vitiello & Stoff, 1997). Aggressive children often misperceive or misinterpret social signals in others, and to judge a social situation correctly we must be able encode information about the situation accurately. This involves the skill of reading subtle cues about facial expression, tone of voice and what type of setting we are in. Then we have to be able to interpret the

situation as hostile, friendly or neutral. Finally, we have to select a goal for our interaction and generate possible responses, such as walk away, talk to the person or be aggressive. When choosing a response, we also have certain expectations about how successful that response will be in getting us what we want (Pliszka, 2003).

Dodge and colleagues classified a large population of third graders and a group of adjudicated juvenile offenders as showing either proactive or reactive aggression and asked them to judge a social situation. They found that reactively aggressive offenders made more encoding errors when judging a social situation compared to proactively aggressive offenders, who expected aggression to reduce aversion (Dodge et al., 1997). It would, therefore, be tempting to conclude that reactive aggressive children have a neuropsychological deficit in encoding facial cues and other subtleties of social interaction and that proactive aggressive children have learned (perhaps from the family environment) that aggression pays off .

When looking at the influence of early family risk factors in the present study, a summary score of risk factors during the first 3 years of life was formed. This variable focuses on abnormal intra-familiar relationship patterns, distorted communication within the family and parental separation/divorce or institutional education outside the family during the first three years of life. The results from the logistic regression analysis indicate that children growing up in an adverse family environment, during the first three years of life, are more likely to develop CD than children growing up in more favourable environment. Those children with a co-morbid CD in our sample had a mean score of 4.21 on the early family risk factor compared to 1.66 in the group with ADHD only. These figures are in harmony with Moffitt´s notion that risk factors seem to operate in an interactive fashion, with the magnitude of aggressive and antisocial behaviour increasing linearly with the aggregation of these risk factors (Moffitt, 1993).

These results are in accordance with previous findings from both clinical and

epidemiological research that adverse social and family environment is a positive predictor of co-morbid ADHD and influences the genesis of CD (Hinshaw, 1987; Rutter & Silberg, 2002; Thapar et al., 2006).

However, there is much heterogeneity in the way individuals respond to these environmental risk factors, and the key question in psychopathology is how does an environmental factor influence the nervous system to generate the symptoms seen in psychiatric diseases (Caspi & Moffitt, 2006).

Among the many aspects of family adversity possibly influencing the development of aggression in ADHD children, are physical abuse and neglect. These two facets of family adversity are usually associated with other types of dysfunction in the parent-child relationship: lack of bonding, eye contact and language stimulation. The "core" of human moral behaviour develops early and depends crucially on the social stimulation provided by a nurturant caregiver. One of the important foundations of human moral behaviour, empathy, that is detectable early in life, is notably reduced in children who have had abusive or neglectful caregivers (Pennington, 2002).

According to Keiley and colleagues, the earlier children experience maltreatment stress, the more likely they are to engage in antisocial behaviour (Keiley et al., 2001). These findings gain support from animal studies showing that early life maltreatment stress can alter monoaminergic neurotransmitter systems and influence aggressive behaviour (Bennett et al., 2002; Bremner & Vermetten, 2001). Animal studies using rats, furthermore indicate that the first two weeks of life are critical in mediating the effects that this maltreatment stress has upon the developing animal, with these two weeks being the equivalent to the first 3 years of human development (Liu et al., 1997). These factors, combined with the negative arousal that accompanies abuse, could interfere with the development of the brain mechanisms underlying social cognition and affect regulation, increasing the risk that the individual develops antisocial or aggressive behaviour (Pliszka, 2003).

The results of this study, therefore add to the body of evidence that early family risk factors, as measured in this study by abnormal intra-familiar relationship patterns, distorted communication within the family, and parental separation during the first three years of life, have a strong influence on co-morbid CD in children with ADHD.

12.2.3 *COMT* Genotype and co-morbid CD

There is converging evidence indicating that neurobiological factors are among the main mechanisms underlying the symptoms of ADHD and that faulty neurotransmission within the monoamine system plays a pivotal role in the etiology of the disorder. Furthermore, ADHD conveys a significant risk for other co-morbid psychiatric disorders, with 40 to 60% of children with ADHD showing signs of antisocial behaviour, evident in CD and ODD (Willcutt, 1999).

One of many genes implicated in the etiology of ADHD is the *COMT* gene, which is responsible for nearly 60% of dopamine degradation in the prefrontal cortex (PFC) and as such plays a key role in modulating dopamine neurotransmission in the PFC (Gogos, 1998; Karoum et al., 1995). Therefore, the *COMT* gene has considerable influence on the optimal functioning of the PFC, which is the cornerstone of behavioural guidance (Grossman et al., 1992). The PFC is, furthermore, very sensitive to its neurochemical environment and changes in catecholamine modulation can have deleterious effects on its ability to guide behaviour (Arnsten & Li, 2005), with results from imaging, neuropsychological and neurobiological studies suggesting that dysfunction of the PFC is a significant predisposition to antisocial behaviour (Raine, 2002).

This covariation between ADHD and CD/ODD is considered to be partly accounted for by a common genetic factor, and based on results from studies linking increased levels of catecholamines with aggressive behaviour (Volavka et al., 2004), the *COMT* Val^{158}Met SNP has been suspected of playing a role in the expression of antisocial behaviour in ADHD (Thapar et al., 2005).

The results of the logistic regression analysis in the present study revealed that the relationship of the *COMT* Val^{158}Met SNP with co-morbid CD in our sample fell just short of significance, with the p value being 0.051. Among children with Met/Met genotype, 33% had co-morbid CD, in the Val/Met group, 16% had co-morbid CD and in the Val/Val group, 25% of the children had co-morbid CD. When the odds of having CD were compared among the genotype groups, using the Met/Met genotype as a reference group, those with Val/Met were significantly less likely to have co-morbid CD compared to Met/Met, p> 0.016. Those with Val/Val genotype were less likely to have co-morbid CD compared to those with Met/Met genotype, the difference was however not significant p= 0.654.

One way to interpret this increased rate of CD among the *COMT* Val^{158}Met SNP homozygous groups is to refer to the tonic-phasic model put forward by Grace (1991), where the optimal function of the PFC is depicted as an inverted U-shaped curve, with either too low or too high DA levels attenuating the regulatory influence of the PFC on behavior (Goldman et al., 1998; Seamans et al., 2004). Those children homozygous for the low activity Met allele would have increased DA in the PFC, and those homozygous for the high activity Val allele would have reduced DA concentrations in the PFC. With either too much or too little DA concentration leading to faulty PFC behavioral control, interfering with the children's ability to control their own behaviour and impairing their ability to consider the future implications of their acts. Such children may have difficulty understanding the negative effect their behaviour has on others and fail to inhibit inappropriate behaviour or adapt behaviour to changing social circumstances, resulting in oppositional or antisocial behaviour (Moffitt, 1993).

The relationship between the *COMT* gene and PFC activity is however, more complex than simply stating that either allele is good or bad, and the precise effect of COMT activity on PFC function is likely to be dependent on where on the inverted U-

shaped curve the individual lies, and that being influenced by complex interaction of other environmental and genetic factors.

The results of the present study that the Met/Met genotype group had the highest rate of CD among the genotype groups are in accordance with many previous studies implicating increased levels of brain catecholamines with aggressive behavior (Volavka et al., 2004). Results from animal studies, indicate that catecholamine agonists, such as L-DOPA and apomorphine, increase aggressive behavior and *COMT* knockout male mice display increased aggression (Gogos, 1998; Lammers & Van Rossum, 1968). Furthermore, Rujescu et al reported that the Met allele was associated with violent suicide attempts and a greater tendency towards external expression of anger across all subjects in a large study including 149 violent suicide attempters and 328 controls (Rujescu et al., 2003). This association between increased levels of brain catecholamines and aggressive behavior has also been supported by work done with the *MAOA* gene. Where elevated aggressive behavior has been observed in *MAOA* knockout male mice (Cases et al., 1995) and inactivation of *MAOA* has been associated with aggressive behavior in humans (Brunner et al., 1993). Furthermore, Caspi et al. (2002) reported an interaction between variation in the *MAOA* gene and childhood maltreatment in the development of antisocial behavior, where those children with a genotype that leads to low *MAOA* expression and were maltreated in childhood were more likely to develop antisocial behavior.

The results of this study are, on the other hand, contradictory to the findings of Caspi et al (2008), where Val/Val homozygouts were significantly more aggressive than those carrying the Met allele. A possible explanation for this discrepancy could lie in the difference in sample characteristics. The present study is based on a clinical sample using DSM-IV criteria for diagnoses of both ADHD and CD, whereas the Caspi study is based on three samples, one clinical and two birth cohorts, varying in both demographics and definition of CD (Caspi et al., 2002; Thapar et al., 2005; Trouton et al., 2002). In the

Caspi study, the age of the participants ranged from 5 years to mid twenties. Considering the important role that the frontal cortex has on guiding behaviour and given the fact that it does not reach full maturity until the late twenties, such an age span could be a confounding issue (Kolb & Whishaw, 1996).

Furthermore, antisocial behaviour is a complicated phenotype, and each method used to measure it is characterized by different strength and limitations, which brings us to the next confounding factor in the Caspi et al (2008) sample. Looking at the ascertainment procedure in the study it is clear that none of the three samples in the Caspi study employed the same procedure, making it questionable if they are actually talking about the same construct when they talk about CD. These ascertainment procedures might be well founded and standardized, but none of them asks the same question or makes the same demands when defining the phenotype of CD or antisocial behaviour. In the Trouton (2002) sample the Childhood Behaviour Checklist was used to define CD status, whereas the Caspi (2002) used a composite index of antisocial behaviour in adolescence and adulthood to define CD status. Finally, in the Thapar (2005) sample a clinical diagnostic interview with a CD symptom count was used, instead of a full blown CD diagnoses according to the DSM-IV. There is, however, a great divide in equating symptoms of aggression to the DSM-IV based definition of conduct disorder. This is evident when we take a closer look at the distribution of CD symptoms in the Thapar sample, where the Met/Met group had on average 0.76 CD symptoms, compared to 0.82 in the Val/Met and 1.23 in the Val/Val group. It is clear that the majority of children have no or very few CD symptoms in this sample and are far away from reaching the 3 symptoms that are needed for DSM-IV diagnoses of CD. This is reflected in the small portion of children in the Thapar sample reaching a DSM-IV diagnoses of CD or only 8.25%, compared to 23.5% in the present study.

Another issue is the way the genotype groups were composed, the sample in the

present study was divided into three genotypes reflecting the functional effects of the *COMT* enzyme, whereas Caspi and colleagues divided their samples into two groups, those homozygous for Val/Val and those carrying a Met allele. This combination of Val/Met and Met/Met genotypes into one group does not reflect the difference in activity level of the enzyme, possibly masking the effect of the Met allele in the process. Therefore, it cannot be excluded that some confounding factors in either study design or demographic of the samples may have been driving the association observed by Caspi and colleagues.

Despite the incongruence between our findings and those of Caspi et al (2008), our results in addition to previous studies implicate the *COMT* gene in the regulation of aggressive behavior, with those individuals homozygous for the Met allele having more severe ADHD symptoms and being more prone to aggressive or antisocial behaviour compared to those with either Val/Met and Val/Val genotype.

12.2.4 Risk factors for ADHD and co-morbid ODD

All children are oppositional from time to time, defying or disobeying parents or teachers. The magnitude or persistence of the behaviours associated with ODD, such as frequent temper outbursts and excessive arguing, can significantly impede adaptive adult-child and child-peer interactions, justifying it's categorisation as a disorder. ODD is one of the most common associated problems among children with ADHD, diagnosed in around 50% of children with ADHD, and is characterized by a recurrent pattern of negativistic, defiant, disobedient and hostile behaviour toward authority figures (APA, 1994).

Despite this considerable overlap between ODD and ADHD there has been little investigation of ODD co-morbid with ADHD (Loeber, 1990), with ODD being studied largely within the context of CD (Connor & Doerfler, 2008). Indeed, most studies on disruptive behaviour disorders have combined children with ODD and CD into a single

generic category, often called “conduct problems”. It has been argued that this practice has contributed to obscured findings and conclusions that are difficult to interpret (Greene et al., 2002).

This is a critical issue considering that the majority of children with ODD do not have CD and may not progress to CD in later years (Hinshaw, 1987).

An improved understanding of ODD therefore requires examination of the clinical correlates of the disorder independent of its association with CD.

Such information can strengthen our understanding of ODD as a meaningful nosological entity and lead to improved treatment approaches aimed at ameliorating the disorder (Greene et al., 2002).

We therefore excluded all children with co-morbid CD from the analysis, 39 in all, when looking at those factors influencing the emergence of co-moribid ODD in our sample.

The initial logistic regression model revealed that none of the gene*environment interaction had a significant impact on the emergence of co-morbid ODD in our sample. Therefore, another model was constructed excluding all interaction terms. In this second model, age, ADHD symptom severity and IQ were significant predictors of co-morbid ODD. Having already discussed the possible effects of ADHD symptom severity upon co-morbid CD, the focus will be on the effects of age and IQ s significant predictors of ODD in our sample.

12.2.5 The effect of IQ on co-morbid ODD

Among the most consistent findings in research focusing on delinquency is the presence of cognitive deficits as significant predictor of delinquency. Results from studies indicate that low IQ is a risk factor for both emergence and continuity of antisocial behaviour across life course in both prospective and cross-sectional studies (Hinshaw, 1992; Moffitt, 1993; Simonoff et al., 2004).

Looking at the influence of IQ on the emergence of co-morbid ODD in the study, the results of the logistic regression show that IQ is a positive predictor of co-morbid ODD in our sample, with those having higher IQ being less likely to have co-morbid ODD. Those with a co-morbid ODD had on average lower full scale IQ compared to the non-co-morbid group, 99.75 versus 102.18 respectively, the difference being not significant.

These results are similar to previous findings and in a review of the neuropsychology of delinquency, Moffitt (1993) pointed out that delinquents showed an overall deficit of a half standard deviation (about 8 points) compared to nondelinquents. However, it is less certain to which extent it is simply lower cognitive ability or specific cognitive deficits, such as poor executive function that are the key factors (Raine, 2002). Finding from studies indicate that that performance IQ is greater than verbal IQ, suggesting that specific language difficulties are prevalent among children with ODD (Lynam et al., 1983). As language development progresses in children they learn to use language as a tool to label and communicate their feelings and thoughts, resulting in more sophisticated mechanisms for self-regulation and affective modulation and by this enabling them to generate strategies aimed at facilitating beneficial interactions with the environment. A possible explanation for this observed correlation between low verbal IQ and delinquency could be that children with specific language difficulties have a propensity to misunderstand rules or find it too difficult to settle conflict with words, resulting in situations that often spiral into aggressive acts (Greene et al., 2002; Koenen et al., 2006). The evidence available to date suggests that language difficulties may be especially important in ODD, but again it is unknown whether such cognitive factors are markers of some other adversity, behave as risk factors themselves or represent key causal mechanisms through which other risk factors operate (Nigg & Huang-Pollock, 2003; Raine, 2002).

12.2.6 Age and ODD

Executive functions represent the farthest reaches of human nature, influencing complex cognition and social behaviour, such as the ability to organize behaviour in order to fulfil goals and intentions, self-awareness and empathy. The neurological substrate for executive functions is related to the frontal lobes, which are slow to mature and are not fully developed until the late twenties (Kolb & Whishaw, 1996). Welsh and colleagues argued for distinct developmental stages of the frontal lobes, where the ability to resist distraction develops around the age of six and impulse control reaching adult level around the age of ten (Welsch et al., 1991). Callahan similarly suggested that the chronologically-delayed development of the frontal lobes was synonymous with the demarcated signs of competent adulthood, such as the ability to anticipate, understand and to be held accountable for the consequences of one's actions (Callahan, 2001).

ODD is typically seen in children before they reach 10 years of age and usually not later than early adolescence and is characterized by a recurrent pattern of developmentally inappropriate levels of negativistic, defiant, disobedient, and hostile behaviour toward authority figures. The frontal lobes play a key role in controlling our actions and reactions to the environment, social understanding and empathy; and with maturity and age playing a central role in their development surprisingly little is known about the role of age when it comes to ODD. Data suggest that the percentage of preschool and early school-age children meeting the criteria for the ODD ranges from seven to 25%, with some studies reporting that early age-of-onset in ADHD is correlated with increased symptom severity and co-morbid psychopathology (Connor et al., 2003).

Loeber and colleagues reviewed the literature and stated than no firm conclusions could be drawn about the prevalence of ODD as a function of age or how age influences the onset of ODD (Loeber et al., 1990). This could possibly be explained by that the age spans studied have been either too narrow or too wide. Another reason could be that

researchers have mainly studied ODD within the context of CD, making it difficult to draw any definite conclusion about ODD and those factors influencing it (Greene et al., 2002). The results from the logistic regression indicate that age was a positive predictor of co-morbid ODD in our sample. Looking further at variable age in the study, it was apparent that those with co-morbid ODD were older than those without ODD, 9.8 versus 9.4 years old, the difference being non significant.

However, it is a bit problematic to interpret these findings, whereas the variable age only indicates the age of the child when it entered the study. Therefore, the variable age only tells us that at the time point when the children entered the study those with co-morbid ODD were on average older than those without co-morbid ODD. It tells us nothing about how age influences the emergence of ODD in our sample. Another confounding factor when interpreting the influence of age on co-morbid ODD is that when gathering information for the study, those children that currently fulfil or had at any time fulfilled the diagnostic criteria for ODD, were grouped together into the variable "lifetime ODD". We do not know how age affects co-morbid ODD in this group labelled "lifetime ODD", did the children receive their diagnoses at an early age, do they still have co-morbid ODD or have they outgrown their ODD symptoms? In hindsight, it would have been sensible to acquire the age when the diagnosis of ODD was confirmed, the variable "age at ODD diagnosis" could then be used to further differentiate those ODD children that outgrow their symptoms compared to those who develop CD or any other psychiatric disorder. For example, do those children that develop CD or any other psychiatric diagnosis get their ODD diagnosis at a very early age compared to those who outgrow their symptoms?

ODD can serve as a partial marker for the development of CD or other psychiatric disorders, but the pathways involved in linking the initial state of having ODD with any of these later manifestations of disorder have yet to be sorted out. Therefore, gaining

information about the clinical correlates of ODD independent of its association with CD can lead to improved understanding of ODD and resulting in improved treatment approaches aimed at ameliorating the disorder before the children embark on the pathway to develop either CD or other psychiatric disorders.

13. Limitations

Our sample included 166 children, thereof only 24% had a DSM-IV diagnoses of CD, which is less than in most clinical samples, so false positive findings cannot be excluded. We only examined the *COMT* Val^{158}Met SNP, therefore, we cannot exclude that other functional variants in the *COMT* gene were driving the results of our findings. Furthermore, whereas the study did not include a control group, the results might only pertain to a clinic-based sample of school-aged children with ADHD. Early family risk factors were assessed retrospectively, so recall bias cannot be excluded. Furthermore, it is likely that larger samples carefully characterized and stratified according to age and gender will be necessary to yield meaningful relations between *COMT* genotype and ADHD.

14. Conclusions

Despite these limitations, the main finding of the study is that the *COMT* Val^{158}Met SNP is associated with ADHD in our sample, with the Met allele being over-transmitted in our sample. Secondly, smoking during pregnancy had significant influence ADHD symptom severity and those with the *COMT* Met/Met genotype had the most severe ADHD symptoms in our sample. Finally, ADHD symptom severity and adverse early family circumstances during the first three years of life are positive predictors of lifetime CD in our sample.

On the other hand, there were no gene*environment interaction influencing the development of CD in our sample or ADHD symptom severity. *COMT* genotype showed a trend toward influencing co-morbid CD in our sample, where those with the Met/Met genotype were significantly more likely to develop co-morbid CD compared to those having Val/Met genotype, Met/Met carriers were also more likely to develop CD compared to those having Val/Val genotype but the difference was not significant.

These findings have implications for the prevention and treatment of ADHD and co-morbid CD, emphasizing the need to address early psychosocial factors and smoking during pregnancy, and offering parents preventive training programs to deal with more severe form of ADHD associated with these risk factors.

It is important to be aware of variables that are correlated with increased co-morbid psychopathology in children with ADHD. Such variables can become targets for clinical interventions that may reduce the overall severity of disease burden in referred youths with ADHD. In addition, the identification of variables associated with increased co-morbid psychopathology in ADHD may stimulate further research effort and facilitate greater understanding of co-morbidity in ADHD. Combining genetic, environmental and neurobiological research has the potential to delineate causal links between ADHD and the developmental course of the disorder, including persistence of ADHD symptoms into adulthood and co-morbidity with associated psychiatric disorders.

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ERKLÄRUNG

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