

**A genetic study of Attention Deficit Hyperactivity Disorder:
Examining environmental influences and phenotypic
variation**

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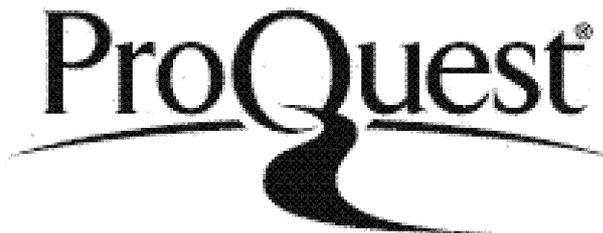
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Abbreviations

ADHD	Attention Deficit Hyperactivity Disorder
CAPA	Child and Adolescent Psychiatric Assessment
CD	Conduct Disorder
ChATTI	Child ADHD Teacher Telephone Interview
COMT	Catechol-O-Methyltransferase
CI	Confidence Intervals
DAT1	Dopamine transporter gene
DRD3	Dopamine Receptor D3 Gene
DRD4	Dopamine Receptor D4 Gene
DRD5	Dopamine Receptor D5 Gene
DSM-IV	Diagnostic Statistics Manual IV
ELBW	Extremely low birth weight ($\leq 1500\text{g}$)
GxE	Gene x Environment interaction
ICD-10	International Classification of Diseases – Version 10
IQ	Intelligence Quotient
LBW	Low birth weight ($\leq 2500\text{g}$)
LRTDT	Logistic Regression Transmission Disequilibrium Test
MAOA	Monoamine Oxidase A
ODD	Oppositional Defiant Disorder
OR	Odds ratio
PIQ	Performance Intelligence Quotient
rGE	Gene x Environment Correlation
TDT	Transmission Disequilibrium Test
VIQ	Verbal Intelligence Quotient
VLBW	Very low birth weight ($\leq 1000\text{g}$)
VNTR	Variable number of tandem repeats

Summary

Candidate gene association studies of Attention Deficit Hyperactivity Disorder (ADHD) reveal mixed findings, possibly due to heterogeneity within the ADHD phenotype and Gene x Environment interactions.

Although specific environmental factors confer risk in the aetiology of ADHD, this is an understudied area, especially regarding possible modifying effects of environmental factors on the ADHD phenotype.

Utilising a clinically ascertained sample of 373 ADHD children and their parents, the contribution of specific genetic and environmental factors and their interaction was investigated. The following variants in dopaminergic genes were genotyped; DRD4 48bp VNTR, DAT1 480bp VNTR, DRD5 (CA)_n microsatellite variant, DRD3 Ser9Gly variant, MAOA 30bp promoter variant and COMT Val¹⁵⁸Met variant.

Family-based analysis revealed no significant association between any of the gene variants and ADHD. Modifying effects on the ADHD phenotype were found. The DRD4 48bp VNTR 7-repeat allele and MAOA 30bp promoter variant low activity allele were associated with antisocial behaviour.

Maternal smoking during pregnancy, birth weight and social class on the ADHD phenotype had significant modifying effects on the total number of hyperactive-impulsive symptoms and antisocial behaviour.

Finally, analysis was conducted testing possible interaction between the gene variants and environmental risk factors. A previously reported interaction between DAT1 genotype and maternal smoking during pregnancy was not replicated. There was evidence of significant interactions between the COMT Val¹⁵⁸Met genotype and social class for both hyperactive-impulsive and conduct disorder symptoms. Significant interaction between the DRD3 Ser9Gly variant and social class was also found for total number of ADHD symptoms. These interactions indicated that where environmental adversity was low, the genetic variant had a greater effect.

This work highlights phenotypic heterogeneity and how this may index underlying aetiological heterogeneity. It also indicates the importance of environmental variables and investigating Gene x Environment interactions in molecular genetic studies of ADHD.

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Thesis Overview

This thesis, ranging over eight chapters, will investigate whether specific gene variants and environmental risk variables contribute to the diagnosis of Attention Deficit Hyperactivity Disorder (ADHD) and have modifying effects on the phenotype.

In the first chapter, a general introduction of ADHD will be followed by a detailed account of the evidence suggesting that genetic influences are important in the aetiology of the disorder. A review of the literature to date of molecular genetic studies of ADHD is included. There will also be a discussion regarding the phenotypic heterogeneity of ADHD and how looking at more homogenous sub-samples may increase the ability to detect genetic risk factors.

The analyses undertaken throughout this thesis utilise a clinically referred sample of 373 six to 16 year olds diagnosed with ADHD and their families. Sample ascertainment and characteristics are described in chapter 2. The specific measures and sub-samples used for each section of analysis are then described in more detail at the beginning of the relevant chapters; 3, 5 and 7.

In chapter 3, the main effects of specific gene variants on the diagnosis of ADHD are investigated, followed by analysis of possible modifying effects of these variants on specific aspects of the ADHD phenotype. Chapter 4 presents a selective literature review of environmental risk indicators for ADHD, focusing on three factors; maternal smoking during pregnancy, birth weight and social class. The possible

modifying effects that these three variables may have on the ADHD phenotype are then analysed in chapter 5.

The concept of Gene x Environment interaction (GxE), previous evidence for its importance in complex disorders (including ADHD) and relevant methodological issues are introduced in chapter 6. The contribution of GxE to the diagnosis of ADHD are assessed in chapter 7. This is followed by analysis based on the environmental factors selected in chapter 5 into of GxE modifying effects on the ADHD phenotype.

A discussion of the findings in chapters 3, 5 and 7 is provided at the end of each respective chapter. Finally, in chapter 8, a general discussion will summarise the findings in all chapters, as well as briefly examining methodological limitations and possible future directions for research.

CHAPTER 1

General introduction, family and genetic studies of ADHD

1.1 Introduction to ADHD as a disorder:

Attention deficit hyperactivity disorder (ADHD) is a childhood onset psychiatric disorder characterised by pervasive and impairing levels of behaviour in three areas; overactivity, inattention and impulsivity. In spite of a wealth of research, the aetiology and causes of the disorder are still largely unknown.

Behaviour problems involving these types of symptoms have been recognised for a number of decades, although the diagnostic criteria and emphasis on such problems have undergone a number of changes. The relevance of each symptom domain, especially hyperactivity; the need for pervasiveness of symptoms across situations; and a variety of causative factors have been differentially emphasised. These changes have resulted in the current similar, but subtly different diagnostic criteria as laid out by the Diagnostic statistics manual version IV (DSM-IV) (American Psychiatric Association, 1994) Attention Deficit Hyperactivity Disorder and the International Classification for Disease version 10 (ICD-10) (World Health Organisation, 1993) Hyperkinetic Disorder (see Appendix (i) for details of diagnostic criteria). These two terms and definitions are frequently used interchangeably, a problem increased as many studies, especially older ones, use previous diagnostic criteria such as DSM-III-R ADHD. In order to adhere to common convention therefore, the general term ADHD will be utilised throughout this thesis to refer to the disorder according to any diagnostic criteria, unless otherwise specified.

Prevalence Rates:

ADHD is one of the most prevalent childhood mental health disorders (Leibson & Hall Long, 2003) and is the most common reason for referral to child mental health

services (Woodward et al., 1997). Although a range of prevalence rates have been reported, a recent and comprehensive epidemiological survey in the UK has been performed (Meltzer et al., 2000), the findings of which have been used to inform government statistics. This study reported prevalence rates of 1.4% for Hyperkinetic Disorder. Interestingly, in the same sample rates of DSM-IV ADHD were found to be higher – 2.2% - (Ford et al., 2003) again illustrating the differences between diagnostic criteria.

Treatment:

A range of different treatments have been utilised for ADHD with some success. The best evidence supports the use of stimulant medication (Brown et al., 2005) which have been demonstrated to have good efficacy in reducing core ADHD symptoms in 75% to 95% of individuals (Solanto et al., 2001). Such pharmacological interventions have been shown to be as effective, if not superior to, intensive behaviour programmes and a combined pharmacological and behavioural approach (MTA Cooperative, 1999).

Gender differences:

A higher proportion of males consistently appear to have the disorder than females; Barkley (1998) notes that boys have a three-fold increase in prevalence for ADHD in epidemiological studies which rises to a six-fold increase in clinical samples.

The reasons why ADHD is identified less frequently in females is generally unknown, although there has been some speculation. It appears that females have a slightly different clinical presentation of the disorder (Biederman & Faraone, 2004). Meta-

analyses have demonstrated that females present with lower levels of hyperactive and impulsive symptoms, as well as lower rates of co-occurring antisocial behaviour disorders (Gershon, 2002; Gaub & Carlson, 1997). Some studies (e.g. Graetz et al., 2005; Gershon, 2002) also indicate that females have higher levels of comorbid anxiety and depressive disorders. However, research seems to conclude that ADHD is the same disorder in males and females (Biederman & Faraone, 2004; Barkley, 1998) as similar patterns of familial transmission of the disorder and comorbid conditions are observed for both genders.

Associated problems:

Considering that the diagnostic criteria for ADHD require impairment of functioning across academic and social settings, it is unsurprising that ADHD is associated with a number of negative outcomes. ADHD children often do not attain the levels of academic achievement predicted by their IQ (Kamphus & Frick, 1996; Frick & Lahey, 1991). Children with ADHD are also more likely to be suspended or excluded from school than their non-ADHD peers, even when the comorbid diagnosis of conduct disorder is taken into account (Barkley, 1998; Fischer et al., 1990).

Peer relationships are also affected. ADHD children have fewer friends than their non-ADHD peers (Hoza et al., 2005), whilst as many as 50% will experience specific peer problems and peer rejection (Guevremont & Dumas, 1994). Relationships with siblings (Mash & Johnston, 1983), teachers (Whalen et al., 1981) and mothers (Edwards, 2001; Cunningham & Barkley, 1979) may also be affected.

Adolescent and Adult outcomes:

Although a childhood onset condition, the effects of ADHD are seen to extend into adolescence and even adulthood. Increasingly, adult ADHD has been recognised and studied (Faraone 2004). Despite some interest in this area, the outcomes are somewhat unclear, largely because of wide variation between individuals; a further demonstration of the heterogeneity of the disorder.

Individuals who have ADHD in childhood are noted to complete significantly fewer years education and fail to complete high school more frequently than non-ADHD controls (Barkley, 2002; Hansen et al., 1999; Mannuzza et al., 1997). In adulthood, those who had ADHD as children are found to be of lower social class than matched controls, despite being matched for parental social class at baseline (Hansen et al., 1999; Mannuzza et al., 1997; Biederman et al., 1993).

Findings suggest children with ADHD are more likely than controls to be in prison, or be involved with the law during adolescence and adulthood (Rasmussen et al., 2001; Satterfield & Schell, 1997; Mannuzza et al., 1997), as well as having higher levels of drug use and abuse (Disney et al., 1999; Mannuzza et al., 1993). However, these findings may be partially, or wholly, mediated by comorbid conduct problems (Disney et al., 1999; Satterfield & Schell, 1997; Moffitt et al., 1990).

Cost of care:

In order to further highlight the relevance of studying ADHD, it seems important to note that the disorder is associated with a significant burden, often financial, to a range of agencies, from mental health services to the individual's family. Individuals

with ADHD have roughly double the healthcare costs of those without the disorder (Leibson & Hall Long, 2003), greater than the costs for other chronic childhood disorders, such as Asthma, Epilepsy and Sickle Cell Anaemia (Leibson & Hall Long, 2003; Shatin et al., 1998). Increased service utilisation and costs in primary care (Guevara et al., 2001), medication (Guevara et al., 2001), Accident and Emergency departments (Leibson et al., 2001; DiScala et al., 1998) and most significantly, although unsurprisingly, mental health services (Shatin et al., 1998) have all been reported.

Interestingly, the (non-ADHD) relatives of ADHD children also have a higher utilisation of healthcare services (Swenson et al., 2003) and there are additional costs to the family through loss of work hours and increased household costs (such as replacing household items and food supplements) (Sayal et al., 2003).

Comorbidity:

ADHD is frequently found to be comorbid or co-occur with a variety of other disorders. Although the presence of some disorders, for example Autistic spectrum disorder, means that a diagnosis of ADHD cannot be made (APA, 1994; WHO, 1993), others are often seen in conjunction with ADHD. Between 43 and 93% of ADHD children are estimated to have a comorbid conduct disorder (Jensen et al., 1997) whilst Biederman and colleagues (1991) estimate that one third have a comorbid internalizing disorder and 25 to 40% are found to have comorbid reading disability (Friedman et al., 2003). These high rates of comorbidity are one of the greatest sources of heterogeneity for the disorder. Jensen and colleagues (1997) note that by not taking comorbid conditions into account has led researchers to have very

heterogeneous samples, whilst just excluding individuals with comorbid conditions would be unrepresentative of the ADHD population.

Indeed, ADHD with comorbid oppositional defiant disorder (ODD) (characterised by behaviours such as defiance, arguing, being deliberately annoying and having temper tantrums, see appendix (ii)) and conduct disorder (CD) (symptoms of which include truancy, stealing, fighting and assault, see appendix (ii)) is thought to be so distinct that the ICD-10 criteria define it as a separate disorder. ICD-10 classifications of Hyperkinetic Disorder are split into those with and without a conduct disorder (Hyperkinetic Conduct Disorder and Hyperkinetic Disorder respectively) (See Appendix (i) for details). No such distinction is present in DSM-IV diagnoses. That such a separation of diagnoses is made further highlights the perceived importance of comorbid conditions.

There is much evidence to suggest that those with comorbid conditions in addition to ADHD fare much worse than those with just one disorder on a wide variety of measures. Increased levels of ADHD symptoms in those with a comorbid conduct disorder have been observed across samples and using multiple informants (Gadow & Nolan, 2002; Woodward et al., 1997; Kuhne et al., 1997; Biederman et al., 1991). Similarly, it appears that comorbidity with ADHD increases problems within other disorders. For example, having a comorbid diagnosis of ADHD increases persistence and severity of conduct symptoms in comparison to those with conduct disorder alone (Loeber et al., 2000; Abikoff & Klein, 1992). The detrimental effects of having ADHD with an additional comorbid condition is also observed to increase risk for additional non-drug psychiatric disorders at follow up 13 years later (Fischer et al.,

2002) and also lead to additional social impairment (Gadow & Nolan, 2002; Kuhne et al., 1997).

However, caution is warranted when looking at comorbidities as there are a number of questions regarding how rates of comorbidity are calculated and, indeed, whether or not child and adolescent psychiatric disorders can indeed be comorbid (e.g. Angold et al., 1999; Caron & Rutter, 1991). Such questions have centred on the sample assessed, methods used for such assessments and even the diagnostic criteria utilised.

Firstly, the sample population studied may result in different conclusions; it has been shown that there is a distinct bias in those attending specialist clinics which affects how representative clinic referred samples can be of the general population. Those individuals attending specialist services have higher rates of comorbid conditions (Woodward et al., 1997). Such a bias may lead to inflated prevalence rates of comorbidity with ADHD reported from studies based on such groups.

Epidemiological samples may also have their limitations; because of small numbers of disordered individuals in the community, researchers may sacrifice detail by collapsing groups, for example males and females, ODD and CD diagnoses and so on. This may mask important issues in the data (Maughan et al., 2004). Further, Faraone and colleagues (2000a) note that epidemiological samples may also exaggerate levels of comorbidity as, in order to increase the numbers of disordered individuals, they utilise relatively low thresholds for disorder. Although the exact prevalence of comorbid conditions may require further investigation, it can be observed that they are of importance as comorbidity can lead to worse clinical manifestation of the disorder and worse prognoses.

Neuroimaging studies:

A number of structural and functional neuroimaging studies comparing ADHD individuals to matched controls have been undertaken, spurred on by the development of non-invasive *in vivo* techniques such as Magnetic Resonance Imaging (MRI) and Electroencephalography (EEG). The findings of such studies are somewhat restricted by limitations including small sample sizes, the efficiency of matching controls and the cost of such procedures (Hale et al., 2000). However, such methods provide a unique opportunity to investigate the pathophysiology of ADHD, not possible using other techniques (Bush et al., 2005).

Structural neuroimaging studies have reported reduced total brain volume in ADHD children compared to controls of up to 5% as well as a loss of caudate asymmetry (e.g. Castellanos et al., 1996). This volume reduction is, however, subject to inter-individual differences and studies require large sample sizes to reach statistical significance (Durston, 2003), whilst findings regarding the specific areas reduced and direction of caudate asymmetry are inconsistent (Hale et al., 2000). Functional neuroimaging studies, looking at individuals both at rest and when performing cognitive tasks, further indicate possible hypofunctionality, of frontalstriatal systems in the aetiology of ADHD. These differences are specifically observed in the dorsal anterior cingulate cortex, caudate and putamen (Bush et al., 2005; Durston, 2003). These findings are generally consistent with current aetiological theories of ADHD, implicating the involvement of systems known to be active during executive functions and attentional processes and which are rich in catecholamines (Bush et al., 2005; Hale et al., 2000). However, further neuroimaging studies are required before conclusions can be drawn.

1.2 Evidence of familiarity and heritability:

Family Studies:

There is a great deal of evidence to suggest that ADHD runs in families and that, at least in part, it has a genetic cause. This evidence comes from a variety of sources using a range of divergent study designs.

Family designs, which mostly compare the families of clinic-referred children with ADHD to psychiatric and normal control families, have found increased rates of ADHD in all family members, including siblings (Smalley et al., 2000; Biederman et al., 1992; Faraone et al., 1991). Family studies have also found that the half siblings of ADHD children reared together have a significantly lower risk of developing the disorder than full siblings (Goodman, 1989). Overall, elevation in the risk of ADHD when having a first degree relative with the disorder is found to be modest, but significant, with relative risks of between 4.0 and 5.4 (Thapar, 2002).

Adoption studies:

Studies utilising other designs have also implicated a genetic component for ADHD. Adoption studies (e.g. Sprich et al., 2000; Albers-Corush et al., 1986; Cantwell, 1975; Cunningham et al., 1975) have found that adopted children with ADHD are more similar to their biological parents on ADHD measures than to their adoptive parents, regardless of whether the ADHD behaviour is identified through the child or the biological parents. Although clearly indicating a genetic contribution to ADHD, adoption studies have been criticised for limitations such as small sample sizes, the fact interviewers are generally not blind to psychiatric or adoptive status (McMahon, 1980) and lack of specificity.

Twin Studies:

One disadvantage of family studies is that they cannot disentangle genetic and environmental influences (Nadder et al., 1998); because biological relatives generally share both genes and an environment, it is difficult to ascertain which is responsible for the concordance in ADHD behaviour.

Twin studies enable such effects to be distinguished (Sherman et al., 1997).

Monozygotic (MZ) twins share all their genes in common, whilst dizygotic (DZ) twins share, on average, only half their genes (the same as other, non-twin siblings). If MZ twins are significantly more similar than DZ twins on a measured variable, then, assuming MZ and DZ twins have equally similar rearing environments, this difference can be attributed to genetic effects. Structural equation modelling allows this effect to be quantified (the heritability estimate), as well as calculating the proportion of the variance attributable to shared, or common, environment and to the environment unique to that individual (including measurement error) (Plomin et al., 1997)

Twin studies have consistently found a large, significant contribution of genes to ADHD in studies across the world, including the UK (Price et al., 2001; Thapar et al., 1995; Goodman & Stevenson, 1989a), Europe (Steffensson et al., 1999; Gjone et al., 1996), Australia (Levy et al., 1997) and the U.S (Sharp et al., 2003; Nadder et al., 1998; Neuman et al., 2001). These twin studies have indicated high heritability of ADHD with between 60 and 88% of the variance in ADHD scores being explained by genetic factors (Thapar & Scourfield, 2002). Estimation of levels of heritability have been found with a range of informants including parents and teachers (Thapar & Scourfield, 2002). Estimations of heritability do not appear to alter regardless of

where the level for extreme scores is set, where defined as a broad category (Price et al., 2001; Thapar et al., 2000; Sherman et al., 1997; Goodman & Stevenson, 1989b) and throughout the normal variation of scores (Levy et al., 1997).

1.3 Genetic Heterogeneity in ADHD:

ADHD is a clinically heterogeneous disorder as illustrated by the frequent presence of comorbid conditions in some individuals, different DSM-IV subtypes of the disorder, as well as variations in associated impairment of functioning and outcomes.

Researchers have been interested in whether or not variations in the presentation of the disorder index genetic heterogeneity.

Symptom dimensions:

Within the DSM-IV classification of the disorder, symptoms of ADHD are split into two groups or dimensions; the hyperactive-impulsive symptoms and the inattentive symptoms (see Appendix (i) for details), a distinction not found in the ICD-10 diagnostic criteria for Hyperkinetic Disorder. The distinction between these dimensions has been deemed important as relating to DSM-IV subtypes of the disorder (predominantly inattentive type and predominantly hyperactive-impulsive type – see appendix (i) for details), but may also be relevant to those individuals with the combined type of the disorder and to those with ADHD scores within the normal range. Researchers have studied whether or not there are differing genetic factors influencing the hyperactive-impulsive and inattentive symptom dimensions. The evidence, mostly from twin studies, indicates that there may be. Sherman and colleagues (1997) found independent genetic effects influencing the two dimensions.

These findings were generally replicated by Hudziak and colleagues (1998) who found shared, as well as distinct, genetic effects. Studies using factor analysis (Neuman et al., 2001; Rohde et al., 2001) have also reported genetically distinct classes for inattentive and hyperactive-impulsive behaviours. Thus it would appear that the two dimensions of ADHD symptoms are, at least in part, genetically distinct. In contrast, the evidence to suggest that the clinically defined DSM-IV subtypes are genetically distinct is less clear. A family study performed by Faraone and colleagues (2000a) found no evidence of familial distinction between the subtypes, a finding mirrored in analysis of sibling pairs (Smalley et al., 2000) and in general population twin studies (Todd et al., 2001).

Comorbidities:

Investigation has been performed looking at whether ADHD with comorbid conditions is genetically distinct from ADHD alone. In the case of ADHD with comorbid conduct disorder, this appears to be the case.

In support of the diagnostic distinction between Hyperkinetic Disorder and Hyperkinetic Conduct Disorder made in ICD-10, family studies have compared the families of those with ADHD with comorbid CD to those with ADHD alone. Across independent samples, it has been demonstrated that family members of those with ADHD and comorbid CD are at greater risk for both disorders than those with ADHD alone (Biederman et al., 1992; Biederman et al., 1991). If ADHD and CD are separate disorders which are transmitted independently, then the risk of CD would not differ between the families of ADHD and non-ADHD control individuals, regardless of the familiarity associated with ADHD itself. This is not seen to be the case. Family

members with ADHD probands are at greater risk of CD in comparison to controls even if CD is not present in the proband (Faraone et al., 1997; Faraone et al., 1991). Such co-aggregation of ADHD indicates that the two disorders are transmitted, at least in part, together. This co-aggregation does not appear to be due to cross-mating between parents with ADHD and CD, as mothers with ADHD involved in family studies, are not significantly more likely to marry individuals with CD (Faraone et al., 2000a; Faraone et al., 1991).

Faraone and colleagues (2000b) have demonstrated that the relative risk of ADHD for the parents of a child with the disorder could be almost doubled, from 5.4 to 9.5 by refining the phenotype to include only those with comorbid CD. Such an increase in relative risk leads to an increase in statistical power for molecular genetic studies (Risch 1990a; 1990b).

This separation of ADHD with comorbid CD and ADHD alone has been seen using other study designs including affected sibling pair (Smalley et al., 2000) and twin samples (Thapar et al., 2001), further supporting such findings. Thapar and colleagues (2001) found a quantitative distinction between ADHD alone and ADHD with comorbid CD with the latter showing higher heritability. These authors concluded that ADHD with comorbid CD shows quantitative differences in genetic loading compared with ADHD alone, but the two have some common aetiology.

1.4 Molecular genetics and ADHD:

Following the body of evidence from adoption, family and twin studies that ADHD is largely genetic in aetiology, researchers have been interested in trying to identify specific susceptibility gene variants for the disorder (Kent, 2004). Like most psychiatric disorders, ADHD is considered to be a complex disorder whereby a large number of genes each contribute a modest effect to the aetiology of the disorder (Faraone et al., 2005). As such, no gene variant is either sufficient or necessary for the disorder to occur. Molecular genetic studies look for susceptibility loci based on either linkage or association methods.

Linkage studies:

Linkage studies identify co-segregation of a genetic marker and a disorder within families by detecting departure from independent assortment and, in parametric designs, estimating the degree of recombination between the marker and the disorder variant. If the disease loci and the studied marker are totally independent, a 50:50 chance of recombination would be expected. The closer two variants are together, the less likely recombination between the two and the further the ratio would be from this equal expected transmission (McGuffin et al., 1994). This deviation from expected transmission can then be quantified, identifying markers close to susceptibility loci. Parametric linkage analysis requires the specification of a number of parameters, including the mode of transmission and degree of penetrance, with the emphasis being on finding single genes with a large effect on the disease phenotype (Sham & McGuffin, 2002). As the mode of transmission for complex disorders such as ADHD is often unknown, such methods may not be suitable (McGuffin et al., 1994). Methods

which do not require specification of such parameters have also been devised, for example by using affected sibling pairs (ASPs). Such methods do not make prior assumptions about the number of genes involved or the mode of transmission, merely looking at whether or not genetic marker and disease are transmitted together more often than chance. This excessive sharing of markers can then be compared across the whole genome (Sham & McGuffin, 2002). Regions which are suggestive of significant linkage can then be investigated by linkage disequilibrium mapping in more detail. Such methods require large samples in order to detect genes of small to moderate effects that are likely to be involved in disorders such as ADHD.

Association Studies:

Association studies compare the allele frequencies of a specific marker in affected and unaffected individuals. Contingency tables can then be created to see if there is a significant difference between the two groups (Sham & McGuffin, 2002). Association studies often take the form of functional candidate gene variant studies, whereby researchers select genes with a plausible *a priori* role in the disease pathogenesis (Thapar & O'Donovan 2002). Significant differences in the marker frequency between affected and unaffected populations may be due to the marker studied being the susceptibility variant; the marker being very close to a susceptibility variant; or differences between the affected and unaffected groups (such as ethnicity), influencing allele frequencies and leading to spurious results. This last phenomenon is known as population stratification (Thapar & O'Donovan, 2002). Although researchers attempt to match samples well, this is a potential problem. One method of avoiding such false positive findings is by creating an internal control group using

genetic information from the parents of affected individuals (Thapar & O'Donovan, 2002).

The Transmission Disequilibrium Test (TDT) (Spielman et al., 1993) is a commonly used method for this. TDT analysis is a test of linkage in the presence of association (Spielman et al., 1993). The transmission of alleles to affected offspring from heterozygous parents are analysed with the transmitted allele considered as the case and the non-transmitted allele creating the control. If an allele is more frequently transmitted than would be expected (that is more than 50% of the time), it may be associated with the disorder. This deviation from expected transmission can then be statistically calculated using a McNemar test (Spielman et al., 1993). TDT analysis may be extended for examining quantitative traits (Waldman et al., 1999) and taking into account missing parent data (Clayton, 1999).

Whole genome linkage analyses of ADHD:

To date, three genome-wide scans of ADHD have been performed in the US (Ogdie et al., 2003; Fisher et al., 2002), Netherlands (Bakker et al., 2003) and Colombia (Arcos-Burgos et al., 2003). A number of regions have been implicated in each individual scan with three regions reaching the level of suggestive linkage (a lod score greater than 2.2) across two scans; 9q (Arcos-Burgos et al., 2003 and Bakker et al., 2003); 11q (Arcos-Burgos et al., 2003 and Ogdie et al., 2003); and 17p (Arcos-Burgos et al., 2003 and Ogdie et al., 2003). Although in their infancy in the study of ADHD, genome-wide scans provide some interesting findings and are likely to lead to the identification of susceptibility loci.

1.5 Candidate gene association studies of ADHD:

In contrast to the relative scarcity of genome-wide scans and other studies utilising linkage designs, a number of functional candidate gene association studies of ADHD have been performed, with some success. Many studies have concentrated on genes coding for receptors and enzymes within the dopaminergic system. The *a priori* justification for this comes largely from three areas; pharmacological treatments of ADHD, neuroimaging studies and animal studies.

Pharmacological treatment:

One of the main sources of evidence implicating involvement of the dopaminergic system in ADHD comes from studies of the therapeutic effects of stimulant medication. Stimulant medications, predominantly Methylphenidate and Dexamfetamine, have been demonstrated to reduce the core symptoms of ADHD in more than 70% of individuals (Thapar & Scourfield, 2002). Although the exact mechanisms through which such medications exert their therapeutic effect are not known, alterations in the dopaminergic system have been observed (Solanto et al., 2001). Empirical evidence suggests that stimulant medications inhibit the dopamine transporter, leading to increases in levels of dopamine in the synapse and also altering dopamine receptors, which control levels of post-synaptic dopamine (Seeman & Madras, 1998). Thus genes coding for the dopamine transporter (DAT1) and dopamine receptors have been suggested as being implicated in the aetiology of ADHD.

Neuroimaging studies:

Although few neuroimaging studies of ADHD have been performed and those that have are beset with limitations such as small sample sizes (often of adults) and possible confounding effects from previous administration of stimulant medication, those studies that have been performed have also implicated the dopaminergic system, specifically DAT1, in the pathophysiology of ADHD (Faraone, 2004). For example, significant increases in DAT1 density have been observed in adults with ADHD compared to controls (Krause et al., 2002; Dresel et al., 2000; Dougherty et al., 1999) although not all studies concur (Van Dyck et al., 2002). Again stimulant medication is seen to have an effect as these increases in dopamine transporter density have also been observed to disappear following continued therapeutic administration of Methylphenidate (Krause et al., 2002).

Animal studies:

Animal models derived from either naturally occurring or artificially produced genetic mutants or with brain lesioning or exposure to toxins can provide a useful paradigm for investigating the aetiology of ADHD. Subject groups are relatively homogenous and can be experimentally manipulated, whilst confounding factors such as comorbid conditions or previous pharmacological treatment, affecting other study groups are not present. However, it is important to remember that animal models may not be fully applicable to ADHD individuals, both because the manifestation of the disorder is not necessarily equivalent to the human presentation and because it is not fully understood what changes other than the production of ADHD-like behaviour experimental manipulation may have. Such animal experiments have also implicated the role of the dopaminergic system in ADHD. For example, neo-natal disruption of

the dopaminergic pathways using 6-hydroxydopamine (6-OHDA) has been seen to result in hyperactive behaviour in rats which is reversed by administration of Methylphenidate (Avale et al., 2003). DAT1 knock-out and knock-down mice (Giros et al., 1996) also exhibit hyperactive behaviour which is reversed by stimulant medication. In Rhesus monkeys, chronic low dose N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine results in behaviours such as attention deficits and lack of persistence in tasks thought to be due to abnormalities in dopamine metabolism (Roeltgen & Schneider, 1991). Again such deficits are, at least in part, ameliorated by administration of Methylphenidate (Faraone, 2004). Although the findings using different models are not entirely consistent (for example, the effects of the 6-OHDA rat are thought to be due to decreases in dopamine transporter density and dopamine neurones (Masuo et al., 2002) whilst in DAT knock-outs the reverse is true with an increase in dopaminergic function (Giros et al., 1996)), all such models implicate the dopaminergic system and it has been proposed that appropriate, intermediate, levels of dopamine are needed for optimal functioning (Davids et al., 2003).

Thus it seems that there is sufficient *a priori* evidence to implicate genes within the dopaminergic system for association studies of ADHD. Genes within the dopaminergic system are not the only ones to have been studied in relation to ADHD. A wide range of variants from other neurotransmitter systems, such as the noradrenergic, glutamatergic and serotonergic systems have also been studied, some of which, including the Serotonergic Transporter gene (5HTT) and SNAP-25, have been associated with the disorder across two or more independent samples. However, to look in depth at all of these genes is beyond the scope of this thesis. Therefore I shall concentrate only on those within the dopaminergic system.

Dopamine receptor 4 (DRD4):

One of the most widely studied variants associated with ADHD is with a variable number tandem repeat (VNTR) polymorphism in exon III of the Dopamine Receptor D4 gene (DRD4). Associations with this marker have been frequently replicated. This marker was initially chosen, in part, because of suggestions that it may be functional, with *in vitro* studies having shown that the 7-repeat version of this variant produced a blunted response to dopamine (Asghari et al., 1995; Van Tol et al., 1992). However, more recent studies have not confirmed the functionality of this variant (Kazmi et al., 2000; Watts et al., 1999).

La Hoste and colleagues (1996) first documented a significant association between the DRD4 7-repeat allele and ADHD, a finding which has since been replicated in both case-control and family-based studies, although not universally (Faraone et al., 2001). A meta-analysis of these findings (Faraone et al., 2001) found significant odds ratios of 1.9 (95% Confidence intervals (CI) 1.4, 2.2) for seven case-control studies and 1.4 (95% CI 1.1, 1.6) for fourteen family-based studies, concluding that this variant confers a small but significant risk for ADHD.

A number of other variants within DRD4 have also been studied, although with less success than the exon III VNTR. For example, a functional variant in the promoter region, -521 (C/T), which has been reported to reduce transcriptional activity by 40% (Okuyama et al., 2000), has been investigated *in vivo*. Although included in significant haplotype association with novelty seeking personality traits (Lee et al., 2003), to date, no studies have found significant association between this variant and ADHD (Mill et al., 2003; Barr et al., 2001).

In an attempt to eliminate sample heterogeneity and explain non-replication for this marker, as well as identify exactly which aspects of the disorder are related to the 49bp VNTR variant, researchers have sub-divided samples based on phenotypic differences. For example, it has been suggested that DRD4 is most relevant to inattention symptoms. Rowe and colleagues (2001) reported high levels of retrospectively recalled inattention symptoms in fathers with the 7-repeat allele and ADHD children, although a study by Kirley and colleagues (2003) failed to find such an association when looking at inattention symptom scores in a clinical ADHD sample. Swanson and colleagues (1998) found association between the 7-repeat allele in a group categorised as having severe ADHD. Curran and colleagues (2001) found high scorers in a population sample on hyperactivity scales were associated with the 7-repeat allele, also suggesting association with severe symptoms of the disorder. However, these findings have not been replicated (Kirley et al., 2004; Mill et al., 2002). Finally, two studies showed that the DRD4 7-repeat allele may confer risk specifically for ADHD children with comorbid conduct problems. In an analysis of pooled data from three studies in the UK and Ireland, Holmes and colleagues (2002) found that non-significant association with DRD4 in the full ADHD sample became significant when looking only at those with comorbid conduct symptoms. In an extension of the Irish sample, Kirley and colleagues (2004) found an association with a diagnosis of comorbid ODD, although the number of informative transmissions for those with comorbid CD were not sufficient to confirm previous findings.

Dopamine Transporter (DAT1):

DAT1 has also been extensively studied. Cook and colleagues (1995) reported evidence of association of a 10-repeat allele of a VNTR in the 3' untranslated region

(UTR) of DAT1 with ADHD. Interestingly, reporter gene studies (Fuke et al., 2001), and studies of RNA expression in human tissues (Mill et al, 2002) have shown that expression is significantly higher for the 10-repeat allele than for the other alleles suggesting this VNTR may be functional, although the evidence is conflicting (van Dyck et al., 2002). Moreover, several molecular genetic studies of DAT1 and ADHD have also found evidence for significant association between the DAT1 10 repeat-allele and ADHD (Chen et al., 2003; Curran et al, 2001; Daly et al., 1999; Waldman et al., 1998). However, others have found a trend (Barr et al., 2001) or no support (Todd et al., 2001; Holmes et al., 2000; Swanson et al., Palmer et al. 1999).

Four pooled-analyses of molecular genetic association studies of DAT1 and ADHD have been reported. Three (Purper-Ouakil et al., 2005; Curran et al., 2001; Maher et al., 2002) found small, non-significant odds ratios whilst Faraone and colleagues (2005) found a significant pooled odds ratio of 1.13 (95% CI 1.03, 1.24). It has been suggested that refining the ADHD phenotype may help to pick apart which aspects of ADHD are associated with DAT1 and this has been performed to some extent; Waldman and colleagues (1998) have suggested that the DAT1 VNTR is most closely associated with hyperactive-impulsive symptoms of ADHD, although this was not replicated in a study of Brazilian children (Roman et al., 2001).

Dopamine receptor 5 (DRD5):

A variant within the Dopamine Receptor D5 (DRD5) has also been associated with ADHD. The 148bp allele of a microsatellite marker with no known function (CA)_n, located 18.5kb 5' to DRD5 was first reported to be associated with ADHD by Daly and colleagues (1999). Since this date, other studies have found mixed findings, some

supporting the association (e.g. Tahir et al., 2000) and others finding no evidence of association (e.g. Payton et al., 2001; Barr et al., 2000). This failure to replicate may be due to a lack of statistical power (Maher et al., 2002), as two pooled analyses of the data (Lowe et al., 2004; Maher et al., 2002) have found small but significant association between the variant and ADHD. Refining the ADHD phenotype may also be of use when studying DRD5, as Lowe and colleagues (2004) suggest the association may be linked primarily to inattentive symptoms of the disorder. In their joint-analysis, excess transmission of the 148bp or 5 allele was found in the inattentive and combined DSM-IV subtypes of the disorder, but not in the hyperactive-impulsive type.

Dopamine receptor 3 (DRD3):

Other genes within the dopaminergic system have also been studied for possible association with ADHD, although with less intensity or success than those previously described. The dopamine receptor 3 gene (DRD3) is a promising candidate for ADHD as mice lacking functional DRD3 receptors demonstrate increased locomotor activity, especially in novel environments (Accili et al., 1996), whilst administration of DRD3 agonists have been seen to reduce spontaneous activity in rats (Daly & Waddington, 1993). Although hotly disputed, a DRD3 Ser9Gly polymorphism may have some functional significance with the G allele showing increased affinity to dopamine in comparison to the A allele, whilst the variant may also influence cellular transduction (Hellstrand et al., 2004; Lundstrom et al., 1996) although further *in vitro* studies are necessary to confirm this. This variant has been studied on a number of occasions (Muglia et al., 2002; Payton et al., 2001; Barr et al., 2000) although none of these groups found evidence of significant association, a finding confirmed by pooled data

analysis (Faraone, 2005). Looking more closely at phenotypic manifestations on ADHD may shed more light on the Ser9Gly variant. Retz and colleagues (2003) found heterozygosity within the variant was associated with impulsivity scores for German forensic patients with a violent history.

Dopamine Receptor 2 (DRD2):

The Dopamine Receptor 2 (DRD2) has also been studied notably the TaqI A1 allele of a restriction fragment length enzyme, thought to be associated with low DRD2 receptor density in humans (Noble et al., 1991). Comings and colleagues (Comings et al., 1996; Comings et al., 1991) report an association of the DRD2 TaqI A1 allele in case-control studies of ADHD children who mostly had comorbid Tourette's syndrome. However, these findings have not been replicated in ADHD only samples (Huang et al., 2003; Kirley et al., 2002; Rowe et al., 1999) or for another polymorphism within the gene (Kirley et al., 2002). Faraone et al. (2005) conclude that there is little or no evidence supporting association between ADHD and DRD2.

Dopamine Beta-Hydroxylase (DBH):

Dopamine Beta-Hydroxylase (DBH) is an enzyme which converts dopamine to noradrenaline. DBH is a promising candidate for ADHD as altered DBH activity has been demonstrated in ADHD adults through neuroimaging studies (Ernst et al., 1999). Most studies have concentrated on the non-coding 5' TaqI polymorphism of this gene. Association studies have had somewhat inconsistent findings regarding possible association of the A2 allele of this variant and ADHD. Some have found positive evidence of association (e.g. Roman et al., 2002; Daly et al., 1999; Comings et al., 1996), whilst others have found no such association (Wigg et al., 2002; Payton et al.,

2001). A recent combined analysis of these findings (Faraone et al., 2005) has, however, found evidence of association between the Taq1 polymorphism and ADHD.

Tyrosine Hydroxylase (TH):

Tyrosine Hydroxylase (TH) is involved in the rate-limiting step in the synthesis of dopamine by catalysing the conversion of tyrosine into dihydroxy-phenylalanine. Five groups have studied variants within this gene. One group (Kirley et al., 2002) found overtransmission of the A allele from fathers for a (TCAT)_n microsatellite in intron 1 but four other studies failed to find evidence of significant association (Tang et al., 2001; Payton et al., 2001; Barr et al., 2000; Comings et al., 1995). However these studies did not assess parental origin effects and so further work may be necessary.

Monoamine Oxidase A (MAOA):

Monoamine Oxidase A (MAOA) is a degradation enzyme located on the X chromosome, which oxidises monoamines including dopamine. A study within the Chinese population found significant association between a dinucleotide repeat in the promoter region of MAOA and ADHD (Jiang et al., 2001), although this finding has not been replicated by either our group (Payton et al., 2001) or a recent Irish study (Domschke et al., 2005). Domschke and colleagues (2005) looked at the functional 914G/T polymorphism and reported a significant association between the G allele, which is associated with increased MAOA activity and ADHD. Significant over transmission was increased when stratifying the sample by parental history of ADHD and also when looking at a three marker haplotype. The 914 G allele was not found to be associated with ADHD in our own sample (Lawson et al., 2003). Sabol and colleagues (1998) reported that a 30bp VNTR within the promoter region of MAOA

affects transcription efficiency. An Israeli family-based and case control study (Manor et al., 2002) reported high activity alleles of this variant to be associated with ADHD. A subsequent study by our group (Lawson et al., 2003) failed to replicate this finding. However, based on reports that MAOA may be implicated in aggressive behaviour in both humans (Brunner et al., 1993) and mice (Cases et al., 1995) with mutations in MAOA, Lawson and colleagues (2003) tested for and found significant increased transmission of the low activity alleles to ADHD individuals with CD symptoms. Because of the relative scarcity of studies regarding MAOA, further investigation into the possible relationship between variants in this gene and ADHD may be warranted.

Catechol-O-Methyltransferase (COMT):

Catechol-O-Methyltransferase (COMT) is an enzyme involved in the degradation of dopamine, making it an attractive candidate for studies looking for susceptibility loci for ADHD. A functional variant of this gene, the Val¹⁵⁸Met polymorphism, has been shown to have one high activity allele (Val) and one low activity allele (Met) (Lachman et al., 1996; Lotta et al., 1995) and has been implicated in pre-frontal cognition (Bellgrove et al., 2005; Diamond et al., 2004; Egan et al., 2001). This variant has been investigated for association with ADHD. Two studies have reported significant association, although not conclusively. Eisenberg and colleagues (1999) found significant association of the high activity Val allele and individuals with the hyperactive-impulsive subtype of ADHD, although this was not replicated by the same group in an independent sample (Manor et al., 2000). Secondly, a study of Han Chinese families demonstrated significant overtransmission of the low activity Met allele in males (but not females) with ADHD (Qian et al., 2003). A number of other

groups (e.g. Payton et al., 2001; Tahir et al., 2000; Hawi et al., 2000; Barr et al., 1999) have failed to detect any association with this variant and ADHD.

1.6 Modifying the ADHD phenotype for molecular genetic studies:

As demonstrated, a number of molecular genetic studies attempting to identify susceptibility variants for ADHD have been performed, with some success. However findings, even for the most promising candidates, have not been entirely consistent with many groups failing to replicate association. The issue of phenotypic definition has been highlighted as of importance; phenotypic heterogeneity both between and within samples may be one reason for non-replication of findings (Thapar et al., 2000; Faraone et al., 2000b). Faraone and colleagues (2000b) note that the aetiology of ADHD is very complex, especially as, although heritability for the disorder is high, the relative risks for first degree relatives are modest. To maximise power to find susceptibility variants and produce replicable results, it has been noted that it is necessary either to have extremely large samples or create more homogeneous samples (which increase locus specific relative risks) (Faraone et al., 2000b).

Increasingly, researchers have concentrated on specific aspects of the ADHD phenotype in an attempt to make samples more homogeneous. Exactly how this should be done is, as yet, unclear, especially as there are many potential ways of doing so. In terms of molecular genetic studies, it seems reasonable that those aspects of the ADHD phenotype investigated should be based on familiarity and heritability (Martin et al., 1997), as well as being feasible in practice and replicable (Faraone et

al., 2000). Although there are a wide range of possibilities, including looking at family history, persistence of symptoms over time and neuropsychological endophenotypes, I shall concentrate on two key areas for which there is some evidence that they index heterogeneity, specifically, ADHD symptom group and comorbid antisocial behaviour.

CHAPTER 2

Sample Ascertainment

This section describes the ascertainment and general characteristics of the family-based sample utilised for all analyses throughout this thesis. A significant proportion of the sample (n = 162, 43%) were clinically assessed by myself as a member of the field team working on the study. Specific descriptions relating to methods and sample characteristics used singularly for different analyses will be described at relevant points in the related chapters.

2.1 Inclusion criteria:

Children aged between six and sixteen years of age with a diagnosis of ICD-10 Hyperkinetic Disorder, DSM-IV ADHD or DSM-III-R ADHD were included in this study. Participants were required to be of British Caucasian origin to the index child's grandparents in order to ensure genetic homogeneity and were required to be living with at least one biological parent to ensure the availability of parental DNA. Because information regarding ADHD symptoms was required whilst the individual was not on stimulant medication, individuals were not included if they had been on stimulant medication for more than a year to avoid potential recall bias as parents were asked to describe their child's behaviour without medication. Exclusion criteria included a full scale IQ below 70, any major neurological condition including Epilepsy, Fragile X syndrome, Pervasive Developmental Disorder and Tourette's syndrome (although those with Tic disorders were not excluded). Ethical approval for this study was obtained from the North West England Multicentre Research Ethics Committee.

2.2 Assessment Procedure:

Children with diagnosed or suspected ADHD were referred by Child and Adolescent Psychiatrists or Paediatricians in the Greater Manchester, South Wales and Avon

areas of the UK. Following assent from parents, an initial telephone screen was performed to ensure eligibility to participate and inform parents about the study. A home visit was then arranged. Prior to this visit, further information sheets for both parents and children, a set of questionnaires and a consent form were sent to the family. These were completed by the parents and collected at the time of the visit.

A trained researcher completed the Child & Adolescent Psychiatric Assessment (CAPA, Angold et al., 1995) with the parents, typically the mother. The CAPA is a semi-structured, interviewer based assessment which has been very well validated and widely used (Angold et al., 1995). It provides information on symptoms and research diagnoses for ICD-10, DSM-IV and DSM-III-R childhood psychiatric disorders. For the purposes of this study, the sections regarding ADHD, oppositional defiant disorder, conduct disorder, anxiety, depression and tic disorders were administered. Interviews were audio-taped and checked for consistency in weekly meetings supervised by a senior child and adolescent psychiatrist. Kappa coefficients illustrated that agreement between raters for individual symptoms was “good” or “very good” (Landis & Koch, 1997) and demonstrated perfect agreement for diagnoses of disorder.

Whilst the interview with the parent was being conducted, the child was assessed in a different part of the house using the Wechsler Intelligence Scale for Children (WISC III-UK; Wechsler, 1992) and the Wechsler Objective Reading Dimension (WORD; Wechsler, 1992) which ascertains Intelligence Quotient and reading ability respectively. Both are widely validated and are regularly used by Educational and Clinical Psychologists across the UK. Written informed assent was obtained from the child prior to assessment.

In order to obtain information regarding pervasiveness of ADHD symptoms, necessary for ICD-10 and DSM-IV diagnoses of the disorder, the Child ADHD Teacher Telephone Interview (ChATTI; Holmes et al., 2004) was performed with the child's teacher. This is a semi-structured interview, based on the CAPA, which assesses ICD-10 and DSM-IV symptoms of ADHD. The ChATTI has demonstrated good levels of reliability and validity (Holmes et al., 2004). For the purposes of this study, for ICD-10 diagnoses pervasiveness was observed if the teacher endorsed one or more symptom in each of the hyperactive, impulsive and inattention symptom areas with associated impairment. For DSM-IV diagnoses, one or more symptoms were required from the inattention dimension and one of hyperactivity or impulsivity with associated impairment, reflecting the slight differences in symptom dimensions between the two classifications.

A range of questionnaire measures were also completed by parents, providing demographic and environmental information as well as questions to further ensure participants met inclusion criteria. This questionnaire package also included information regarding maternal smoking and drinking during pregnancy, the weight of the child at birth and family occupational status.

DNA samples were obtained from the index child and both parents, where available, through venous blood samples or buccal cell mouthwash samples. When one parent (usually the father) was not available, where possible a full biological sibling, screened using the CAPA to ensure they did not have ADHD, also provided a DNA sample. Failing this, parent-child duos were used. DNA was extracted from blood and buccal cell samples using standard procedures (as described in Payton et al., 2001).

2.3 Sample Characteristics:

The final sample consisted of 373 individuals with mean age nine years, two months (Standard Deviation two years, one month). 335 (90%) were male and 38 (10%) were female. The mean full scale IQ of the sample was 90 (Standard Deviation 12.1). Individuals were assessed separately for each of the ICD-10, DSM-IV and DSM-III-R ADHD diagnoses. The presence and absence of these diagnoses for each participant are detailed in Table 2.1. Individuals may have met diagnostic criteria for more than one diagnosis (e.g. ICD-10 HKD, DSM-IV ADHD combined type and DSM-III-R ADHD). However, individuals may have met criteria for only one diagnosis. For example, an individual meeting diagnostic criteria for DSM-IV ADHD Hyperactive-impulsive type, may not meet criteria for any other disorder. ICD-10 Hyperkinetic Disorder was diagnosed in 61% (n=213); 68% (n=245) had DSM-IV Combined type, 11% (n=42) had DSM-IV Hyperactive-impulsive type and 5% (n=20) had DSM-IV Inattentive type, whilst 97% (n=362) had a diagnosis of DSM-III-R ADHD. ICD-10 diagnoses of common comorbid conditions were present with the following prevalence; ODD 47% (n=147), CD 13% (n=46), anxiety disorders 5% (n=20), depression 1% (n=3) and tic disorders 10% (n=38).

Table 2.1: Summary of ADHD diagnoses for all individuals

	Present	Absent
ICD-10 Hyperkinetic Disorder	213 (61%)	160 (39%)
DSM-IV ADHD Combined Type	245 (68%)	128 (32%)
DSM-IV ADHD Hyperactive-impulsive Type	42 (11%)	331 (89%)
DSM-IV ADHD Inattentive Type	20 (5%)	353 (95%)
DSM-III-R ADHD	362 (97%)	11(3%)

The mean number of DSM-IV and ICD-10 ADHD symptoms within the sample ranged from 7 to 18 with a mean of 14.74 and a standard deviation of 2.45. Number of inattentive symptoms ranged from 0 to 9 with a mean of 6.99 and standard deviation of 1.70. For hyperactive-impulsive symptoms, the mean number of symptoms was 7.74 with a standard deviation of 1.49 and a range of 2 to 9. For DSM-IV and ICD-10 ODD symptoms, total number of symptoms ranged from 0 to 8 with a mean of 3.86 and a standard deviation of 2.49. Total number of DSM-IV and ICD-10 CD symptoms ranged from 0 to 8 with a mean of 0.91 and standard deviation of 1.42.

DNA was obtained from 243 trios and 117 parent-child duos, for whom an unaffected sibling was available for 34. Total sample size varied for genetic analyses. The number of individuals available for genotyping differed for each variant as genotyping was ongoing throughout sample collection.

CHAPTER 3

Molecular genetic analysis and results

3.1 Introduction:

Family, adoption and twin studies have suggested that there is a substantial heritable component in the aetiology of ADHD. Genes within the dopaminergic system have been implicated both theoretically and through previous molecular genetic studies. Issues of non-replication between samples and attempts to discover which aspects of the ADHD phenotype are associated with specific variants have been addressed by analysis of specific aspects of the ADHD phenotype. Such analyses have had some success but further investigation is warranted.

3.2 Aims:

To further develop such findings, this part of the thesis investigated variants within the dopaminergic system in a large sample of ADHD children using a family-based design. Based on the findings of previous studies, whilst accepting that not all variants have been genotyped in this sample, the following variants were investigated:

- DRD4 48bp VNTR
- DAT1 480bp VNTR
- DRD5 (CA)_n microsatellite marker
- DRD3 Ser9Gly variant
- MAOA 30bp promoter VNTR
- COMT Val¹⁵⁸Met polymorphism

Some of these variants have previously been analysed within this sample (See Lawson et al., 2003; Payton et al., 2001; Holmes et al., 2000). However, in order to confirm the robustness of the previous findings and to ensure that all individuals genotyped

have been included in the analysis, all variants have been reanalysed (using a slightly different statistical programme). It is important to note that association studies rarely look at only one variant, recognising that, especially in complex disorders where variants are likely to be of small effect, the chances of finding association with a significant variant are small. Instead, researchers often undertake systematic analysis of haplotypes across each gene. However, the purpose of this thesis is not solely to identify susceptibility variants for ADHD, rather to look at the phenotype of the disorder and other possible aetiological factors in greater depth. Therefore, I shall concentrate on analysing a small number of variants which have previously been investigated with replicated findings of association with the disorder.

Secondary analysis tested whether these variants have a modifying effect on the ADHD phenotype. Based on previous discussion of interesting phenotypic issues, the following phenotypes have been analysed:

- Inattentive symptoms
- Hyperactive-impulsive symptoms
- CD and ODD symptoms and diagnosis.

These aspects of the phenotype were investigated for all variants. Only when there was a clear *a priori* reason to perform other analyses, based on previous literature, were additional analyses undertaken.

3.3 Methods:

Association Analysis:

Association analysis between candidate gene markers and ADHD was performed using the TRANSMIT programme (version 2.5) (Clayton, 1999). Using a traditional TDT design, TRANSMIT tests for association between a genetic marker and disease by analysing transmission from parents to affected offspring. A Chi-square value is then calculated to test whether there is a significant over transmission of any alleles. This specific programme was chosen as it enables individuals to be included in the analysis when parental genotypes are unknown and includes information from unaffected siblings to narrow down the range of possible parental genotypes. Such methods are advantageous for this investigation as the sample included parent-child duos, for some of whom information from unaffected siblings was also available.

Files including all family members were created for each variant. Commands were added into the programme to include all possible alleles (-all) and to use the robust estimate of the variance of the vector score (-ro). All significance tests were then bootstrapped to include 10000 bootstrap samples (-bs10000). For multiallelic markers, two further tests were performed. In the first, the -agg5 command replaced that looking at all haplotypes to consider only those alleles with a frequency of greater than 5%. Those with frequency below 5% were aggregated. Finally, the original analysis was re-run with the hypothesised risk allele and all the other alleles aggregated (a risk versus non-risk analysis).

Possible deviations from Hardy Weinberg Equilibrium in probands and their parents were investigated using the in house programme HardyW (version 1.1; McGuffin and

Hamshere, 2001) and accessed through the Department of Psychological Medicine folder (PSYCM departmental) in the Networked Applications section of the Cardiff University computer system. For multiallelic markers, the risk allele was compared to all other alleles. Deviation from Hardy Weinberg Equilibrium might be indicative of selection, assortative mating, inbreeding or genetic drift and would violate the assumptions of equal transmission necessary for the test. Calculations for the whole sample, probands only, mothers only and fathers only were performed.

Statistical power calculations were performed using the Genetic Power Calculator (<http://statgen.iop.kcl.ac.uk/gpc/dtdt>). For this analysis, the population prevalence for ADHD was set at 0.014 in accordance with the findings of Meltzer and colleagues (2000). The relative risk for each genotype was taken from published meta-analyses, joint-analyses or pooled data analyses. For those variants where previous joint analyses have not been performed (MAOA 30bp promoter variant, COMT Val¹⁵⁸Met variant and DRD3 Ser9Gly variant), the risk ratio for the DRD4 48bp VNTR from the meta-analysis by Faraone and colleagues (2001) was used. Statistical power to detect an alpha effect significant at the 5% level was calculated.

Testing for modifying effects on phenotype measures:

To investigate the possible association between each candidate variant and the quantitative phenotypic measures, logistic regression TDT (LRTDT) was used. Described by Waldman and colleagues (1999), LRTDT is an extension of traditional TDT analyses (performed by such programmes as TRANSMIT) using logistic regression, which additionally enables the influence of one or more continuous or categorical variables to be ascertained. This method, implemented in standard

statistical programmes (in this case SPSS version 11), maintains the desirable features of TDT analysis, whilst also having the advantages of regression analysis.

Like traditional TDT, LRTDT utilises information regarding transmission of risk or non-risk alleles from heterozygous parents to offspring. Additional information from the proband who is being investigated (e.g. number of ADHD symptoms) can then be included. This means that any given proband may contribute one, two or no records of data, depending on how many heterozygous parents they have (this is the same as in traditional TDT analysis). The transmission of risk or non-risk alleles is the dependent variable whilst the phenotypic (or other) information act as independent variables.

Standard logistic regression analysis is then performed to ascertain whether or not there is a significant association between the genetic marker and phenotypic variable. This analysis was run in the SPSS programme (version 11, Norusis/SPSS Inc., 2001). Initially the analysis was run including the relevant phenotypic variable as the only independent variable. Where this variable significantly predicted transmission, possible confounding factors including age at assessment and gender as well as any other important covarying phenotypic variables uncovered in analysis, were added.

Genotyping information:

Genotyping of the variants was performed by a different member of our group at various time points during sample collection (as evidenced by differing sample sizes). The information regarding genotyping methods has been taken directly from previous publications by our group; Holmes and colleagues (2000); Payton and colleagues (2001) and Lawson and colleagues (2003). The genotyping primers and conditions, as well as references used are detailed in Table 3.1.

Table 3.1: Details of genotyping conditions references where obtained:

Variant	Primers	Conditions described by	Genotyped by	Further details in
DRD4 48bp VNTR	5' GCGACCGTGGTCTACTCG 3' 5' AGGACCCCTCATGGCCTTG 3'	Okuyama et al. (1999)	A Payton	Holmes et al. (2000)
DAT1 480bp VNTR	5' CTCCTGGTGTAGGGAACGGCCTG 3' 5' CTCCTGGAGGTCACCGGCTCAAGG 3'	Daly et al (1997)	D. Turic	Langley et al. (2005)
DRD5 (CA)_n microsatellite variant	5' CGTGTATGATCCCTGCAG 3' 5' GCTCATGAGAAGAATGGAGTG 3'	Sherrington et al. (1993)	A. Payton	Payton et al. (2001)
DRD3 Ser9Gly variant	5' GCTCTATCTCCAACCTCTCACA 3' 5' AAGTCTACTCACCCTCCAGGTA 3'	Lanfelft et al. (1992)	A. Payton	Payton et al. (2001)
MAOA 30bp promoter variant	5' ACAGCCTGACCCGTGGAGAAG 3' 5' GAACGGACGCTCCATTCGGA 3'	Sabol et al. (1998)	D. Turic	Lawson et al. (2003)
COMT Val¹⁵⁸Met variant	5' ACT GTG GCT ACT CAG CTG TG 3' 5' CCT TTT TCC AGG TCT GAC AA 3'	Lachman et al. (1996)	D. Turic	Turic et al. (2005)

3.4 Association analysis:

DRD4 48bp VNTR:

This analysis included 130 families. There were no deviations from Hardy Weinberg Equilibrium for this variant. The allele frequencies and results can be observed in Table 3.2. No significant over transmission of the 7-repeat allele (or any other allele) was observed (Global $\chi^2= 4.97$, df 5, p=0.52). These findings remained regardless of whether all alleles are included in the analysis or just those with a frequency of more than 5% (Global $\chi^2=4.21$ df 3, p=0.23). Again, when transmissions of the 7-repeat “risk” allele were compared to transmissions of all other alleles, no significant over transmission was observed ($\chi^2=0.02$, df 1, p=0.87).

Table 3.2: TRANSMIT results for DRD4 48bp VNTR (all alleles)

Allele	Frequency	Observed	Expected	χ^2	P-value
2	0.11	24	28	1.80	0.28
3	0.05	9	12	1.84	0.28
4	0.62	169	162	1.83	0.26
5	0.004	1	1	0.008	0.73
6	0.002	0	0.5	1.14	0.26
7	0.22	57	56	0.02	0.86
Global				4.97	0.52

DAT1 480bp VNTR:

259 families provided information for this analysis. No significant over transmission of the 10-repeat risk allele or any other alleles was observed (Global $\chi^2=2.83$ df 3, p=0.50) See Table 3.3 for details. The frequency of the 10-repeat risk allele was 74%. Findings remained negative when only those alleles with frequencies greater than 5% were analysed (Global $\chi^2=0.64$ df 2, p=0.73) and also when transmission of the 10-

repeat risk allele was compared to all other alleles ($\chi^2=0.28$ df 1, $p=0.60$). No deviations from Hardy Weinberg Equilibrium were detected for this variant.

Table 3.3: TRANSMIT results for DAT1 VNTR (all alleles)

Allele	Frequency	Observed	Expected	χ^2	P-value
8	0.001	0	0.5	1.20	0.26
9	0.25	132	129	0.25	0.63
10	0.74	382	386	0.37	0.57
11	0.005	4	3	1.31	0.28
Global				2.83	0.50

DRD5 (CA)_n Microsatellite marker:

121 families provided information for this analysis. No deviations from Hardy Weinberg Equilibrium were observed. For this variant, there was a trend towards significant over-transmission of the previously implicated 148bp, 5 allele. There was no evidence of association in the global significance of the test when all alleles were compared (Global $\chi^2=6.52$ df 12, $p=0.87$), or when only those alleles with frequencies above 5% were included (Global $\chi^2=4.22$ df 6, $p=0.59$). When transmission of the 5 risk allele was compared to all other variables ($\chi^2=2.83$ df 1, $p=0.08$), there was a trend towards significant association (see Table 3.4). Unfortunately, this sample had insufficient individuals with the inattentive or hyperactive-impulsive subtype to attempt to replicate the analysis of DRD5 by Lowe and colleagues (2004).

Table 3.4: TRANSMIT results for DRD5 (CA)_n Microsatellite (risk allele 5 vs. all other alleles)

Allele	Frequency	Observed	Expected	χ^2	P-value
Non-risk alleles	0.57	123	130	2.83	0.08
Risk allele 5	0.43	110	101	2.83	0.08
Global				2.83	0.08

DRD3 Ser9Gly variant:

323 families contributed information to this analysis. No deviations from Hardy Weinberg equilibrium were detected. The risk allele, allele G, had a frequency of 0.34. No significant over transmission of either allele was observed. These findings are summarised in Table 3.5.

Table 3.5: TRANSMIT results for DRD3 Ser9Gly polymorphism:

Allele	Frequency	Observed	Expected	χ^2	P-value
A	0.67	368	372	0.29	0.65
G	0.33	198	194	0.29	0.65
Global				0.29	0.65

MAOA 30bp promoter variant:

107 families contributed information to this analysis. No deviations from Hardy Weinberg Equilibrium were observed. For this analysis, alleles were split into those with high activity (alleles 3.5, 4 and 5) and those with low activity (allele 3). It was then treated as a biallelic marker. The low activity alleles had a frequency of 33%. No significant association was found between the low activity allele and ADHD ($\chi^2=2.53$ df 1, p=0.15). See table 3.6 for details.

Table 3.6: TRANSMIT results for MAOA 30bp promoter variant (high vs. low activity alleles)

Allele	Frequency	Observed	Expected	χ^2	P-value
High activity	0.67	138	144	2.53	0.15
Low activity	0.33	76	70	2.53	0.15
Global				2.53	0.15

COMT Val¹⁵⁸Met variant:

246 families contributed to this analysis. No deviations from Hardy Weinberg Equilibrium were observed. No significant associations was found between this variant and ADHD ($\chi^2=0.02$ p=0.89). These findings are summarised in Table 3.7.

Table 3.7: TRANSMIT results for COMT Val¹⁵⁸Met variant

Allele	Frequency	Observed	Expected	χ^2	P-value
Met	0.52	253	254	0.02	0.89
Val	0.48	239	238	0.02	0.89
Global				0.02	0.89

Statistical power calculations:

As can be seen in Table 3.8, the statistical power for each variant differed substantially. Adequate power was obtained for the DRD3 Ser9Gly variant and the COMT Val¹⁵⁸Met variant, but not for the DRD4 48bp VNTR, DAT1 480bp VNTR, DRD5 (CA)n microsatellite variant and MAOA 30bp promoter variant.

Table 3.8: Statistical power calculations for TRANSMIT analysis.

	Risk allele frequency	Relative risk Aa genotype	Relative risk AA genotype	Reference for relative risk	No. of trios	Power at 5%
DRD4 48bp VNTR	0.22	1.4	1.96	Faraone et al., 2001	130	0.38
DAT1 480bp VNTR	0.74	1.27	1.54	Maher et al., 2002	259	0.29
DRD5 (CA)n microsatellite	0.43	1.24	1.54	Lowe et al., 2004	121	0.22
DRD3 Ser9Gly	0.33	1.4	1.96	Faraone et al., 2001	323	0.83
COMT Val¹⁵⁸Met	0.48	1.4	1.96	Faraone et al., 2001	246	0.75
MAOA 30bp promoter	0.33	1.4	1.96	Faraone et al., 2001	107	0.39

3.5 Analysis of modifying effects on manifestation of ADHD:

Hyperactive-impulsive symptoms (Table 3.9):

A trend towards significant association was observed between transmission of the DRD5 148bp 5 allele and having fewer hyperactive-impulsive symptoms (Beta=-0.35, 95% CI -0.32, 0.01, p=0.08). This trend remained when covariates of age at time of assessment and gender were added to the model. No significant associations were observed between this variable and any of the other gene variants investigated. These findings are summarised in Table 3.9.

Table 3.9: LRTDT analysis of hyperactive-impulsive symptoms - all gene variants.

	No. informative transmissions	Beta (95% CI)	Standard error	Wald	df	P-value
DRD4 7-repeat allele	77	0.15 (-0.06, 0.19)	0.15	0.99	1	0.32
DAT1 10-repeat allele	140	0.11 (-0.05, 0.14)	0.11	1.01	1	0.32
DRD5 148bp 5 allele	87	-0.35 (-0.32, 0.01)	0.20	3.11	1	0.08
DRD3 G allele	190	-0.02 (-0.09, 0.07)	0.09	0.05	1	0.98
MAOA Low activity 3 allele	64	-0.23 (-0.15, 0.14)	0.17	0.02	1	0.89
COMT Val allele	152	0.17 (-0.02, 0.17)	0.11	2.24	1	0.13

Inattentive symptoms (Table 3.10):

No evidence of association was observed between total number of inattentive symptoms and any of the variants studied (See Table 3.10 for details).

Table 3.10: LRTDT analysis of inattentive symptoms - all gene variants.

	No. informative transmissions	Beta (95% CI)	Standard error	Wald	df	P-value
DRD4 7-repeat allele	77	0.10 (-0.10, 0.18)	0.16	0.36	1	0.55
DAT1 10-repeat allele	140	-0.10 (-0.12, 0.04)	0.10	1.10	1	0.29
DRD5 148bp 5 allele	87	-0.04 (-0.15, 0.11)	0.15	0.07	1	0.79
DRD3 G allele	190	0.05 (-0.05, 0.09)	0.08	0.34	1	0.56
MAOA Low activity 3 allele	64	0.03 (-0.12, 0.09)	0.16	0.03	1	0.86
COMT Val allele	152	0.10 (-0.04, 0.17)	0.09	1.12	1	0.29

Conduct disorder symptoms (Table 3.11):

Significant association was observed between the total number of CD symptoms and overtransmission of the low activity allele of the MAOA 30bp promoter variant (Beta=0.43, 95% C.I.=0.00, 0.37, p=0.05). Age at time of assessment, gender and total number of ADHD symptoms were not significantly associated with transmission of this variant and so were not added to the model as covariates. Trends towards significant association were observed for transmission of the risk alleles for the DRD4 48bp VNTR (Beta=0.22, 95% C.I.=-0.02, 0.32, p=0.09) and for the DAT1 480bp VNTR (Beta=0.22, 95% C.I.=-0.01, 0.20, p=0.08). A trend for significance was observed with lower numbers of conduct disorder symptoms and transmissions of the DRD5 148bp risk allele (Beta= -0.39, 95% C.I.=-0.37, 0.03, p=0.09). Again, age at time of assessment, gender and total number of ADHD symptoms were not significantly associated with these three variants and so were not added to the model

as covariates. Finally, no evidence for association was found between the DRD3 Ser9Gly variant and the COMT Val¹⁴⁸Met variant. These findings are summarised in Table 3.11.

Table 3.11: Results for LRTDT analysis of conduct disorder symptoms with all gene variants.

	No. informative transmissions	Beta (95% CI)	Standard error	Wald	df	P-value
DRD4 7-repeat allele	77	0.22 (-0.02, 0.32)	0.10	4.45	1	0.09
DAT1 10-repeat allele	140	0.22 (-0.01, 0.20)	0.13	2.99	1	0.08
DRD5 148bp 5 allele	87	-0.39 (-0.37, 0.03)	0.23	2.83	1	0.09
DRD3 G allele	190	0.20 (-0.02, 0.18)	0.12	2.52	1	0.11
MAOA Low activity 3 allele	64	0.43 (0.00, 0.37)	0.22	3.83	1	0.05*
COMT Val allele	152	0.05 (-0.07, 0.11)	0.11	0.21	1	0.65

* = Significant at the $p \leq 0.05$ level

Oppositional Defiant Disorder symptoms (Table 3.12):

Total number of ODD symptoms was significantly associated with transmission of the risk alleles for the DRD4 VNTR (Beta=0.34, 95% C.I.=0.008, 0.18, $p=0.04$) and the MAOA variant (Beta=0.30, 95% C.I.=0.02, 0.22, $p=0.01$). Age at time of assessment, gender and total number of ADHD symptoms were not significantly associated with transmissions of either of these alleles and were therefore not added to the models as covariates. No evidence of association was observed between total number of ODD symptoms and any other variants analysed. See Table 3.12 for details.

Table 3.12: LRTDT analysis of Oppositional Defiant Disorder symptoms - all gene variants.

	No. informative transmissions	Beta (95% CI)	Standard error	Wald	df	P-value
DRD4 7-repeat allele	77	0.34 (0.008, 0.18)	0.20	2.81	1	0.04*
DAT1 10-repeat allele	140	0.08 (-0.03, 0.10)	0.07	1.16	1	0.28
DRD5 148bp 5 allele	87	0.009 (-0.08, 0.09)	0.10	0.008	1	0.93
DRD3 G allele	190	0.002 (-0.05, 0.05)	0.06	0.001	1	0.97
MAOA Low activity 3 allele	64	0.30 (0.02, 0.22)	0.11	6.71	1	0.01**
COMT Val allele	152	-0.02 (-0.06, 0.05)	0.06	0.07	1	0.80

* = Significant at the $p \leq 0.05$ level

** = Significant at the $p \leq 0.01$ level

Diagnosis of Conduct Disorder (Table 3.13):

A trend towards significance was observed between transmission of the DRD3 Ser9Gly Gly allele and a diagnosis of conduct disorder (OR=2.44, 95% C.I.=0.94, 6.30, $p=0.07$). Age at time of assessment, gender and total number of ADHD symptoms were not significantly associated with allele transmission for this variant and so were not included in the model as covariates. No other evidence for association was found between diagnosis of CD and any of the other variants analysed. Details of these findings are summarised in Table 3.13.

Table 3.13: LRTDT analysis of diagnosis of Conduct Disorder - all gene variants.

	No. informative transmissions	Beta (95% CI)	Standard error	Wald	df	P-value
DRD4 7-repeat allele	77	1.24 (-0.71, 1.26)	0.85	2.13	1	0.14
DAT1 10-repeat allele	140	0.21 (-0.35, 0.52)	0.51	0.17	1	0.68
DRD5 148bp 5 allele	87	-1.90 (-1.70, 0.14)	1.14	2.78	1	0.10
DRD3 G allele	190	0.89 (-0.03, 0.80)	0.49	3.37	1	0.07
MAOA Low activity 3 allele	64	1.02 (-0.18, 1.06)	0.73	1.94	1	0.16
COMT Val allele	152	0.007 (-0.39, 0.40)	0.45	0.00	1	0.99

Diagnosis of Oppositional Defiant Disorder (Table 3.14):

No evidence of association was observed between diagnosis of ODD and any of the variants studied. These findings are summarised in Table 3.14.

Table 3.14: LRTDT analysis of diagnosis of Oppositional Defiant Disorder - all gene variants.

	No. informative transmissions	Beta (95% CI)	Standard error	Wald	df	P-value
DRD4 7-repeat allele	77	0.18 (-0.17, 1.26)	0.48	0.15	1	0.70
DAT1 10-repeat allele	140	0.03 (-0.35, 0.52)	0.34	0.006	1	0.94
DRD5 148bp 5 allele	87	1.24 (-1.70, 0.14)	0.85	2.13	1	0.14
DRD3 G allele	190	-0.21 (-0.03, 0.80)	0.30	0.47	1	0.49
MAOA Low activity 3 allele	64	0.69 (-0.18, 1.06)	0.52	1.80	1	0.18
COMT Val allele	152	0.07 (-0.39, 0.40)	0.33	0.04	1	0.84

3.6 Discussion:

Main Association analysis:

No significant over transmission of alleles was observed for any of the variants using TRANSMIT analysis. A trend for association was, however, observed for the 5-repeat risk allele of the DRD5 (CA)_n microsatellite marker ($p=0.08$). These findings are in keeping with previous analyses of this data set (see Lawson et al., 2003; Payton et al., 2001; Holmes et al., 2000). This analysis used a slightly different statistical programmes and in some cases an expanded sample from that previously reported.

The failure to find significant association between proposed risk alleles (based on previous research) and ADHD in this sample may stem from the small sample size genotyped and heterogeneity between samples. It may be that specific phenotypic aspects of ADHD which are related to these variants are less prevalent in this sample than in other samples, thus masking any significant associations.

LRTDT analysis:

Symptom groups:

Looking at hyperactive-impulsive symptoms, the only finding approaching significance was a trend for fewer symptoms when the putative 148bp risk allele of the DRD5 (CA)_n variant was transmitted. This finding has not been previously reported, although in their joint analysis, Lowe and colleagues (2004) found no evidence of overtransmission of the risk allele to those with the DSM-IV hyperactive-impulsive subtype. No support was found for the findings of Rowe and colleagues (2001) for association between the DRD4 48bp VNTR and hyperactive-impulsive

symptoms. Similarly, no support was found for Waldman and colleagues (1998) finding of association between the DAT1 480bp VNTR and hyperactive-impulsive symptoms, although it was in keeping with the findings of Roman and colleagues (2001) in this regard. LRTDT analysis found no evidence of association between number of inattentive symptoms and any of the gene variants analysed. This is not consistent with Lowe and colleagues (2004) joint-analytic findings that the DRD5 148bp risk allele is associated with risk for the DSM-IV inattentive and combined subtypes of ADHD.

A reason for failing to detect association with hyperactive-impulsive scores, inattentive symptoms scores and total number of ADHD symptoms (except for a trend for under transmission of the DRD5 148bp risk allele and hyperactive-impulsive symptoms) may be due to limited variation in scores within this sample. Because all individuals within this sample meet diagnostic criteria for ADHD, there are floor effects as each individual, by the nature of their inclusion, have a certain number of symptoms. This can be observed in the relatively high mean scores for each of the symptom groups, considering the maximum possible is nine (mean scores 7.74 and 6.99 for hyperactive-impulsive and inattentive symptoms respectively).

Antisocial behaviour:

From these analyses, it appears that comorbid antisocial behaviour provides the most interesting findings. The MAOA low activity allele was most consistently associated with antisocial behaviour measures, specifically the number of CD symptoms (Beta=0.43, 95% CI 0.00, 0.37, p=0.05) and number of ODD symptoms (Beta=0.30, 95% CI 0.02, 0.22 p=0.01). No associations between this variant and the diagnoses of

either ODD or CD were observed, although previous analysis of this sample found significant association when the diagnosis of CD was broadened to include all those with one or more symptom of the disorder (Lawson et al., 2003).

The DRD4 7-repeat allele also appears to be associated with comorbid antisocial behaviour. Significant association between this allele and total number of ODD symptoms (Beta=0.34, 95% CI 0.008, 0.18 p=0.04) was observed, whilst a trend for association with number of CD symptoms (Beta=0.22, 95% CI -0.02, 0.32 p=0.09) was also observed. These findings are also generally in agreement with those found in analysis of this sample (Holmes et al., 2002) where association with a broader diagnosis of CD was found. Interestingly, the findings of Kirley and colleagues (2004) for association between the DRD4 risk allele and a diagnosis of ODD was not replicated. Indeed, the diagnosis of ODD was not associated with any of the variants analysed.

A trend towards association between the DAT1 480bp variant and number of CD symptoms was also observed (Beta=0.89, 95% CI -0.03, 0.80, p=0.08). This finding has not been previously reported to my knowledge but may further suggest the utility of examining conduct disorder symptoms in ADHD.

Finally, a trend towards association between the DRD3 G allele and diagnosis of Conduct Disorder was observed (OR = 2.44, 95% CI 0.94, 6.30 p = 0.07), a finding not previously reported. It is possible that these results represent main effects of the risk allele on antisocial behaviour, rather than modifying effects on the ADHD phenotype. Such an argument may have some credence as MAOA, particularly, has

been associated with aggression and antisocial behaviour (Manuck et al., 2000; Shih et al., 1999; Vanyukou et al., 1995). This is not a hypothesis which can be tested in this sample as there are no participants with antisocial behaviour but without ADHD. However, the purpose of this investigation was to look at modifying influences on the ADHD phenotype rather than to examine antisocial behaviour. Therefore, further work ascertaining whether specific gene variants influence antisocial behaviour in those with ADHD only, or in the population in general is required.

Methodological issues to be considered:

Overall, it would appear that further analyses testing whether specific dopaminergic gene variants modify specific aspects of the ADHD phenotype have yielded significant findings where main effects on ADHD were not observed through TRANSMIT analysis alone. Although independent replications of these findings are clearly necessary, this indicates that such further analysis may be a beneficial strategy in molecular genetic studies of ADHD.

A number of general issues regarding these findings (and lack of significant findings) warrant discussion. Firstly, failure to detect association, both through initial TRANSMIT analysis and with the secondary LRTDT analysis are not conclusive of no association. As has been demonstrated by statistical power calculations (see table 3.8), some variants had insufficient power to detect previously reported effect sizes. This is not entirely surprising and has been observed across molecular genetic studies of ADHD (to which this sample is of a comparable size). Indeed, for a number of variants, individual studies have failed to find association, but joint analyses, including data from this group, have found significant evidence for association with

the DRD4 48bp VNTR, the DAT1 480bp VNTR and the DRD5 (CA)_n microsatellite variant (Faraone et al., 2001; Faraone et al., 2005 and Lowe et al., 2004 respectively). Encouragingly, it has been demonstrated that looking at specific aspects of the ADHD phenotype (as in this LRTDT analysis) significantly increases statistical power to detect an effect (Faraone et al., 2000b). However, a lack of statistical power may still explain the non-significant findings in this study.

This issue of statistical power may be particularly relevant when looking at comorbid conduct disorder symptoms and especially the clinical diagnosis of comorbid conduct disorder. The mean number of CD symptoms for individuals in this group is very low (mean = 0.91 s.d 1.42) whilst only 13% of individuals have a diagnosis of conduct disorder. This number is further reduced as only those with informative transmissions are included in the analysis (in the case of the COMT Val¹⁵⁸Met variant, only 12 individuals with a diagnosis of CD contributed to the analysis). The use of only informative transmissions further exacerbates the situation, as a study from our group (West et al., 2002) has demonstrated that probands with missing fathers have higher rates of comorbid antisocial behaviours. This group of individuals are less likely to provide informative transmissions as they have missing parental gene data making it less likely that the origin of the child's alleles can be deduced. It may, therefore, be advantageous to perform case-only analysis of this sample in an attempt to maximise the sample size. It should also be noted that in analyses such as LRTDT (but not TRANSMIT) which do not utilise information regarding allele frequencies, predicting transmissions where information is missing from one parent can introduce bias (Curtis & Sham, 1995). However, as very few transmission could be predicted in this way, this is unlikely to have significantly altered findings.

A second possible explanation for these findings is that one, or all, of the variants investigated are not themselves susceptibility or modifying variants for ADHD, but are in a region close to a susceptibility variant. Therefore, in some samples linkage disequilibrium of the marker studied with the susceptibility variant would lead to significant association but may not do so in a second sample. This possible explanation for these findings further highlights the utility of looking at a number of variants and haplotypes across a gene rather than a single variant, as in this case. It is also possible that the original findings of significant association were false positives, although this proposal is less likely for those variants where pooled or meta-analyses have been performed.

Finally, it is also important to bear in mind that a large number of calculations were performed on this data and so some of the significant findings could be false positives resulting from multiple testing. This is clearly possible, although there were strong *a priori* hypotheses for all the analyses performed. Each of the variants studied have been previously implicated by studies, across samples or in meta-analyses and for all variants except the DAT1 480bp VNTR, over transmission of the risk allele in the expected direction was observed (albeit not significantly). Furthermore, the phenotypic factors chosen for investigation were based on past findings. As this can be described as mostly exploratory analysis which has not previously been undertaken, perhaps the issue of multiple testing is less important but the need for independent replications of findings needs to be highlighted.

CHAPTER 4

Environmental influences on ADHD: Selective literature review

4.1 Introduction:

In addition to the contribution of genetic influences, researchers have also been interested in environmental factors which may be involved in the aetiology of ADHD (Barkley, 1998). Twin studies have demonstrated that, in addition to there being genetic effects, shared and individual environment accounts for between 12 and 40% of the available variance in twin ADHD scores (Thapar et al., 2000; Sherman et al., 1997; Goodman & Stevenson, 1989a). Identification of such environmental factors may have important public health implications (Biederman et al., 1995a), whilst identification of subgroups based on such correlates and clinical characteristics may lead to improvements in specified health care provision (Scahill et al., 1999).

Pre- peri- and postnatal factors have been identified as conferring risk for the disorder, whilst exposure to psychosocial adversity throughout childhood is also associated with both symptoms and the diagnosis of ADHD. Factors during pregnancy such as exposure to toxins including nicotine, alcohol and illicit substances (Taylor & Warner Rogers, 2005); pregnancy complications such as maternal bleeding (Milberger et al., 1997); and maternal hypothyroidism (Taylor & Warner Rogers, 2005) have all been linked to ADHD behaviours in childhood. Parental psychopathology has also been found to be significantly associated with ADHD, although caution is warranted when attributing such associations to genetic or environmental causes. Parental ADHD has been found to increase risk of ADHD behaviour problems in the child (Biederman et al., 1995b), whilst maternal stress has been reported to lead to childhood behaviour problems whether experienced during or after pregnancy (O'Connor et al., 2002). The family environment has also been found to be associated with childhood ADHD with family conflict (e.g. Burt et al., 2003) and extreme neglect (e.g. Rutter et al., 2003)

both thought to be significant. Exposure to toxins, both in the local environment and diet have been implicated as risk factors for ADHD, although much more work in this area is necessary (Taylor & Warner Rogers, 2005). However, it is difficult to know which of these associated factors are causal risk factors. Some may also arise secondary to the child's (or parent's) psychopathology, for example. Finally, some environmental risk factors are distal to the psychopathophysiology and are better considered as risk indicators, rather than as the causal risk factors.

Based on available data, this thesis will now discuss in detail three selected environmental variables; maternal smoking during pregnancy, birth weight and social class.

4.2 Maternal smoking during pregnancy:

One prenatal environmental risk factor which has been widely studied is maternal smoking during pregnancy. It is estimated that around 25% of women in the UK and USA smoke during pregnancy (DiFranza & Lew, 1995; Owen et al., 1998; ONS, 2001; U.S. Dept. Health & Human Services, 2001). It is widely accepted that there are ante-natal, peri-natal and post-natal disadvantages associated with maternal smoking during pregnancy including spontaneous abortion (Walsh, 1994), sudden infant death syndrome (Anderson et al., 2005), low birth weight (Bhutta et al., 2001) and growth problems (Cornelius et al., 2002). More recently, there have been reports that smoking during pregnancy is associated with disruptive behaviour in children, including ADHD. This evidence will now be considered.

Population-based studies

Over the past twenty years 13 population based epidemiological studies investigating possible associations between maternal smoking during pregnancy and ADHD behaviours in offspring have been performed. Of these studies, 11 reported a significant association (nine based on prospectively ascertained measures of smoking by the mother and two on retrospective measures). The only prospective study which did not demonstrate significant association used performance on neurocognitive tasks rather than symptoms of ADHD as an outcome measure.

One of the largest, best-designed studies was based on a longitudinal cohort involving over 1000 children from New Zealand who were followed for over a time span of more than 15 years (Fergusson et al, 1993). Smoking behaviour in mothers in all three trimesters of pregnancy was assessed; at birth and every subsequent year until the child was five years of age. Both conduct and ADHD problems were assessed at three age points (eight, ten and twelve years) by both mothers and teachers (to avoid potential bias caused by mothers reporting on both their smoking and their child's behaviour). Fergusson et al. (1993) found that smoking during pregnancy was significantly associated with ADHD symptom scores in a dose-dependent fashion (i.e. the more a mother smoked, the greater the number of ADHD symptoms). This association remained significant even when a wide range of potential confounding factors including social adversity, maternal characteristics (maternal age and education and a measure of emotional responsiveness) and the child's disruptive and conduct disorder behaviour (from parent Rutter and Connor's questionnaires), were taken into account using multiple regression analysis. Importantly, there were no major differences in results for maternal and teacher measures of ADHD, suggesting

that there is no bias caused by mothers rating both their own and their child's behaviour.

The largest prospective population-based study was based on a cohort sample of 9357 Finnish children (Kotimaa et al, 2003). This research group found hyperactivity (based on Rutter ADHD scales) in eight year olds to be significantly associated with maternal smoking during pregnancy. This study had the advantage of a very large sample. Previous authors have commented that population based studies are often insufficiently powered to detect a relationship between maternal smoking during pregnancy and ADHD (Williams et al., 1998). However, although a number of confounding factors were included in this study, it did not include the effects of conduct disorder symptoms or birth weight.

These epidemiological studies, however, tell us little about the potential impact of maternal smoking during pregnancy on diagnosis of ADHD. Although one population based study (Rodriguez & Bohlin, 2005), did look at diagnoses of DSM-IV ADHD, as only seven individuals (out of a sample of 280) met diagnostic criteria for ADHD (not including impairment of functioning) these results are not sufficient. Findings on the association between maternal smoking during pregnancy and clinical diagnosis of ADHD come from clinical case-control studies.

Clinical studies:

There have been six case-control studies which have looked at associations of maternal smoking during pregnancy with a diagnosis of ADHD. The majority have utilised clinic referred individuals and compared children with ADHD diagnosed

using DSM criteria to controls. Most report a significant association between ADHD diagnosis and maternal smoking during pregnancy; Mick and colleagues (2002) reported over a two-fold increase in exposure to smoking during pregnancy in ADHD cases in comparison to controls (OR 2.1, 95% CI 1.1, 1.4, $p=0.02$). Similarly, Landgren and colleagues (1998) in a Swedish study of six-year olds found that those with poor motor control and attention deficits were more likely to have been exposed to cigarette smoking in utero.

To better summarise the findings, in this chapter I derived a pooled estimate of the odds ratios for the association of maternal smoking during pregnancy and ADHD based on a selection of studies. All published case-control designs using DSM diagnoses of ADHD (and one of Deficits in Attention Motor control and Perception Disorder - DAMP) where odds ratios were reported or could be derived from the papers were included in this analysis. This resulted in the inclusion of five studies (see table 4.1). A pooled odds ratio was calculated using the “meta” function within the statistical package STATA (version 8; STATA Corp., 2003). Using the more conservative random effects model, a pooled odds ratio of 2.39 (95% CI 1.61, 3.52) was obtained (see table 4.2). This analysis has been pictorially depicted in figure 4.1. No heterogeneity between individual studies was detected using Cochran’s Q statistic ($Q = 2.24$, $df 4$, $p = 0.691$).

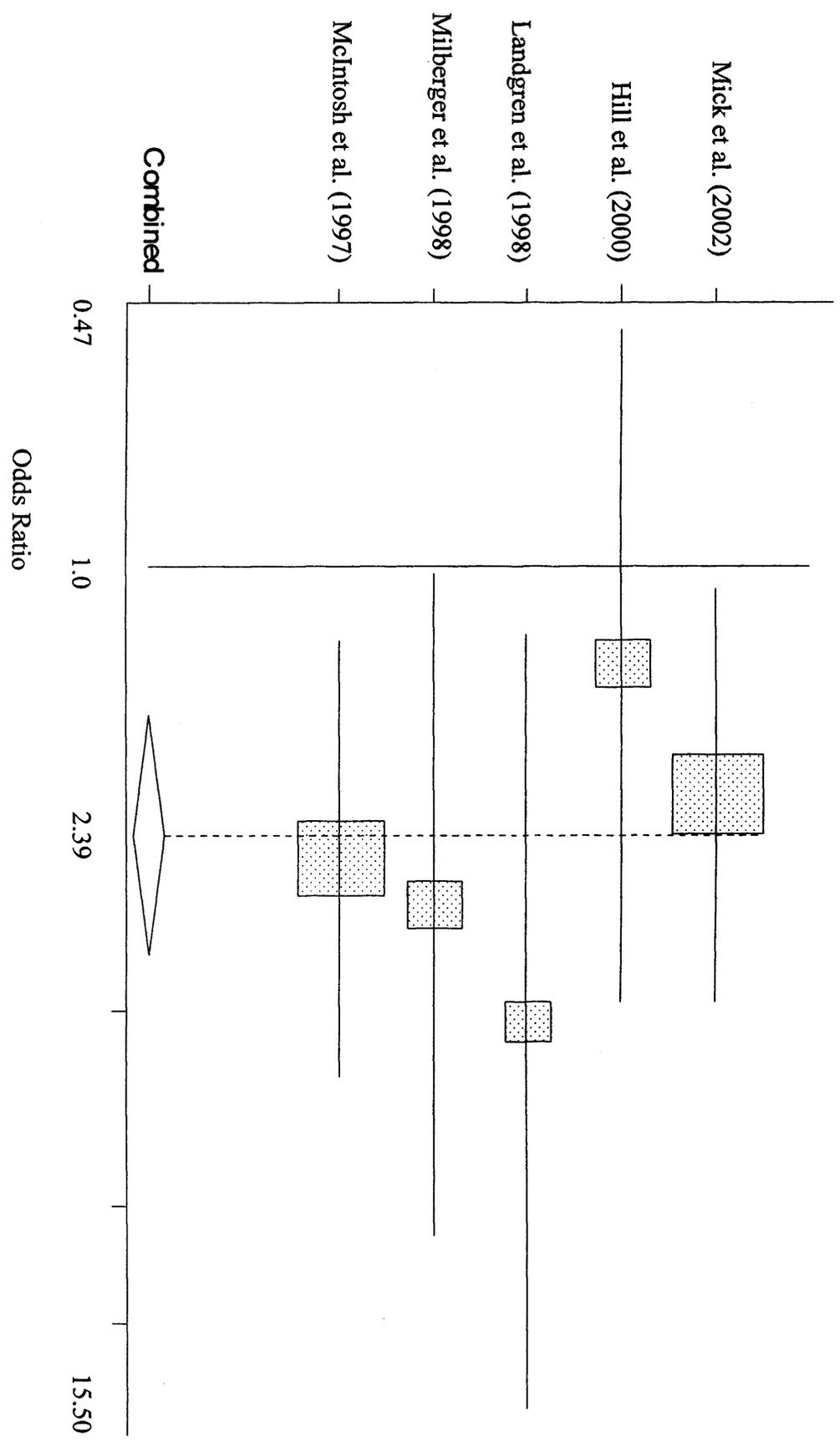
Table 4.1: Studies included in pooled odds ratio analysis:

Study	Sample size	Odds Ratio	95% Confidence Intervals	
			Lower	Upper
Mick et al. (2002)	522	2.10	1.10	4.10
Hill et al. (2000)	150	1.38	0.47	4.09
Landgren et al. (1998)	113	3.00	1.10	8.80
Milberger et al. (1998)	271	4.4	1.20	15.5
McIntosh et al. (1995)	209	2.58	1.27	5.23

Table 4.2 Results of pooled odds ratio for case-control studies:

Method	Pooled Odds ratio	95% CIs		z-value	p-value	No. of studies
		Lower	Upper			
Random effects	2.38	1.61	3.52	4.37	<0.0001	5

Figure 4.1 : plot of studies included in estimate of the pooled odds ratio:



This analysis gives a pooled odds ratio which provides a better global sense of the findings of case-control studies. It does not attempt to be a stringent meta-analysis and was based only on data readily available from published papers. As such, no raw data were obtained directly from the authors. From this analysis it can be seen that, overall, the evidence from case-control studies strongly supports an association between maternal smoking during pregnancy and ADHD.

Conduct Disorder:

Antisocial behaviour and conduct disorder symptoms have frequently been observed to co-occur with maternal smoking during pregnancy. For example, in their epidemiological study, Weissman and colleagues (1999) observed a four-fold increase in the rate of conduct disorder in the offspring of mothers who smoked during pregnancy. This and other studies imply that there is a significant association between conduct disorder and maternal smoking, making it imperative that such behaviours are taken into account when assessing the relationship between maternal smoking during pregnancy and ADHD. Although a surprising number of studies fail to do this, many do. Two population-based studies measured conduct problems and ADHD separately, (Fergusson et al., 1993; Thapar et al., 2003) and were thus able to illustrate significant associations between these two outcomes independently. The continued significance of maternal smoking during pregnancy as a risk factor for ADHD when controlling for conduct problems, has also been found in studies of clinical samples (Mick et al., 2002), thus making a strong argument for the association between ADHD and maternal smoking during pregnancy.

Maternal psychopathology:

Maternal psychopathology is another factor which may confound results. Maternal mental health problems may influence recall or reporting of smoking behaviour or the child's problems. Furthermore, maternal psychopathology may influence smoking behaviour and interactions with the child, which means that both current mental health and that at time of pregnancy must be taken into account.

Those studies that have measured maternal psychopathology (e.g. Mick et al., 2002; Maughan et al., 2001; Williams et al., 1998) have included the effects of current maternal anxiety and depression. Controlling for maternal depression and parental ADHD did not alter the significant association between the maternal smoking during pregnancy and child ADHD found by Mick and colleagues (2002).

Postnatal smoking:

Also of interest is the possible relationship between post-partum smoking and ADHD behaviour. Studies which have attempted to address this problem have had mixed results. For example, Eskenazi & Trupin (1995) found both smoking prenatally and up to the child's fifth birthday affected activity levels. Williams and colleagues (1998) estimated that up to 25% of child behaviour was due to smoking during pregnancy whilst an additional but somewhat smaller risk was conferred by concurrent smoking. Fergusson and colleagues (1993) found that smoking following pregnancy up to the age of five had no significant effect on ADHD behaviour when smoking during pregnancy was taken into account. The problem of accounting for smoking after birth is exacerbated both because studies do not assess smoking behaviour of other adults in the house and also because it is possible that the detrimental effects of smoking

actually occur due to exposure through breastfeeding (Weissman et al., 1999). Mixed findings on the influence of post-natal smoking mean that further investigation is necessary before conclusions can be drawn.

Retrospective recall:

One frequently highlighted limitation of some studies assessing the effects of smoking during pregnancy on ADHD is the use of retrospective measures of smoking behaviour (Mick et al.; 2002, Ernst et al., 2001; Thapar et al., 2003). It has been suggested that there may be a bias in mother's retrospective recall of their smoking during pregnancy, especially as the study may be conducted many years later. A number of studies have attempted to correct for this potential limitation using a variety of methods and most have concluded that retrospective recall does not appear to be a problem. Mick and colleagues (2002) aimed to reduce potential bias by asking mothers about their smoking behaviour on two separate occasions; once when asking about the child's behaviour and again when asking about the mother's own behaviour. They found no differences in their findings based on the different sources of smoking information, suggesting that any recall bias is generally stable. Furthermore, significant associations between maternal smoking during pregnancy and ADHD have been found for both studies using retrospective recall (e.g. Mick et al., 2002) and also for prospective studies (e.g. Fergusson et al., 1993).

Interestingly, it seems that mothers are relatively accurate in reporting their smoking behaviour, at least if such questions are asked during the pregnancy. Some studies, (e.g. Eskenazi & Trupin, 1995, Buka et al., 2003) have tested levels of cotinine (a chemical made from nicotine by the body used to assess exposure to cigarette smoke)

in the bloodstream and shown high accuracy of reported smoking behaviour. For example, Buka and colleagues (2003) found over 95% accuracy in mothers classifying themselves as non-smokers.

Genetic factors:

Finally, it has been suggested that an alternative explanation for findings of association between maternal smoking during pregnancy and ADHD may be that there are genetic effects which influence both smoking during pregnancy and offspring ADHD behaviour.

A twin study by Thapar and colleagues (2003) attempted to pick apart the genetic and environmental influences of smoking during pregnancy on ADHD. Thapar and colleagues found a significant, direct environmentally mediated association of smoking during pregnancy on teacher-rated questionnaire based measures of ADHD behaviour. This association remained significant even when a number of possible confounding variables were taken into consideration. Although it is possible that additional maternal genetic characteristics also account for observed associations between smoking during pregnancy and ADHD, this study illustrates that there is a true environmental effect.

From the findings reviewed, it would appear that there is strong evidence implicating maternal smoking during pregnancy as a risk factor for ADHD. Case-control and epidemiological studies suggest that maternal smoking during pregnancy increases risk for both ADHD symptoms and for a diagnosis of the disorder. A pooled odds ratio of 2.39 (95% CI 1.61, 3.52) derived from case-control studies further

demonstrates this association, implicating over a two-fold increase in ADHD when mothers smoke during pregnancy.

4.3 Low Birth weight

Definitions & Prevalence of low birth weight:

The average birth weight for a singleton child in the UK is 3300g (www.hefa.gov.uk).

The definition of low birth weight (LBW) is weighing under 2500g at birth. Recent data suggest that 7.6% of UK births (including stillbirths) meet this criteria (Stevenson et al., 1999). This classification of LBW has been further subdivided into low birth weight (LBW), <2500g; very low birth weight (VLBW), <1500g; and extremely low birth weight (ELBW), <1000g (Teplin et al., 1999). A number, but not all of these children have also been born prematurely which is defined as being born before 37 weeks (Morrison & Rennie, 1997). It is reported in the UK that 6% of surviving neonates weigh below 2500g with 1% being of VLBW and 0.4% ELBW (Stevenson et al., 1999; Stjernqvist & Svenningsen, 1999). The increasing survival rate of small babies has led to research interest in the developmental, cognitive and behavioural outcomes for such individuals. One, quite extensive, area of research addresses the incidence of ADHD in LBW children. The findings of such investigation shall now be discussed.

LBW as a risk factor for ADHD:

The vast majority of studies take a case-control approach to investigate the behavioural implications of being LBW, often using a regional cohort of LBW individuals. For example, Pharoah and colleagues (1994) obtained a cohort of 233

babies born consecutively in five health districts in Merseyside who weighed 2000g or less. At the age of eight or nine years, these children were compared to a group of matched controls from the same mainstream school. Using parent and teacher Rutter questionnaires, 14% of LBW children were classified as hyperactive, in comparison to only 6% of controls. ($\chi^2=9.39$, df 1, $p=0.002$). Similarly, Botting and colleagues (1997) looked at VLBW or premature children at age 12 years in comparison to matched controls from within the child's primary school. VLBW status was significantly associated with a diagnosis of ADHD, even when controlling for IQ, school achievement and a variety of demographic factors.

However, not all studies have reported significant association between LBW and ADHD. Taylor and colleagues (2000) found association between birth weight status and parent rated ADHD symptoms, but not with teacher ratings, in their study of ELBW and VLBW children aged 11 years compared to children born at full term. Conversely, Teplin and colleagues (1991) found significantly poorer teacher rated selective attention ($p<0.01$) but no differences in parent-rated hyperactivity. However these studies did not look at diagnoses of ADHD.

Degree of low birth weight:

Some researchers have also looked at samples with a range of birth weights, investigating whether or not the degree of low birth weight affects ADHD behaviour. Pinto-Martin and colleagues (2004) studied a cohort of 645 nine year old children who had been born weighing below 2000g in New Jersey hospitals. Birth weight was considered as a continuous measure and the sample was also split into ELBW, VLBW and LBW (below 2000g) groups. Pinto-Martin and colleagues found a negative

correlation between birth weight and ADHD symptoms. Similar associations were also seen whereby children from smaller birth weight groups had more ADHD symptoms. McCormick and colleagues (1996) also examined children from all birth weight categories and found similar results. Hyperactivity increased as birth weight reduced regardless of whether birth weight was considered as continuous or categorical measure ($p < 0.0001$). These findings remained significant in multiple regression analysis controlling for demographic factors. Association between low birth weight and ADHD has also been observed in samples of children selected for their ADHD status. In one study, 252 children from an ADHD clinic were compared to 231 non-ADHD paediatric controls (Mick et al., 2002). ADHD children were significantly more likely to have been born weighing below 2500g than controls (OR=3.1 CI 1.03, 9.3 $p=0.04$), even when controlling for smoking or alcohol use during pregnancy, maternal age, IQ and parental ADHD.

Overall, low birth weight appears to be significantly associated with ADHD behaviours and the clinical diagnosis. This view is confirmed in the results of a meta-analysis of case-control studies (Bhutta et al., 2002). Analysis of 16 studies of ADHD and low birth weight, regardless of gestational age, provided a combined calculated risk ratio of 2.64 (CI 1.85, 3.78 $p < 0.001$). The conclusion appears to be that birth weight is significantly associated with the diagnosis of ADHD.

Comorbid behaviour:

Not much work has been undertaken specifically looking at rates of comorbid disorders among ADHD children who were of low birth weight. However, studies have frequently found that rates of all psychiatric disorder are higher in low birth

weight children than normal controls. Conversely, other researchers have noted that the increased rate comes mostly from increases in only ADHD behaviours (Szatmari et al., 1990) with some even concluding that the increased rates of disorder in low birth weight children is specific to ADHD (Botting et al., 1997). A number of researchers have found no increase in the rates of antisocial behaviour in low birth weight children (Saigal et al., 2003; Botting et al., 1997; Breslau et al., 1996), a surprising finding considering the high rates of comorbidity found between the two disorders. Not all studies have mirrored these findings; Stevenson et al. (1999) found significant increases of conduct problems as well as ADHD in their sample. The reasons for this are unclear and so this issue requires further investigation.

Rates of attrition:

Although the majority of studies have been designed to use a regional or even national cohort of low birth weight children, rates of attrition are often quite high by the age of the behavioural assessment. For example, McCormick and colleagues (1996) report an overall drop-out of 35% which rises to 55% for the VLBW group. However, a number of studies have managed to maintain high rates at follow-up, often of over 90% (Pinto-Martin et al., 2004; Gross et al., 2001; Pharoah et al., 1994) suggesting that this is not a universal or insurmountable problem.

Multiple births:

Children from multiple births generally have lower birth weights than singletons; twins have an average birth weight of 2500g and triplets of 1800g (www.hefa.gov.uk). This means twins and triplets are more likely to be classed as low birth weight than their singleton counterparts. Furthermore, children from multiple

births may have different outcomes from singletons and may not be comparable. None of the studies looking at rates of ADHD in low birth weight children exclude those from multiple births and generally do not record the number from such families. This oversight may have an impact on the conclusions drawn regarding birth weight and ADHD. Unfortunately, the data are not available from the papers of previous studies to redress this limitation.

Neurosensory and cognitive impairments:

Low birth weight has been observed to have a number of negative outcomes other than just ADHD. Children born of low birth weight have been widely observed to have increased levels of neurosensory impairments including Cerebral Palsy (Taylor et al., 2000) and also to have lower IQs (Bhutta et al., 2002). It is possible that low IQ and neurological problems account for the relationship between LBW and ADHD. However, a number of studies have corrected for IQ or repeated analyses without individuals with neurosensory impairments or low IQs (e.g. Saigal et al., 2003; Botting et al., 1997; Ross et al., 1991) and still found association between ADHD and low birth weight.

Other risk indicators:

A number of factors, including demographic factors and parenting styles appear to be associated with both ADHD and low birth weight and so it is possible that these other factors are actually responsible for the associations observed between ADHD and low birth weight. The association between social class and ADHD is discussed in detail in section 4.4. More strikingly, both low birth weight and ADHD have been associated with maternal smoking during pregnancy (ADHD and maternal smoking during

pregnancy have been discussed previously in section 4.2). Reports suggest that maternal smoking during pregnancy is related to around a 200g reduction in birth weight (National Cancer Institute, 1999). Although not all studies of low birth weight and ADHD control for these factors (e.g. Pinto-Martin et al., 2004; Hille et al., 2001), many did allow for the effect of these other factors including maternal smoking during pregnancy (e.g. Mick et al., 2002; McCormick et al., 1996). Continuing significant findings having taken these factors into account imply that association between low birth weight and ADHD is not merely to do with a third environmental factor.

4.4 Social class

ADHD is associated with poverty, lower income and social class. These factors are sometimes called indicators of risk as it is unlikely that they exert a direct causal effect on ADHD. The evidence for these factors being associated with ADHD behaviours will now be discussed.

Epidemiological studies:

Almost all epidemiological studies have found association between social class and ADHD. In a UK study of 2462 six and seven year olds, Taylor and colleagues (1991) found that children with inattentive and hyperactive symptoms were significantly more likely to come from families with lower social class, especially those with ADHD symptoms which were pervasive across home and school settings. More recently, a governmental survey into the mental health of children and adolescents, also in the UK, suggested that diagnosis of ADHD is associated with family income

and social class (Meltzer et al., 2000). The report also suggested that children with the disorder were significantly more likely to come from families where neither parent was working. Community based studies from other countries have also reported associations between ADHD and low social class. In a study based in Manizales, Columbia, Pineda and colleagues (1999) randomly sampled 540 four to seventeen year olds. Children classified as being of low social class were found to have significantly higher levels of DSM-IV ADHD symptoms.

Data from the Smoky Mountains Study of Youth have also revealed a significant increase of ADHD (and other psychiatric disorders) in children living in poverty, compared to non-poor children (Costello et al., 1996). A two-fold increase in diagnosis of DSM-IV ADHD was observed in children who fell below the national poverty line.

Clinically referred samples:

Researchers have also found that social class and poverty are associated with ADHD in clinical samples. For example, in a study of 280 ADHD individuals and 242 healthy controls taken from Paediatric clinics, Biederman and colleagues (2002) reported that clinically referred children came from families with significantly lower social class. Similarly, in a study primarily designed to look at the psychiatric, psychosocial and cognitive functioning of female adolescents with ADHD, Rucklidge and Tannock (2001) found significant association between the disorder and social class. Comparison between 59 thirteen to sixteen year olds with ADHD (of both sexes) and 48 non-ADHD controls referred from Paediatric clinics showed that

ADHD children came from families with significantly lower social classes, regardless of gender.

Negative clinical studies:

Contrary to the evidence described, not all studies have found an association between social class and ADHD. A review of consecutive non-emergency primary care admissions across North America found no such increase in attentional and hyperactivity problems in low social class families, as defined by the family requiring Medicare (Wasserman et al., 1999). Similarly, a systematic review of physician administrative and payment claim files in a Canadian province over two year period found no income bias in the diagnosis of ADHD or prescription of stimulant medication (Brownell et al., 2001). Indeed, Brownell and colleagues found, if anything, an increase in ADHD diagnoses in higher income families residing in rural areas.

However, these findings may be due to patterns of recognition of the disorder and also the availability of healthcare for poorer families. Both studies acknowledge these findings may, in part, be due to the fact low income families seeking medical attention may do so through routes not included in their studies. This is partially thought to be due to the cost of healthcare. Certainly cost of treatment for mental health disorders in the USA has been highlighted as a barrier to care for poor children, especially those without medical insurance, by the US Surgeon General at the 2000 conference on children's mental health (US Public Health Service: Report of the Surgeon General's Conference on children's mental health: A national action agenda. Washington DC,

Department of Health and Human Services, 2000), reinforcing this as a source of ascertainment bias.

Reverse causation:

It is possible that, rather than low social class increasing risk for ADHD behaviours and diagnosis, child ADHD causes families to have lowered income and social class. There certainly appear to be financial constraints and disadvantages associated with having a child with pervasive ADHD symptoms. Even in the UK where there is no charge for health care, one study (Sayal et al., 2003) has reported that the parents of ADHD children have increased financial burden with higher proportions having to change jobs, working hours, or stop working altogether.

However, it is unlikely that all of the association between social class and ADHD can be explained by such reverse causation. A number of longitudinal studies have been undertaken where social class is recorded prior to ADHD behaviour being assessed (e.g. Bor et al., 1997; Chandola et al., 1992) and continued to observe a significant association. Indeed, low income in these studies has been ascertained at the birth of the child (or before), thereby certainly occurring before the onset of ADHD behaviours. Thus, although a proportion of the association between social class and ADHD may be the result of increased financial burden for parents caused by the child's behaviour, it seems that low social class precedes the onset of ADHD.

This issue also raises questions as to whether or not childhood ADHD in lower social class families is due to the adverse effects of being in a lower income family, or because family liability for mental health problems causes the family (and therefore

their offspring) to be less well off. Although no studies have attempted to answer this question looking at offspring ADHD behaviour, naturalistic experiments have enabled these two hypotheses to be explored in other childhood psychiatric disorders. In one study (Costello et al., 2003), a longitudinal epidemiological study included a proportion of native Americans, a large number of whom fortuitously underwent an improvement in financial circumstances. During the course of the study, a casino was opened, the profits of which were partially shared between all native Americans. Prior to the opening of the casino, children from families whose income fell below the poverty line showed significantly higher prevalence of DSM-IV psychiatric symptoms. When followed up after the casino opened, 14% of study families had moved out of poverty, 53% remained poor, whilst 32% had never been poor. The ex-poor families reported prevalence of symptoms of externalising disorders (CD & ODD) similar to families who were never poor, whilst families continuing to fall below the poverty line had significantly higher symptom rates. Changes may be due to the richness of the home environment and presence of teaching or other materials. ADHD symptoms were not assessed in this study as participants were aged 14 years at follow up and prevalence rates for the disorder were too small to analyse. Although not conclusive, this study suggests low income causes childhood psychiatric problems, at least in part, rather than family liability leading to both poverty and childhood symptoms.

Taking other problems into account:

Although there appears to be some support for association between ADHD and low income or social class, these studies do not take into account the possible confounding effects of comorbid disorders (Szatmari et al., 1989). Low social class has also been

found to be associated with a range of childhood mental health problems other than ADHD, especially antisocial problems (e.g. Costello et al., 2001, Fergusson et al., 1993). However, few studies have examined the influence of family income in the presence of more than one disorder concurrently. Taylor and colleagues (1991) found association between ADHD problems and low income even in the group without comorbid conduct problems; indeed, the association was strongest in the group with only inattentive problems. However, more work is needed in this area before conclusions can be drawn.

Researchers have found that psychosocial factors are highly correlated (e.g. Costello et al., 1997), making it difficult to pick apart the risk associated with each individual factor. Of note, social class cannot be the actual mechanism through which ADHD occurs and thus acts only as an easily identifiable and measurable indicator of risk (e.g. Rutter, 1999). However, to date, no studies have successfully identified a proximal, causal pathway between an environmental risk factor and ADHD. More distal factors, such as social class are therefore of use in identifying at risk groups (even if that specific variable is just a proxy for the true risk). The use of associated risk indicators may also reveal variation in behaviour manifestations of the disorder through modifying effects of specific symptom groups or comorbid behaviours within the ADHD phenotype. In addition to having public health implications in themselves, use of distal variables to identify specific at risk groups may lead to the discovery of more proximal associations and, hopefully, specific pathways. Thus it would appear that there is strong evidence for the environmental variables of maternal smoking during pregnancy, birth weight and social class being independently associated with ADHD. The influence of these variables may now be assessed in this sample.

CHAPTER 5

Analysis of environmental factors modifying the ADHD phenotype

5.1 Introduction:

Heterogeneity within ADHD may affect detection of environmental risk factors in the same way that it does for genetic susceptibility variants. Phenotypic differences between samples may lead to non-replication of findings, especially as individual environmental factors may confer risk for specific aspects of ADHD, rather than the disorder as a whole. For example, environmental factors such as maternal smoking during pregnancy, birth weight and social class may have modifying effects on ADHD symptom severity, subtype and comorbid antisocial behaviour. It is the intention of this thesis to investigate the modifying effects of selected environmental factors.

5.2 Aims:

The aim for this chapter is to examine association between selected environmental factors and specific phenotypic aspects of ADHD. Based on previous evidence and available data, the following environmental factors will be investigated:

- Maternal smoking during pregnancy
- Birth weight
- Social class

Based on previous discussion of clinically important aspects of the ADHD phenotype, the following outcomes will be analysed:

- Hyperactive-impulsive symptoms

- Inattentive symptoms
- Total number of ADHD symptoms.
- Conduct disorder and oppositional defiant disorder symptoms and diagnosis

5.3 Method:

Sample:

For this part of the analysis, 356 individuals were included. The specific sample sizes for each analysis are present in the tables and differ slightly for each analysis due to missing data on specific items.

Environmental measures:

Maternal smoking during pregnancy: (n = 356) Maternal smoking during pregnancy was retrospectively assessed by maternally rated questionnaire at the time of the interview using two questions; firstly, a yes/no answer was required to the question “Did you smoke during pregnancy?” Secondly, mothers who answered “yes” to the first question (n= 163, 46%) described the average number of cigarettes smoked daily according to two choices; “less than 10 a day” (n=76, 48%) or “more than 10 a day” (n=84, 52%). This meant that a dichotomous variable of those who did and didn’t smoke during pregnancy could be obtained (smoking yes/no), as could an ordinal variable (smoking quantity).

Birth weight: (n = 340) The question “What did your child weigh at birth?” was also answered retrospectively by mothers by paper questionnaire administered at time of interview. Answers in pounds and ounces were converted into total weight in grams.

This provided a continuous measure of birth weight. The mean birth weight was 3286g with a standard deviation of 621.23g.

Social class: (n = 345) Mothers stated the occupation of the main earner in the household in questionnaires. Social class was obtained from this description based on the UK Standard Occupational Classification (2nd Edition) (OPCS, 1995), based on the Registrar General's Social Class. Because of a variable number of participants in each category and in an attempt to make categories which were more meaningful, these were then split into three categories; high, medium and low social class. High social class (n=73, 22%) consisted of families from professional and managerial jobs in classes 1 and 2; medium social class (n=101, 30%) was made up of families from skilled occupations in classes 3 non-manual, and 3 manual and also from partially skilled workers in class 4; whilst low social class (n=161 47%) consisted of families from unskilled jobs in class 5 and unemployed or unclassified individuals. Because of recognised difficulties in classification (OPCS, 1995), families who stated the occupation of the main earner in the household as being in the Armed Forces (n=4 1%) were not included in the analyses.

Additional measures used in analysis: In addition to the use of gender and age at time of assessment as potential covariates, based on previous research, a number of possible confounding variables were also assessed.

Maternal alcohol use during pregnancy: (n = 213) Mothers stated whether or not they drank alcohol during pregnancy (no=149, yes=74) and then further stated the quantity “one drink every two to three months” (n=20), “about one drink a month” (n=15), “about one drink a week” (n=18) and “two or more drinks a week” (n=11).

Intelligence Quotient (IQ): (n = 356) As previously described, the child's full scale IQ was assessed using the WISC III UK (Wechsler, 1992) which also enables separate calculations of performance and verbal IQ. The mean full scale IQ was 90 with a standard deviation of 12.1.

Maternal ADHD: (n = 354) A measure of current maternal ADHD symptoms was obtained using the total score on the maternally rated Barkley Adult ADHD scale (Barkley & Murphy, 1998).

Paternal ADHD: (n = 106) Similarly to maternal ADHD, current paternal ADHD symptoms were obtained using the total score on the paternally rated Barkley Adult ADHD scale (Barkley & Murphy, 1998).

Statistical analysis:

Normality of distribution for each of the dependent variables (phenotypic measures) was assessed using measures of skewness and kurtosis. Where skewness and kurtosis exceeded the generally accepted levels of 1 and 3 respectively (Field, 2000), the data were transformed according to the recommendations of Tabachnick & Fidell (2001). In brief, the square root was taken for small skews whilst the natural log (plus one if the values included zero) was calculated for larger skews. Negatively skewed data were also reflected (reversing each value to make the data positively skewed by adding a constant which is larger than any of the values in the variable). Following transformation, skewness and kurtosis were within the accepted levels.

Linear and logistic regression analyses, using the enter method, were undertaken with the phenotypic measures as dependent variables. Missing data were dealt with using the pairwise option. Where significant associations were detected, covariates were

added to the model when they too were significant, or had a trend towards significance. These additional covariates included other environmental factors studied where appropriate to ascertain independent effects.

For the birth weight variables, because of differences in national mean birth weight for singletons and those from multiple births, all individuals from multiple births (n = 9) were removed from the analysis. All analyses were implemented using the programme SPSS (version 11) (Norusis/SPSS Inc., 2001).

5.4 Results:

Hyperactive-impulsive symptoms

The total number of hyperactive-impulsive symptoms was negatively skewed. The raw values were reflected and then square rooted to approximate normality. The reflection reverses the raw values. Therefore, negative beta coefficients and t-values actually relate to an increase in the number of hyperactive-impulsive symptoms and vice versa. Linear regression analysis showed significant increase in hyperactive-impulsive symptoms when mothers smoked during pregnancy ($t = -2.80, p = 0.005$), increasing with the quantity of cigarettes smoked during pregnancy ($t = -2.80, p = 0.006$) and also with lower social class ($t = -2.74, p = 0.006$). No significant association was observed between hyperactive-impulsive symptoms and birth weight (see table 5.1 for details).

Table 5.1: Univariate linear regression analyses for environmental measures

predicting reflected square root of hyperactive-impulsive symptoms:

	Unstandardised		Standardised	t	p-value
	Beta	Standard error	Beta		
Smoking yes/no	-0.13	0.05	-0.15	-2.80	0.005**
Smoking quantity	-0.08	0.03	-0.15	-2.80	0.006**
Birth weight (g)	0.00003	0.0001	0.05	0.85	0.40
Social class	-0.27	0.10	-0.15	-2.74	0.006**

**= Significant at $p \leq 0.01$

The covariates age at time of assessment ($t = 2.10, p = 0.04$), verbal IQ ($t = 2.43, p = 0.02$), and conduct disorder symptoms ($t = -3.42, p = 0.001$) also predicted total number of hyperactive-impulsive symptoms. Gender, performance IQ, maternal ADHD, paternal ADHD and maternal alcohol consumption during pregnancy were not significant predictors.

Using the significant variables from the univariate analyses and the covariates, multiple regression analysis was performed (see Table 5.2). Where two measures of similar constructs were found to be significant (as in the case of the two smoking variables), the more significantly associated variable was selected to avoid problems of collinearity. Even taking these covariates into account, a significant association with maternal smoking during pregnancy and social class was observed (see Table 5.2) (of note, the transforming of the hyperactive-impulsive symptoms due to non-normality included reflecting the data which accounts for the negative, rather than positive t value).

Table 5.2: Multiple regression analysis for reflected square root predicting hyperactive-impulsive symptoms

	Unstandardised		Standardised	t	p-value
	Beta	Standard error	Beta		
Constant	2.28	0.42	-	5.39	<0.001**
Smoking yes/no	-0.31	0.16	-0.11	-1.96	0.05*
Social class	-0.22	0.10	-0.12	-2.19	0.03*
CD symptoms	-0.14	0.06	-0.14	-2.51	0.01*
Age at assessment	0.007	0.003	0.12	2.19	0.03*
Verbal IQ	0.003	0.002	0.09	1.71	0.04*

* = Significant at $p \leq 0.05$ ** = Significant at $p \leq 0.01$

Inattentive symptoms:

The total number of inattentive symptoms was normally distributed. None of the environmental measures analysed using univariate linear regression were significantly associated with inattentive symptoms (see table 5.3 for details). Therefore, no further analyses were undertaken.

Table 5.3: Univariate linear regression analyses for environmental measures predicting inattentive symptoms:

	Unstandardised		Standardised	t	p-value
	Beta	Standard error	Beta		
Smoking yes/no	0.06	0.18	-0.02	-0.36	0.72
Smoking quantity	0.06	0.10	-0.03	-0.59	0.56
Birth weight (g)	0.00001	0.0001	-0.006	-0.11	0.91
Social class	0.07	0.12	0.03	0.58	0.56

Total number of ADHD symptoms:

This variable was normally distributed. A significant association was observed between total number of ADHD symptoms and social class ($t = 2.03$, $p = 0.04$). No evidence of association was observed for any of the other environmental measures and thus multiple regression analysis was not undertaken. These findings are summarised in Table 5.4.

Table 5.4: Univariate linear regression analyses for environmental measures

predicting total number of ADHD symptoms:

	Unstandardised		Standardised	t	p-value
	Beta	Standard error	Beta		
Smoking yes/no	0.39	0.26	0.08	1.49	0.14
Smoking quantity	0.19	0.15	0.07	1.27	0.20
Birth weight (g)	0.0002	0.0001	-0.04	-0.69	0.49
Social class	0.34	0.17	0.11	2.03	0.04*

* = Significant at $p \leq 0.05$

Conduct Disorder symptoms:

The total number of CD symptoms was positively skewed. The data were transformed using the natural log (plus 1) to approximate normality. Maternal smoking during pregnancy ($t = 3.94$, $p = 0.0001$), quantity of cigarettes smoked ($t = 3.13$, $p = 0.002$), and social class ($t = 2.55$, $p = 0.01$) significantly predicted number of CD symptoms. A trend towards significance was also observed for birth weight ($t = -1.89$, $p = 0.06$). See Table 5.5 for details.

Table 5.5: Univariate linear regression analyses for environmental measures predicting natural log (plus 1) of conduct disorder symptoms:

	Unstandardised		Standardised	t	p-value
	Beta	Standard error	Beta		
Smoking yes/no	0.23	0.06	0.21	3.94	0.0001**
Smoking quantity	0.11	0.03	0.17	3.13	0.002**
Birth weight (g)	-0.0004	0.001	-0.10	-1.89	0.06
Social class	0.10	0.04	0.14	2.55	0.01*

* = Significant at $p \leq 0.05$

** = Significant at $p \leq 0.01$

The covariates of gender ($t = 6.75, p = 0.007$), Verbal IQ ($t = -2.86, p = 0.004$) and total number of ADHD symptoms ($t = 2.13, p = 0.03$) also significantly predicted the number of conduct symptoms in univariate linear regression analysis. Age at time of assessment, performance IQ, maternal ADHD, paternal ADHD and maternal alcohol consumption during pregnancy did not significantly predict CD symptoms. Multiple regression analysis including those variables which had been significant in univariate analyses, showed that maternal smoking during pregnancy ($t = 3.34, p = 0.001$) continued to independently predict the total number of CD symptoms whilst a trend towards significance was found for social class ($t=1.72, p=0.09$). These findings are summarised in Table 5.6.

Table 5.6: Multiple regression analysis predicting natural log (plus 1) of Conduct

Disorder symptoms

	Unstandardised		Standardised	t	p-value
	Beta	Standard error	Beta		
Constant	0.55	0.33	-	1.70	0.09
Smoking yes/no	0.21	0.06	0.18	3.34	0.001**
Social class	0.07	0.04	0.09	1.72	0.09
Gender	-0.28	0.10	-0.15	-2.84	0.005**
Verbal IQ	-0.004	0.003	-0.09	-1.68	0.09
ADHD symptoms	0.02	0.01	0.10	1.85	0.07

** = Significant at $p \leq 0.01$

Oppositional Defiant Disorder Symptoms:

The total number of ODD symptoms was normally distributed. Maternal smoking during pregnancy ($t = 2.20$, $p = 0.03$) and quantity of cigarettes smoked ($t = 1.95$, $p = 0.05$) significantly predicted the total number of ODD symptoms. No other significant associations were observed between this measure and the other environmental measures (see Table 5.7 for details). None of the covariates (age at time of assessment, gender, maternal ADHD, paternal ADHD, IQ, total number of ADHD symptoms and maternal alcohol consumption during pregnancy) were significantly associated with the total number of ODD symptoms. Therefore, additional multiple regression analysis was not conducted.

Table 5.7: Univariate linear regression analyses for environmental measures

predicting Oppositional Defiant Disorder symptoms:

	Unstandardised		Standardised	t	p-value
	Beta	Standard error	Beta		
Smoking yes/no	0.58	0.26	0.12	2.20	0.03*
Smoking quantity	0.29	0.15	0.10	1.95	0.05*
Birth weight (g)	0.0003	0.0001	-0.08	-1.37	0.71
Social class	0.19	0.17	0.06	1.14	0.26

* = Significant at $p \leq 0.05$

Diagnosis of Conduct Disorder:

40 individuals (11%) in this analysis met DSM-IV diagnostic criteria for CD.

Maternal smoking during pregnancy (OR = 3.14, 95% C.I.1.54, 6.41 $p = 0.002$), quantity of cigarettes (OR = 1.68 95% C.I., 1.18, 2.39, $p = 0.004$) smoked and social class (OR = 1.95 95% C.I. 1.18, 3.12, $p = 0.009$) significantly predicted diagnosis of conduct disorder in univariate logistic regression analysis. None of the other environmental measures were associated with CD (see Table 5.8 for details).

Table 5.8: Univariate logistic regression analyses of environmental measures

predicting diagnosis of Conduct Disorder:

	Beta	Standard error	Wald	df	Odds ratio (95% CI)	p-value
Smoking yes/no	1.15	0.36	9.95	1	3.14 (1.54, 6.41)	0.002**
Smoking quantity	0.52	0.18	8.25	1	1.68 (1.18, 2.39)	0.004**
Birth weight (g)	0.0001	0.0001	0.05	1	1.00 (0.99, 1.006)	0.82
Social class	0.67	0.26	6.78	1	1.95 (1.18, 3.23)	0.009**

** = Significant at $p \leq 0.01$

The covariate of verbal IQ (OR = 0.96, 95% C.I. 0.93, 0.99, p = 0.004) significantly predicted diagnosis of conduct disorder. Multiple regression analysis of all significant variables revealed significant association of the diagnosis of conduct disorder with maternal smoking during pregnancy (OR = 2.26, 95% C.I. 1.22, 5.61, p = 0.01) and social class (OR = 1.91, 95% C.I. 1.08, 3.35, p = 0.03). A trend was also observed for verbal IQ (OR = 0.97, 95% C.I. 0.94, 1.00, p = 0.06). Full details of this analysis are in Table 5.9.

Table 5.9: Multiple logistic regression analyses of environmental measures predicting diagnosis of Conduct Disorder:

	Beta	Standard error	Wald	df	Odds ratio (95% CI)	p-value
Smoking yes/no	0.96	0.39	6.11	1	2.62 (1.22, 5.61)	0.01**
Social class	0.64	0.29	5.01	1	1.91 (1.08, 3.35)	0.03*
Verbal IQ	-0.03	0.02	3.67	1	0.97 (0.94, 1.00)	0.06
Constant	-1.35	1.72	0.61	1	0.26	0.43

* = Significant at $p \leq 0.05$

** = Significant at $p \leq 0.01$

Diagnosis of Oppositional Defiant Disorder:

177 individuals (47%) met the diagnostic criteria for ODD. None of the environmental measures studied significantly predicted diagnosis of ODD, see Table 5.10 for details. Therefore, no further multiple logistic regression analysis was performed.

Table 5.10: Logistic regression analyses of environmental variables on diagnosis of

Oppositional Defiant Disorder:

	Beta	Standard error	Wald	df	Odds ratio (95% C.I.)	p-value
Smoking yes/no	-0.12	0.14	0.74	1	0.88 (0.74, 1.70)	0.39
Smoking quantity	-0.12	0.14	0.81	1	0.88 (0.85, 1.37)	0.37
Birth weight (g)	0.0001	0.0001	0.03	1	1.00 (1.00, 1.00)	0.86
LBW	0.30	0.11	0.32	1	0.94 (0.54, 2.38)	0.57
Social class	-0.008	0.14	0.004	1	0.99 (0.76, 1.30)	0.99

5.5 Discussion:

From the results obtained, it appears that specific environmental factors do have independent effects on phenotype manifestation in a clinical ADHD sample. Total number of hyperactive-impulsive symptoms was predicted by maternal smoking during pregnancy ($t = -1.96, p = 0.05$). This indicates that children whose mothers smoked during pregnancy were more hyperactive and impulsive than those who did not. Quantity of cigarettes smoked also significantly predicted hyperactive-impulsive symptoms ($t = -2.80, p = 0.006$). This dose-dependent effect is similar to those seen by other researchers (e.g. Thapar et al., 2003), but this is specifically for a clinical sample. Social class also significantly predicted hyperactive-impulsive symptoms ($t = -2.19, p = 0.03$). Furthermore, this association has not previously been reported for only hyperactive-impulsive symptoms. This finding, in contrast to the lack of association observed between any of the environmental measures and inattentive symptoms, may indicate that smoking during pregnancy is related specifically to hyperactive-impulsive symptoms, rather than all symptoms of ADHD (in a clinical

sample at least). However, it is important to remember that this lack of findings for inattentive symptoms could be due to a lack of statistical power to detect effects. The effect of environmental influences on ADHD subtype symptoms has not been previously examined and so requires further investigation.

A significant association was observed between social class and total number of ADHD symptoms ($t=2.03$, $p=0.04$). No independent associations were observed between the other environmental measures and overall ADHD symptom severity or the total number of inattentive symptoms.

The failure to detect association between ADHD symptom severity and inattentive symptoms may, as previously discussed, be due to the reduced variance in number of symptoms due to this being a sample clinically diagnosed with the disorder and therefore individuals had to have a certain number of symptoms to be included.

Furthermore, this analysis does not test for main effects of environmental measures on the occurrence of ADHD (which has been demonstrated by other studies, as reviewed), rather whether or not these measures have additional, modifying effects on the ADHD phenotype. Because this analysis is within a clinical sample, it may even be argued that any independently significant findings, such as the effect of maternal smoking during pregnancy on hyperactive-impulsive findings are even more interesting as the power to detect them is small.

Finally, independent significant effects of maternal smoking during pregnancy ($t = 3.34$, $p = 0.001$) on CD symptoms were found, even when taken together with other

covariates. This shows that smoking during pregnancy is associated with the number of CD symptoms in individuals diagnosed with ADHD.

Similarly, a diagnosis of CD was also significantly associated with maternal smoking during pregnancy (OR=2.62, 95% C.I. 1.22, 5.61 $p = 0.01$) and social class (OR=1.91, 95% C.I. 1.08, 3.35 $p = 0.03$) in multiple logistic regression analysis. This indicates that there is more than a 2.5-fold increase in the risk of a diagnosis of CD for those ADHD children whose mothers smoked during pregnancy. Furthermore, for children who are in the low social class group and whose mothers smoked during pregnancy, there is an even greater risk of a comorbid diagnosis of CD.

Significant associations between maternal smoking during pregnancy ($t = 2.20$, $p = 0.03$), quantity of cigarettes smoked ($t = 1.95$, $p = 0.05$) and total number of ODD symptoms were also observed. Interestingly, no significant associations were found between diagnoses of ODD and any of the environmental measures.

It is possible that, rather than showing an association between ADHD with comorbid CD symptoms and the environmental measures, this analysis is instead revealing main effects of these factors on antisocial behaviour in general. This is certainly plausible as associations between maternal smoking during pregnancy, social class and antisocial behaviour have previously been reported (e.g. Costello et al., 2001; Weissman et al., 1999). Unfortunately, this is not something which can be tested in this sample as neither a normal or conduct disordered control group are available. However, the aim of this study was not to find main effects of environmental measures.

Clearly there are a number of limitations. As previously mentioned, modifying effects rather than main effects of environmental factors on ADHD are examined. These findings are also dependent upon the reliability and validity of the environmental measures used. The measures of maternal smoking during pregnancy and birth weight were rated retrospectively by mothers and recall may be biased. This may not be a problem as retrospective recall of smoking during pregnancy has been shown to be relatively accurate (Mick et al., 2002) whilst studies comparing maternal retrospective recall of birth weight to antenatal records have been shown to be highly correlated ($r = 0.98$) (Olson et al., 1997). However, as mothers rated the environmental measures and reported on their child's behaviour through the CAPA interview, bias may have potentially been introduced. The measure of social class may also have its problems. Social class is a general measure which encompasses many different environmental factors (e.g. income, family structure). Calculating social class based on only parental occupation may not be entirely valid. Indeed, more recent editions of the Occupational Classification system (ONS, 2000) require further information for social class to be assigned.

A variety of other possible covariates were not measured and therefore could not be included in the analysis. For example, no measure of parental antisocial behaviour was obtained. This may be especially pertinent as mothers who have antisocial behaviour may be more likely to smoke during pregnancy. These factors would have to be included before any firm conclusions about causality could be made.

Finally, as previously mentioned, these environmental measures are likely to be distal (indirect) rather than proximal (direct) risk factors, although animal studies suggest potential neurotoxic effects of maternal smoking during pregnancy (Weitzman et al., 2002). However, such risk indicators do have advantages as they are relatively simple to ascertain and findings can thus be easily replicated and can be informative in identifying individuals who are at greater risk for specific outcomes.

Despite these limitations, it appears that environmental measures of maternal smoking during pregnancy, social class and, to some extent, birth weight are significantly associated with number of hyperactive-impulsive symptoms and comorbid antisocial behaviour problems in children with ADHD. These findings will now be taken forward into my next set of analyses, looking for Gene x Environment interactions.

CHAPTER 6

Gene x Environment interaction: Selective literature

review

6.1 Introduction:

Gene x Environment interaction (GxE) has been defined as genetically influenced individual differences in the sensitivity to specific environmental features (Rutter & Silberg, 2002).

Traditionally, researchers have not accounted for GxE and have dealt with any possible interactions by either ignoring them or rescaling measures to eliminate them (Wachs & Plomin, 1991). However, more recently, there has been a wider recognition of the importance of GxE (Rutter & Silberg, 2002; Aiken & West, 1991). Wachs and Plomin (1991) comment that, although ignoring GxE may lead to more parsimonious explanations of data, it may neglect potentially important influences.

There is evidence to suggest that interactions between genetic and environmental factors are common and contribute substantially to the aetiology of disorders (Wachs & Plomin, 1991). Because interactions are subsumed under the genetic estimate (heritability estimate) in traditional twin studies (Rutter & Silberg, 2002), the extent of their influence is hard to gauge. However Rutter and Silberg (2002) amongst others, have suggested that if both genetic and environmental factors are thought to have an effect on an outcome, then their interplay is a legitimate topic of study.

6.2 Previous examples of GxE in complex disorders:

Animal studies:

Some work has been performed looking at GxE effects, although it is relevant to note that such study is not methodologically simple, nor are there sufficient guidelines to inform researchers (Aiken & West, 1991). Animal studies have demonstrated GxE in a number of situations. In one well renowned experiment, Cooper and Zubek (1958) looked at maze learning in two strains of rats, bred to be either 'maze-dull' or 'maze-bright'. Cooper and Zubek found that rearing environment had differing effects on the genetically different rats; when raised in an 'enriched' environment, the maze-solving ability of the maze-dull rats improved, whilst no effect was observed on the maze-bright rats. Conversely, when reared in an 'impoverished' environment, a negative effect was observed on the maze-solving ability of maze-bright rats, whilst no effect was observed for the maze-dull rats. This illustrates an interaction as individual differences were seen in sensitivity to the rearing environment depending upon the genetic strain of the rats. GxE effects have also been shown in animal studies looking at specific genotypes. In a series of studies, Suomi (1997) looked at serotonin function in monkeys raised by either their mothers or peers. In peer raised monkeys, a risk factor for aggressive behaviour and reactive, impulsive temperament, those with the short allele of a functional serotonin promoter polymorphism (5HTTLPR) had lowered concentration of a primary serotonin metabolite. No differences were observed for maternally raised monkeys.

General Medicine:

GxE has also been demonstrated in general medicine. For example, Nelson and colleagues (2002) have reported an interaction between sunburn and a polymorphism

in the XRCC1 gene which has differing effects on non-melanoma skin cancer. Looking at main effects alone, a significant increase in risk for squamous cell carcinoma was observed for individuals who reported experiencing three or more painful sunburns in their lifetime, whilst a significant decrease in risk was associated with the Gln/Gln genotype of the XRCC1 Arg399Gln polymorphism. However, analysis revealed an interaction between three or more sunburns and the previously protective Gln/Gln genotype which resulted in almost a seven-fold increase in risk (OR 6.8, $p=0.02$). Similarly, in their study looking at predictors of low birth weight, Wang and colleagues (2002) looked at possible interactions between maternal smoking during pregnancy and two maternal metabolic genes which are involved in the metabolism of chemicals in cigarette smoke. Comparing the birth weights of 741 infants, 174 whose mothers smoked during pregnancy and 567 whose mothers had never smoked, a mean reduction in birth weight of 377g (95% CI -413, -147) was associated with smoking during pregnancy. Interactions between maternal smoking during pregnancy and maternal genotypes were observed; mothers who smoked and had the Aa or aa genotype of CYP1A1 or the absence of the GSTT1 deletion polymorphism had babies who were born even lighter (mean reductions of 520g and 642g respectively) and who were at higher risk of being born low birth weight (OR=3.2 95% CI 1.6, 6.4 and OR=3.5, 95% CI 1.5, 8.3 respectively). The greatest reduction in birth weight was observed for infants of those mothers who smoked during pregnancy and had both risk genotypes; a 1285g reduction in birth weight ($p < 0.001$), a reduction greater than that of just adding together the independent effects of the two risk factors. This finding of an interaction is interesting, not least because it suggests a possible mechanism (the maternal metabolic system) through which smoking could be exerting its effects on infant birth weight.

Psychiatric disorders:

GxE effects are also beginning to be detected in complex psychiatric disorders. For example, Mayeux and colleagues (1995) demonstrated an interaction between head injury and Apolipoprotein-epsilon-4 (APOE-4) influencing risk for Alzheimer's disease. In a study of 236 community-dwelling elderly persons, Mayeux and colleagues reported a two-fold increase in risk for Alzheimer's disease in those individuals with the previously reported APOE-4 risk allele, whilst no independent risk was associated with head injury. However, a 10-fold increase in risk was observed in those with both a head injury and the APOE-4 allele. Although replications of this finding are necessary, not least to overcome issues of low power, this study does illustrate ability to detect GxE effects in complex, psychiatric disorders. More recently, a group based in the UK and New Zealand have been interested in finding GxE in complex psychiatric disorders and traits with some success. Utilising a well characterised, longitudinally studied epidemiological sample from Dunedin, New Zealand, Caspi and colleagues (2002) initially reported an interaction between MAOA 30bp promoter variant genotype and maltreatment during childhood on antisocial behaviour, in 466 males. Individuals with the MAOA low activity allele who had experienced probable or severe maltreatment were significantly more likely to have a conviction for violent offences; more symptoms of antisocial personality disorder and score higher on a composite 'antisocial index' at age 26. Trends towards significance in the expected direction were observed for diagnoses of CD ($p=0.06$) and 'disposition towards violence' ($p=0.10$). Conversely to the study by Mayeux and colleagues (1995), Caspi and colleagues found a main effect of environmental exposure, but not of the genotype was observed. Interestingly, Caspi and colleagues (2002) reported that the effect of maltreatment is only significant in

the presence of low MAOA activity. For example, when looking at the percentage of males convicted of a violent crime (11% of the whole sample), there was a significant effect of maltreatment in the low MAOA activity group (beta=1.20, $p<0.001$) but no significant effect in the high MAOA activity group (beta=0.37, $p=0.17$).

Several groups have attempted to replicate this reported interaction with mixed results. For example, Foley and colleagues (2004) did report significant interaction between MAOA genotype and maltreatment on antisocial behaviour in their study of twin pairs (aged eight to 17 years). It is important to note, however, that the measures of maltreatment used by Foley and colleagues were quite distinct from those utilised in Caspi and colleagues original study, whilst the prevalence of severe maltreatment (the only ones showing an interaction) were very small (only one individual with low MAOA activity experienced the highest level of maltreatment). A second study (Haberstick et al., 2005) found no evidence of interaction between maltreatment and MAOA genotype in a larger sample of 774 males. Thus it appears that this interesting finding of Caspi and colleagues (2002) requires further investigation and replication, although it does potentially demonstrate an interesting interaction.

This same group (Caspi et al., 2003) has also reported significant interactions between stressful life events and a gene in the promoter region of the serotonin transporter gene (5HTTLPR) on depressive symptoms. Again utilising the Dunedin cohort, these researchers found a significant increase in subsequent depressive symptoms with number of life events according to possession of the number of short alleles in the 5HTTLPR variant. A main effect of the environmental, but not the genetic factor was

seen. Caspi and colleagues interpreted these findings as the long genotype having a protective effect against the effects of life events.

Interestingly, this investigation builds on a previous study by Silberg and colleagues (2001) of genetic risk and life events predicting depressive symptoms in adolescent females. Using structural equation modelling in a study of adolescent female twin pairs, Silberg and colleagues reported a significant interaction between maternally rated independent negative life events (those deemed beyond the individual's control) and genetic risk, measured by their co-twin's depression score, on the number of depressive symptoms an individual reported. The Caspi and colleagues (2003) study further develops this finding by proposing a specific genetic variant which may be involved in the interaction.

The proposed GxE between 5HTTLPR genotype and life events influencing depression (Caspi et al., 2003) has been the subject of a number of replication studies which vary in their similarity to the original study. Some have not successfully replicated the finding, despite using similar designs (Gillespe et al., 2005). Others have found evidence of significant interaction using a variety of different environmental stressors including childhood maltreatment and lack of social support (Kaufman et al., 2004) and a composite of "family environmental risk" comprising of family social adversity, parental education and family adverse life events (Eley et al., 2004) and unemployment (Grabe et al., 2004). This perhaps illustrates how using different, distal, environmental risk measures influence the same outcome. A recently published study (Kendler and colleagues, 2005) has utilised a more similar method to that of Caspi and colleagues (2003) and also reports a significant interaction between

5HTTLPR genotype and life events predicting diagnosis of depression. Although further investigation and replications are required, the majority of the evidence does appear to support the interaction reported by Caspi and colleagues (2003).

Finally, Caspi and colleagues (2005) have recently published findings of a significant interaction between COMT Val¹⁵⁸Met genotype and adolescent cannabis use, influencing psychotic symptoms at 26 years of age. The findings of this group, although subject to the usual limitation of requiring further work and repeated independent replications, is extremely interesting and encouraging for researchers interested in possible GxE in complex psychiatric disorders.

GxE in ADHD:

In the area of ADHD, three studies of possible GxE effects have been published. The first (Kahn et al., 2003), investigated interactions between DAT1 480bp VNTR and maternal smoking during pregnancy on ADHD symptoms, in a population sample of 161 five year olds. Although no main effects of either maternal smoking during pregnancy or DAT1 genotype were observed, significant increases in number of maternally reported hyperactive-impulsive symptoms were observed for those individuals whose mothers smoked during pregnancy and who were homozygous for the DAT1 10 allele, when compared to those who were not exposed to prenatal cigarette smoke and had only one or no copies of the 10 allele ($t=7.5$, $p<0.01$). No such interaction was found for inattentive symptoms, although interactions were also seen for symptoms of oppositional defiant disorder. Replications of these findings are warranted.

Secondly, Seeger and colleagues (2004) found a significant interaction between season of birth and DRD4 48bp VNTR on diagnosis of ADHD with comorbid conduct disorder. In a study of 64 children with ADHD and conduct disorder compared to 163 healthy controls, no main effects of either DRD4 genotype or season of birth were found. Children were divided into those born in the autumn or winter and those born in the spring or summer. The latter were considered to be the risk group as the dopamine-melatonin system, subject to seasonal variations, would have been less active during gestation and individuals would therefore have been exposed to less dopamine during pregnancy – in line with a hypodopaminergic theory of the disorder. In accordance with the hypothesis, an interaction was observed whereby those with the DRD4 7-repeat allele born in the spring or summer had a significantly increased risk of being diagnosed with ADHD and conduct disorder (OR=2.83, 95% CI 1.23, 6.53, $p=0.01$), whilst being born in the autumn or winter was associated with a significant 5.4-fold decrease in risk ($\chi^2=9.55$, $df\ 1$, $p=0.002$). This is an interesting finding, however, further replications are necessary.

Finally, analysis by our own group (Thapar et al., in press) has revealed a significant interaction between birth weight and COMT Val¹⁵⁸Met genotype, modifying number of conduct disorder symptoms in ADHD individuals. Utilising a sub-set of the sample described here, Thapar and colleagues found main effects of both COMT Val¹⁵⁸Met genotype ($t=3.14$, $p=0.002$) and birth weight ($t=-3.08$, $p=0.002$), whilst also finding a significant interaction between the two, whereby being of lower birth weight and having the previously implicated Val/Val genotype conferred risk for greater numbers of conduct disorder symptoms ($t=2.75$, $p=0.006$). This finding remained significant when covariates, including maternal smoking during pregnancy and verbal IQ, were

entered into the regression equation. This finding is especially interesting both because it demonstrates a GxE modifying the ADHD phenotype and also because the COMT Val¹⁵⁸Met variant has been implicated in the Caspi and colleagues (2005) study into cannabis use and symptoms of psychosis, possibly suggesting that genetic variants interact with different environmental risk factors in a variety of ways to influence a number of disparate outcomes.

Thus it would appear that it is important to look for GxE in ADHD. This is, however, an understudied area and it is interesting that, despite the presence of a number of variables, both genetic and environmental, which have been consistently associated with ADHD, little work, bar that described here, has been undertaken.

6.3 Methodological issues:

As previously discussed, it would appear that GxE effects are both worthy of investigation and possible to detect in complex diseases including ADHD. However, there are a number of important methodological issues which must be considered before such investigations can be undertaken. Perhaps the most important is the fact GxE as defined here is not the only way in which genetic and environmental effects can combine to influence an outcome. Gene-environment correlation (rGE) may also influence outcomes. rGE has been defined as genetic effects on individual differences in liability to be exposed to environmental factors (Rutter & Silberg, 2002), so an individual with a specific genetic factor may be more likely to find themselves in certain environmental situations. rGE can work in three different ways. Firstly, active rGE, where the individual seeks out environments suited to their genotype. Secondly, passive rGE, whereby the individual is exposed to environmental situations by virtue

of sharing genes with their parents who provide environments for their offspring. Finally, there is evocative rGE, whereby specific genotypes elicit responses from others which reinforce specific environmental exposures (Scarr & McCartney, 1983).

In psychiatry, the possible presence of rGE is potentially a major problem as it is likely that important risk factors are going to be person and therefore possibly genotype dependent. However, separating rGE from GxE is not a simple task. Researchers have attempted to control for rGE in their studies of GxE. For example, in their study of negative life events, genetic risk and depressive symptoms, Silberg and colleagues (2001) looked at only independent life events; occurrences which the adolescents had no influence over. Although this may not account for all rGE (for example, a parent losing their job was deemed independent, but may still contribute to passive rGE), controlling for at least some rGE is an important step. It is also possible, to some extent, to test for rGE for example through twin designs. The testing of rGE is improved by having measured genotypes, an added advantage of analysing specific molecular genetic markers. Clearly rGE is important when studying GxE, although some researchers have argued that it may not be possible or relevant to separate the two as they are not distinct in nature (Wachs, 1991).

Even beyond the issue of rGE, interactions, as defined here by Rutter and Silberg (2002), are not the only type of interaction between genetic and environmental factors. The definition used here refers to a statistical interaction tested by looking for a departure from a multiplicative model (Weiss, 1999). There are other ways to look for interactions, for example, looking for departure from an additive model. The type of interaction detected is, therefore, dependent upon the statistical model used.

Biological interactions have also been studied. Biological interactions are concerned with co participation of two risk factors in the same causal mechanism of a disease (Weiss, 1999). By this definition, a possible causal mechanism therefore has to be defined (Weiss, 1999). In this way, biological interactions can be described as looking at how one factor moderates the effect of another. There has been much interest in both statistical and biological interactions, with epidemiological studies being especially concerned with the latter. To study one or the other is not incorrect but, in the study of complex psychiatric disorders, interest has centred on looking at statistical interactions. This shall therefore be the emphasis in this thesis.

Other factors also need to be considered when looking at GxE. Power to detect effects is an extremely important issue as power to detect GxE is less than that for main effects (Chronbach, 1991). By virtue of the fact GxE is concerned with individual differences within a population, rather than generalisations across the population (Chronbach, 1991), power to detect GxE may also be reduced as interactions may account for only a small proportion of the variance in scores (Plomin & Hershberger, 1991). This is not to dismiss the importance of GxE; in complex disorders each risk factor, be it a specific genotype, environmental exposure or interaction, will account for only a very small proportion of the variance (Rutter et al., 2001). Indeed, van den Oord and Neale (2004) argue that where genes exert a small effect, it is advantageous to study them in the context of their interplay with the environment. Furthermore, variance accounted for must be taken in context; a 10% change in death rates may account for only 1% of the overall variance but is still clearly significant (Rutter & Pickles, 1991). It is therefore necessary to increase power to detect GxE as much as possible. One way in which this can be done is by increasing sample sizes. Power

may also be increased by ensuring accurate measurement of genetic, environmental and outcome variables (Plomin & Hershberger, 1991). Plomin and Hershberger (1991) further comment that accurate measurement of genetic factors will be greatly improved by the study of specific genetic markers through the use of DNA (as opposed to using estimates of familial loading through twin and other designs).

Wachs and Plomin (1991) have commented that there are a number of different methods for testing GxE and that different methods may lead to different findings. One widely discussed issue is whether or not main genetic and environmental effects should be placed in the same model as the interaction term. Putting main effects into the model may lead to a failure to detect GxE as the main effects absorb some of the variance associated with the interaction (Rutter & Pickles, 1991). Given the small proportion of the variance likely to be attributable to GxE discussed previously, this is an important consideration. Not putting main effects into the model, however, will inflate the interaction term. Furthermore, if main effects account for almost all the variance, then the influence of any GxE effect is irrelevant (McCall, 1991). Thus it would appear that, even though it may be a conservative approach which may lead to type 2 errors, it can be argued that it is necessary to look for significant alteration in the proportion of the variance accounted for by the GxE term, over and above main effects in order to claim to have found significant GxE (Kraemer et al., 2001).

A further issue is whether or not significant genetic and environmental main effects need to be present before their interaction can be examined. We have seen examples where interactions have been found in the presence of both genetic and environmental main effects (Nelson et al., 2002), only genetic effects (Mayeux et al., 1995), only

environmental effects (Caspi et al., 2003; Caspi et al., 2002) and where main effects of neither are present (Kahn et al. 2003; Seeger et al., 2004). It seems logical that, even in the absence of observed main effects, an interaction should be conceptually plausible. Interestingly, Rutter and Pickles (1991) propose that it is not necessary to fully understand the mechanisms through which an interaction works in order to statistically test for it. Indeed, they suggest that the discovery of interactions should be the starting point for identifying such mechanisms. They state that if we already understand the biological pathways through which an interaction works, then we will no longer be interested in the interaction. Therefore, it appears that although some plausible influence is necessary, exact conceptualisation of the mechanisms through which GxE arise is not. By not restricting study of interactions to those with hypothesised mechanisms, the range of possible GxE effects which can be investigated is increased immensely.

This inevitably throws up issues of possible multiple testing. Although GxE effects are believed to be common (Rutter & Silberg, 2002), it is also evident that testing for such interactions between all available genetic and environmental factors will result in artifactual findings (Chronbach, 1991). This problem is further exacerbated as correcting for multiple testing can alter findings of GxE (Chronbach, 1991). Given that study of GxE in complex diseases including ADHD is in its infancy, perhaps it seems legitimate to perform many tests in an exploratory manner without correcting for multiple testing. These results can then be presented with the caveat that any significant findings are merely suggestive and require further confirmation through independent replication. With this in mind, it seems suitable to suggest that if

variables have previously been postulated as genetic or environmental risk variables, then testing for their possible interaction is justified.

Given that in complex diseases both genetic and environmental influences are central, it is important to look at GxE in ADHD in accordance to the definition of Rutter and Silberg (2002). In such study, accurate measurement of predictors and outcomes is paramount. It is preferable if rGE is not present and the use of molecular genetic data will encourage this. Main effects of genetic and environmental influences must be included in any models, whilst change in variance, rather than just a significant p-value, is necessary for any interaction to be meaningful – however small this alteration is. It seems that, due to the exploratory nature of any current investigation, strict hypotheses and corrections for multiple testing are too rigorous, enabling interactions to be investigated wherever there is prior justification for genetic or environmental main effects found by these or other researchers.

CHAPTER 7

Analysis of GxE in a clinical ADHD sample

7.1 Introduction:

GxE effects are likely to be common (Rutter & Silberg, 2002) and contribute to the aetiology of complex disorders including ADHD. GxE may also mediate manifestation of the disorder. For example, as demonstrated by the study by Kahn and colleagues (2003), GxE may influence only particular aspects of the ADHD phenotype.

7.2 Aims:

1). GxE effects on the diagnosis of ADHD

The first aim of this chapter is to test for interactions between dopaminergic genetic markers and environmental factors which have previously been suggested to be independently associated with ADHD. To this end, the following genetic markers (previously studied for main effects in this sample in chapter 3) are examined:

- DRD4 48bp VNTR
- DAT1 480bp VNTR
- DRD5 (CA)_n microsatellite marker
- DRD3 Ser9Gly variant
- MAOA 30bp promoter VNTR
- COMT Val¹⁵⁸Met polymorphism

The following environmental factors which have previously been found to be positively associated with ADHD (reviewed in chapter 5) will be investigated:

- Maternal smoking during pregnancy
- Birth weight
- Social class.

2). Modifying effects of GxE on the ADHD phenotype:

a). The second aim is to replicate the findings of Kahn and colleagues (2003) by testing whether the DAT1 480bp VNTR interacts with maternal smoking during pregnancy to influence hyperactive-impulsive and ODD symptoms in a clinical sample.

b). Finally, the third aim is to further investigate the modifying influences of GxE on the ADHD phenotype. In this sample, an interaction between birth weight and COMT Val¹⁵⁸Met genotype influencing total number of conduct disorder symptoms has previously been reported (Thapar et al., in press). This analysis will not, however make up part of this thesis. In order to focus this analysis, GxE will only be tested where specific environmental factors have been independently, significantly associated with, or shown a trend towards significant association with, specific aspects of the ADHD phenotype in this sample (see chapter 5). That is, I will only test for GxE where I have found main environmental effects. Therefore, possible interactions will be investigated between the following genes:

- DRD4 48bp VNTR
- DAT1 480bp VNTR
- DRD5 (CA)n microsatellite marker
- DRD3 Ser9Gly variant
- MAOA 30bp promoter VNTR
- COMT Val¹⁵⁸Met polymorphism

and the following environmental and phenotypic variables:

For Maternal smoking during pregnancy –

- Total number of hyperactive-impulsive symptoms
- Total number of CD symptoms

For social class –

- Total number of hyperactive-impulsive symptoms
- Total number of ADHD symptoms
- Total number of CD symptoms

Although diagnosis of conduct disorder was also independently associated with maternal smoking during pregnancy and social class in previous analysis (see chapter 5), as only a few individuals had this diagnosis, it was not possible to investigate GxE for this phenotypic measure in this sample.

7.3 Methods:

1). Testing GxE for diagnosis of ADHD: Conditional logistic regression analysis of cases and pseudocontrols is equivalent to matched case-control analysis and enables the detection of GxE in family-based trios (Cordell, 2004; Cordell et al., 2004). The presence or absence of the risk allele in the proband is matched to three pseudocontrols, derived from the parental genotypes which were not transmitted to the child. Thus there are four pieces of information for each individual.

Environmental information is inputted for each case and pseudocontrol. Because the pseudocontrols are derived from the proband information, all four observations for each individual will contain the same environmental information. An example of the coding and SPSS input file for two hypothetical individuals can be seen in Table 7.1.

Figure 7.1: Example of coding and SPSS input files for conditional logistic regression analysis for two hypothetical individuals

Basic information utilised:

Individual 1:

Father's Genotype 1 2

Mother's Genotype 1 2

Child's Genotype 2 2 (possible untransmitted genotypes 1 2, 1 2, 1 1)

Environmental Status: Mother smoked during pregnancy (coded as 1)

Individual 2:

Father's Genotype 1 2

Mother's Genotype 2 2

Child's Genotype 1 2 (possible untransmitted genotypes 1 2, 2 2, 2 2)

Environmental Status: Mother did not smoke during pregnancy (coded as 0)

SPSS input file for these individuals:

Individual	Actual genotype or Pseudocontrol?*	Genotype	Environmental Status
1	1	2 2	1
1	0	1 1	1
1	0	1 2	1
1	0	1 2	1
2	1	1 2	0
2	0	1 2	0
2	0	2 2	0
2	0	2 2	0

* = 1 denotes actual genotype transmitted, 0 denotes pseudocontrol derived from untransmitted genotypes

Using these data, a chi-square test was performed to investigate whether or not the risk variables (G and GxE) were present significantly more frequently in probands than in the pseudocontrols and were therefore associated with the disorder.

For this analysis, observations with the reported risk allele were assigned the value of 1 and those without the risk allele were assigned the value of 0. Similarly, individuals who were exposed to the risk environment (e.g. whose mothers smoked during pregnancy) were given the value of 1 and those who were not exposed to environmental risk (e.g. whose mothers did not smoke during pregnancy) a value of 0. Birth weight was used as a continuous variable. Genetic and environmental variables were multiplied to provide the GxE term.

The difference in the variance explained when the main genetic effects alone were placed into the model and when the GxE term was added was analysed. This analysis was performed using the Cox's regression function in SPSS version 11 (Norusis/SPSS Inc, 2001).

To compare the effects of high, medium and low social class, two dichotomous variables were created. In accordance with Aiken and West (1991), using a method previously utilised in the analysis of GxE (for example by Kendler et al., 2005), dummy variables were created, using low social class as the reference category. The two dummy variables (one comparing low and high social class and the second comparing low and medium social class) were then added to the model as the GxE term as before.

This type of GxE analysis has the advantages of TDT analysis, being able to control for such potential problems as population stratification. However, there are two disadvantages of this method. Firstly, as with any family-based method, the main effects of the environment can not be assessed (because the environmental measures are the same for cases and pseudocontrols). Secondly, only information from parent-child trios (not duos) can be utilised as both parental genotypes must be known before pseudocontrols can be constructed.

2). Replication of Kahn and colleagues (2003): To exactly replicate the methods used by Kahn and colleagues (2003), multivariate linear regression analysis was performed. In accordance to the method used by Kahn and colleagues (2003), the main and interaction effects of DAT1 genotype and maternal smoking during pregnancy were entered as three dummy variables to represent DAT1 10/10 risk genotype and smoking, DAT1 risk genotype and not smoking, DAT1 non-risk genotype and smoking and the reference category of DAT1 non-risk genotype and not smoking. Backwards elimination method linear regression was used to obtain the final model (Kahn and colleagues, 2003). These analyses were performed using SPSS version 11.

3). GxE effects on different aspects of the ADHD phenotype: To investigate possible GxE effects on specific aspects of the ADHD phenotype, linear and logistic regression were performed using the enter method. Using the same method as Caspi and colleagues (2003, 2002), the risk allele and exposure to the environmental risk factor was given the value of 1 and the non-risk allele and non-exposure to the environmental risk factor was given the value of 0. Genetic and environmental factors

were multiplied to provide the GxE term. For the COMT Val¹⁵⁸Met polymorphism, the genotype was analysed because this was the method used by previous analyses using this variant (Caspi et al., 2005; Thapar et al., in press) and values of 0, 1 and 2 were assigned for Met/Met, Val/Met and Val/Val individuals respectively. In the case of the DAT1 480bp VNTR, the number of individuals without a copy of the 10-repeat allele (n=18) was too small to analyse. Therefore, in accordance with Kahn and colleagues (2003), those individuals homozygous for the 10-allele were compared to all others. All analyses were repeated to look at allele and genotype, where feasible. The results remained the same.

For regression analysis, in the first block, main effects of the genetic and environmental measures were entered into the model. In the second block, the GxE term was added. The change in proportion of variance explained by the different models is reported. Any models where the interaction term was significant were then retested with the addition of any covariates associated with the outcome measure (for details see chapter 5) added to block one of the model. The results of these analyses are reported. Because transforming data can mask significant interaction effects (Rutter & Silberg, 2002), the outcome measures were not transformed.

7.4 RESULTS:

1). Tests of GxE for the diagnosis of ADHD

Table 7.1: Results from pseudocontrol GxE analysis for all gene variants:

	Main genetic effects			Interaction with maternal smoking during pregnancy yes/no			Interaction with birth weight (in grams)			Interaction with social class: High, medium, low		
	n	χ^2 (df 1)	p-value	n	χ^2 (df 1)	p-value	n	χ^2 (df 1)	p-value	n	χ^2 (df 2)	p-value
DRD4 48bp VNTR	95	0.24	0.62	92	1.76	0.19	92	0.20	0.65	87	0.54	0.76
DAT1 480bp VNTR	166	0.12	0.73	161	1.63	0.20	157	1.28	0.26	144	3.60	0.17
DRD5 CA(n) microsatellite	78	0.002	0.96	75	2.78	0.10	73	2.22	0.14	67	4.32	0.12
DRD3 Ser9Gly variant	175	1.33	0.25	171	0.45	0.50	169	0.001	0.97	157	2.76	0.25
MAOA VNTR	139	0.001	1.00	137	0.67	0.42	138	1.52	0.22	121	5.74	0.06
COMT Val¹⁵⁸Met variant	127	1.08	0.30	124	0.46	0.50	124	2.26	0.13	112	0.65	0.72

1). Testing GxE effects for the diagnosis of ADHD

Conditional logistic regression analysis revealed a trend towards significant interaction between the MAOA high activity alleles and higher social class predicting the diagnosis of ADHD ($\chi^2=5.74$, df, 2, $p=0.06$). Bi-variate analysis showed that where individuals were not exposed to environmental risk (where social class was high) the high activity alleles had a greater influence on diagnosis of disorder than for those individuals with exposure to environmental risk (low social class) (OR=5.25, df 1, $p=0.02$). These findings are detailed in table 7.1.

2). Modifying GxE effects on the ADHD phenotype:

a). Replication of Kahn and colleagues (2003)

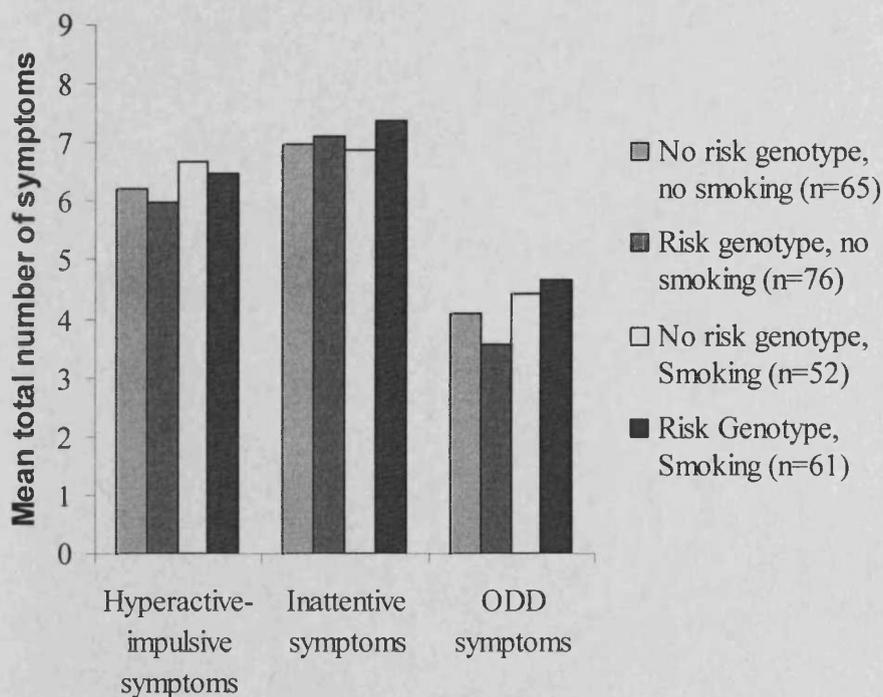
No support was found for the interaction between DAT1 480bp genotype and maternal smoking during pregnancy reported by Kahn and colleagues (2003). These findings are summarised in Table 7.2 and graphically represented in figure 7.2. Main effects of maternal smoking during pregnancy were found (see Smoke-DAT +/- or -/- cells in Table 7.2), as described for this sample in chapter 5. This analysis directly replicated the method of Kahn and colleagues (2003) and so the DAT1 480bp VNTR 10/10 genotype was taken as conferring risk. Similar, negative, findings were also observed when, as is consistent with previous literature, the 10 repeat allele was considered as a risk allele and also where analysis by genotype was performed.

Table 7.2: DAT1 x Maternal smoking during pregnancy testing for GxE:

	Hyperactive-impulsive symptoms		Inattentive symptoms		ODD symptoms	
	Beta (S.E)*	p-value	Beta (S.E)*	p-value	Beta (S.E)*	p-value
Interaction effects:						
Smoke-DAT +/+	0.44 (0.19)	0.11	-0.25 (0.30)	0.40	0.55 (0.45)	0.22
Smoke-DAT +/- or -/-	0.79 (0.27)	0.006	0.24 (0.32)	0.44	0.33 (0.47)	0.48
No smoke DAT +/+	0.02 (0.26)	0.94	-0.16 (0.29)	0.57	-0.54 (0.43)	0.21
No smoke DAT +/- or -/-	Reference category	Reference category	Reference category	Reference category	Reference category	Reference category

* (S.E.) = standard error

Figure 7.2: Graph of DAT1 x Maternal smoking during pregnancy testing for GxE:



b). Testing GxE effects on different aspects of the ADHD phenotype

1). Hyperactive-impulsive symptoms:

a). Maternal smoking during pregnancy

Table 7.3: Interaction analysis of all gene variants and maternal smoking during pregnancy predicting hyperactive-impulsive symptoms:

	n	Change statistics and significance when GxE added to main effects model		
		r ² change	t	p-value
DRD4 48bp VNTR	124	<0.001	0.03	0.98
DAT1 480bp VNTR	263	<0.001	-0.10	0.92
DRD5 (CA)n microsatellite	85	0.02	1.56	0.12
DRD3 Ser9Gly variant	229	0.01	1.89	0.06
MAOA 30bp promoter VNTR	156	0.008	1.13	0.26
COMT Val¹⁵⁸Met variant	307	<0.001	0.07	0.95

The results of analysis testing for interaction between maternal smoking during pregnancy and each gene variant are summarised in table 7.3. The only GxE effect for total number of hyperactive-impulsive symptoms with maternal smoking during pregnancy which approached significance was with DRD3 Ser9Gly variant (r^2 change for model with interaction term added = 0.01, F change = 2.44, df 1, p = 0.06).

However, when the covariates found to be significantly associated with the total number of hyperactive-impulsive symptoms (age at time of assessment, maternal alcohol consumption, verbal IQ, CD symptoms and social class – see chapter 5 for details) were entered into the model, the interaction was no longer significant (r^2

change for model with interaction term added = 0.003, F change = 0.51, df 1, p = 0.48).

b). Social class

Table 7.4: Interaction analysis of all gene variants and social class predicting hyperactive-impulsive symptoms:

	n	Change statistics and significance when GxE added to main effects model		
		r ² change	t	p-value
DRD4 48bp VNTR	118	0.001	0.34	0.73
DAT1 480bp VNTR	256	0.007	1.35	0.18
DRD5 (CA)n microsatellite	82	0.008	-0.93	0.35
DRD3 Ser⁹Gly variant	171	0.003	-0.83	0.41
MAOA 30bp promoter VNTR	151	0.004	0.72	0.47
COMT Val¹⁵⁸Met variant	229	0.03	-2.68	0.008**

** = Significant at the p≤0.01 level

A significant change in the variance explained by the interaction between the COMT Val¹⁵⁸Met genotype and social class on total number of hyperactive-impulsive symptoms was found (r² change for block 2 = 0.03, F change = 7.17, df 1, p = 0.008). Details for all these results are summarised in table 7.4. Multivariate analysis including previously found significant covariates (age at time of assessment, maternal alcohol consumption, verbal IQ, CD symptoms and social class – see chapter 5 for details) was performed.

Table 7.5: Interaction analysis of COMT Val¹⁵⁸Met variant, Social class and significant covariates predicting hyperactive-impulsive symptoms:

	Change statistics			
	r ² change	F change	df	P-value
Model with main effects of G, main effects of E and covariates	0.11	3.57	7	0.001
Model with GxE added	0.02	5.47	1	0.02*

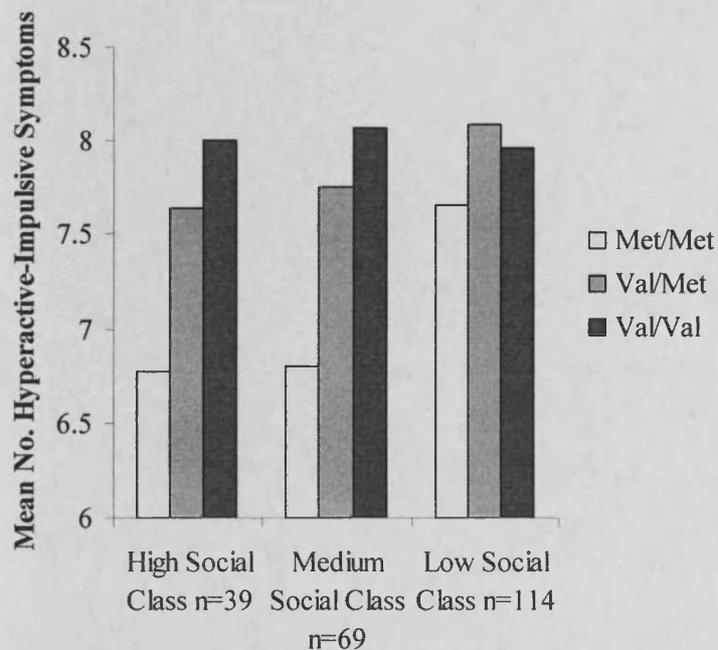
Model with main effects, covariates and GxE added	Unstandardised coefficients		Standardised coefficients	t	p-value
	Beta	S.E.	Beta		
Constant	7.59	1.06	-	7.18	<0.001
Maternal alcohol consumption	-0.16	0.08	-0.13	-1.94	0.05
Age at assessment	0.006	0.004	-0.11	-1.64	0.10
Verbal IQ	0.01	0.008	-0.10	-1.41	0.16
CD symptoms	0.05	0.08	-0.05	0.73	0.47
Smoking during pregnancy	0.37	0.21	0.12	1.75	0.08
Social class	0.96	0.37	0.51	2.61	0.01
COMT genotype	1.54	0.52	0.70	2.93	0.004
COMTxSocial class	-0.82	0.35	0.71	-2.34	0.02*

* = Significant at the p≤0.05 level

The interaction between social class and COMT genotype continued to be significant (r² change for block 2 = 0.02, F change = 5.47, df 1, p = 0.02), indicating that where individuals were exposed to environmental risk (being of low social class) the COMT val¹⁵⁸met variant had less of an effect. The results are somewhat surprising in that, although the mean number of symptoms is higher in those with the Val/Val genotype for the high and medium social class groups, there appears to be no consistent pattern, with the Val/Val genotype not conferring risk for those in the low social class group. These findings are detailed in table 7.5 whilst a graphical illustration can be found in figure 7.3.

Figure 7.3: Interaction between COMT Val¹⁵⁸Met genotype and social class

predicting total number of hyperactive-impulsive symptoms:



2). Total number of ADHD symptoms

a). Social class

Table 7.6: Interaction analysis of all gene variants and social class predicting total ADHD symptoms:

	n	Change statistics and significance when GxE added to main effects model		
		r ² change	t	p-value
DRD4 48bp VNTR	118	<0.001	0.23	0.82
DAT1 480bp VNTR	256	0.007	1.35	0.18
DRD5 (CA)n microsatellite	82	0.04	-2.05	0.04*
DRD3 Ser⁹Gly variant	171	0.02	-2.13	0.03*
MAOA 30bp promoter VNTR	151	0.002	0.57	0.57
COMT Val¹⁵⁸Met variant	229	0.005	-1.04	0.30

* = Significant at p≤0.05 level

Significant interactions between social class and both the DRD5 (CA)_n microsatellite marker (r^2 change for model with interaction term added = 0.04, F change = 4.22, df 1, $p = 0.04$) and the DRD3 Ser9Gly polymorphism (r^2 change for model with interaction term added = 0.02, F change = 4.55, df 1, $p = 0.03$) were found to influence total number of ADHD symptoms (details of all interaction analyses can be seen in table 7.6). When significant covariates were entered into the model (gender, maternal alcohol consumption and CD symptoms – see chapter 5 for details), this interaction disappeared for the DRD5 (CA)_n microsatellite variant (r^2 change for model with interaction term added = 0.04, F change = 2.27, df 1, $p = 0.14$), but continued to be significant for the DRD3 Ser9Gly variant (r^2 change for model with interaction term added = 0.02, F change = 3.84, df 1, $p = 0.05$). Where environmental risk was high (those with low social class) the G allele had less of an influence. For a graphical illustration of this findings, see figure 7.4. No other significant interactions were found for this outcome (see table 7.7 for details).

Table 7.7: Interaction analysis of DRD3 Ser9Gly variant, Social class and significant covariates predicting total number of ADHD symptoms:

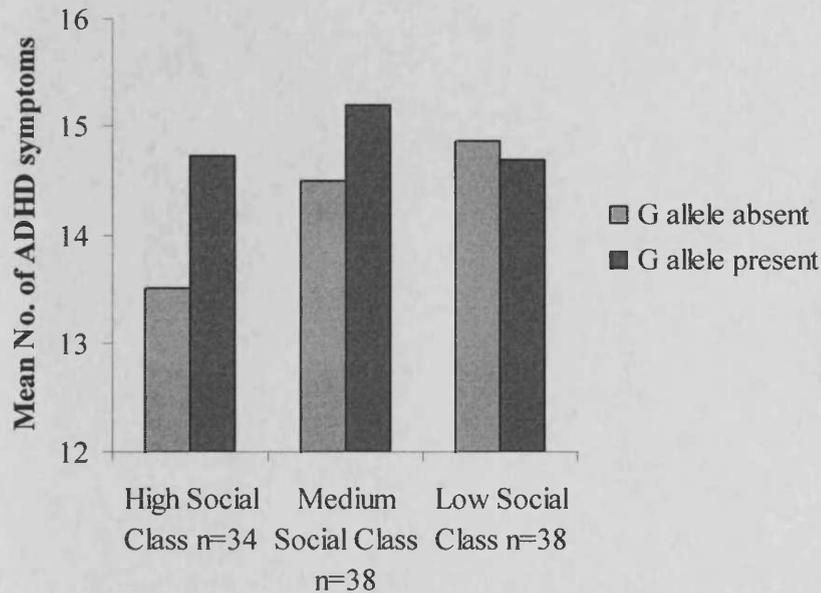
	Change statistics			
	r^2 change	F change	df	P-value
Model with main effects of G, main effects of E and covariates	0.05	1.71	5	0.13
Model with GxE added	0.02	3.84	1	0.05*

Model with main effects, covariates and GxE added	Unstandardised coefficients		Standardised coefficients	t	p-value
	Beta	S.E.	Beta		
Constant	12.48	0.94	-	13.32	<0.001
Maternal alcohol consumption	-0.23	0.15	-0.12	-1.52	0.13
Gender	1.01	0.60	0.13	1.68	0.10
CD symptoms	0.20	0.13	0.11	1.48	0.14
Social class	0.83	0.39	0.67	2.11	0.04
DRD3 G allele	1.33	0.75	0.27	1.77	0.08
DRD3xSocial class	-1.04	0.53	-0.37	-1.96	0.05*

* = Significant at the $p \leq 0.05$ level

Figure 7.4: Interaction between DRD3 Ser9Gly variant and social class predicting

total number of ADHD symptoms:



3). Conduct Disorder symptoms:

a). Maternal smoking during pregnancy

Table 7.8: Interaction analysis of all gene variants and maternal smoking during pregnancy predicting Conduct Disorder symptoms:

	n	Change statistics and significance when GxE added to main effects model		
		r ² change	t	p-value
DRD4 48bp VNTR	124	0.03	2.10	0.04*
DAT1 480bp VNTR	263	<0.001	-0.10	0.92
DRD5 (CA)n microsatellite	85	0.02	-1.54	0.13
DRD3 Ser9Gly variant	229	0.01	2.04	0.04*
MAOA 30bp promoter VNTR	156	0.004	0.80	0.43
COMT Val¹⁵⁸Met variant	307	<0.001	-0.23	0.82

* = Significant at the p≤0.05 level

Interactions between maternal smoking during pregnancy and both the DRD3 Ser9Gly variant and the DRD4 148bp VNTR were significant for the total number of CD symptoms (for details of all interaction results, see table 7.8).

Table 7.9: Interaction analysis of DRD4 48bp VNTR, maternal smoking during pregnancy and significant covariates predicting CD symptoms:

	Change statistics			
	r ² change	F change	df	P-value
Model with main effects of G, main effects of E and covariates	0.12	2.41	6	0.03
Model with GxE added	0.02	3.09	1	0.08

Model with main effects, covariates and GxE added	Unstandardised coefficients		Standardised coefficients	t	p-value
	Beta	S.E.	Beta		
Constant	1.32	1.31	-	1.01	0.31
Gender	-0.65	0.41	-0.15	-1.61	0.11
Verbal IQ	-0.009	0.01	-0.78	-0.84	0.40
ADHD symptoms	0.05	0.05	0.08	0.90	0.37
Social class	0.16	0.16	0.09	0.97	0.33
DRD4 7-repeat allele presence	-0.001	0.34	-0.001	-0.004	0.99
Smoking during pregnancy	0.20	0.33	0.04	0.36	0.72
DRD4xSmoking	0.92	0.53	0.25	1.76	0.08

In multivariate analysis including all the significant covariates previously found to be associated with total number of CD symptoms (gender, ADHD symptoms, verbal IQ and social class – see chapter 5 for details), the interaction with the DRD3 Ser9Gly variant disappeared (r^2 change for model with interaction term added = 0.006, F change = 1.66, df 1, p = 0.20). The interaction between DRD4 and maternal smoking continued to have a trend whereby those individuals with the 7-repeat allele whose mother smoked during pregnancy had more conduct disorder symptoms (r^2 change for model with interaction term added = 0.02, F change = 3.09, df 1, p = 0.08). For details of this analysis, see table 7.9.

b). Social class:

Table 7.10: Interaction analysis of all gene variants and social class predicting Conduct Disorder symptoms:

	n	Change statistics and significance when GxE added to main effects model		
		r ² change	t	p-value
DRD4 48bp VNTR	118	0.01	-1.32	0.19
DAT1 480bp VNTR	256	0.003	0.83	0.41
DRD5 (CA)n microsatellite	82	0.01	-1.09	0.28
DRD3 Ser9Gly variant	171	<0.001	0.20	0.84
MAOA 30bp promoter VNTR	151	<0.001	-0.19	0.85
COMT Val¹⁵⁸Met variant	229	0.06	-3.68	<0.001**

* = Significant at the p≤0.01 level

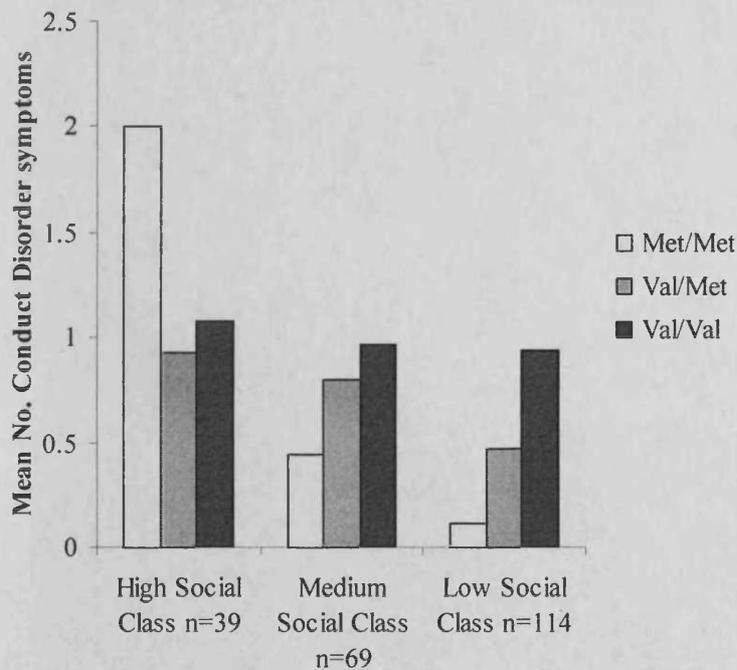
COMT Val¹⁵⁸Met significantly interacted with social class resulting in a significant change to the variance explained in the number of CD symptoms an individual had. When the covariates of gender, verbal IQ, total number of ADHD symptoms, birth weight and maternal smoking during pregnancy were added to the model, the interaction term continued to significantly increase the variance explained (r² change for block 2 = 0.07, F change = 19.41, df 1, p <0.001). These findings are summarised in table 7.11 whilst a graphical representation of the interaction can be found in figure 7.5. No other interaction terms significantly predicted total number of CD symptoms (see table 7.10 for details).

Table 7.11: Interaction analysis of COMT Val¹⁵⁸Met variant, social class and significant covariates predicting CD symptoms:

	Change statistics			
	r ² change	F change	df	P-value
Model with main effects of G, main effects of E and covariates	0.11	4.34	6	<0.001
Model with GxE added	0.07	19.41	1	<0.001

Model with main effects, covariates and GxE added	Unstandardised coefficients		Standardised coefficients	t	p-value
	Beta	S.E.	Beta		
Constant	0.10	0.97	-	-0.10	0.92
Gender	-0.75	0.28	-0.17	-2.68	0.008
Verbal IQ	-0.02	0.007	-0.14	-2.13	0.04
ADHD symptoms	0.04	0.04	0.07	1.04	0.30
Smoking during pregnancy	0.54	0.18	0.19	3.02	0.003
Birth weight	0.00002	<0.001	0.04	0.56	0.57
Social class	1.45	0.31	0.81	4.64	<0.001
COMT genotype	2.09	0.45	0.98	4.67	<0.001
COMTxSocial class	-1.31	0.30	-1.19	-4.41	<0.001

Figure 7.5: Interaction between COMT Val¹⁵⁸Met genotype and social class predicting total number of conduct disorder symptoms:



7.5 Discussion:

1). Main effects of GxE on diagnosis of ADHD:

There was no evidence of significant GxE for any of the genetic variants tested together with maternal smoking during pregnancy, birth weight or social class. A trend towards a significant interaction between the MAOA 30bp promoter variant activity and social class ($\chi^2=5.74$, df 2, $p=0.06$) was observed. The results showed a significant difference between those individuals from high and low social class families (OR=5.25, df 1, $p=0.02$) with the high activity alleles having a greater risk effect in those from low social class families. Previous association findings for this variant have differed across studies with one (Manor et al., 2002) finding significant overtransmission of the high activity alleles (as implicated in this analysis) whilst other studies including our own (Domschke et al., 2005; Lawson et al., 2003) finding no such evidence. It is possible that such inconsistencies are due to heterogeneity across samples, partly indexed by social class. However as the interaction finding presented here is only a trend, caution is warranted, and in light of the number of tests performed, it is likely that this is a chance finding. It is necessary to reassert that this analysis cannot test for main effects of the environmental variables.

The possibility that the negative results and the trend towards significance for the MAOA variant and social class are false negative and positive findings must be considered. First, multiple statistical tests were performed, increasing the chance of type one errors. Second, statistical power must also be considered an issue for all these analyses; the number of cases for each analysis were modest, especially considering that larger samples may be required to detect GxE (Chronbach, 1991). The sample size was further reduced because only parent-proband trios could be

utilised in this analysis. This sample does contain mother-proband duos (with or without unaffected siblings) who are excluded. This may be a significant limitation of this analytical method as ADHD probands were different from trios in that they show increased numbers of conduct disorder symptoms (West et al., 2002). The issue of missing fathers and the associated loss of power is not problematic in the analysis of the MAOA 30bp promoter variant. Because the MAOA gene is located on the X chromosome, for male offspring, only mothers provide genetic information.

Therefore, for male probands, all pseudocontrols can be determined from maternal genotype alone. As the vast majority of this sample is male (90%) and where parental genotypes were missing, it was almost exclusively from fathers (for 98%), very little information was lost for the analyses on the MAOA 30bp promoter variant. The use of a case-control sample may increase power to detect effects, however, it may introduce bias through population stratification which is not an issue for this sample.

Overall, this work represents preliminary and exploratory investigation into possible GxE influencing ADHD. A possible interaction between MAOA 30bp promoter variant activity and social class was revealed. Further investigation, preferably with larger samples to increase statistical power, is necessary both in an attempt to replicate this finding and also to allow further testing for GxE effects between other the variants and environmental variables.

2). Modifying GxE effects on the ADHD phenotype:

a). Failure to replicate Kahn and colleagues (2003) findings

Counter to the findings of Kahn and colleagues (2003), no evidence for significant interaction between DAT1 genotype and maternal smoking during pregnancy was revealed for either hyperactive-impulsive symptoms ($\beta=0.02$, $p=0.94$) or ODD symptoms ($\beta=-0.54$, $p=0.21$), despite a comparable sample size. Analysis in this thesis revealed main effects of maternal smoking during pregnancy on both outcome measures which were not found by Kahn and colleagues (2003) (see chapter 5 for details).

This is clearly not a direct replication of the Kahn and colleagues (2003) study, which may explain the differences in results. Kahn and colleagues (2003) utilised a community sample of children with ADHD scores across the normal range. This sample consisted of clinically referred children with diagnosed ADHD who, by definition, had high numbers of ADHD symptoms and ODD symptoms. Thus it may be that interaction between DAT1 genotype and maternal smoking during pregnancy is relevant only to those with ADHD scores within the normal range and not the clinical population. Similarly, as mentioned previously, the range of ADHD symptom scores is truncated in a clinical sample and this lack of variation will have reduced power to detect any interaction. This may not be relevant for the analysis of ODD symptoms. The possibility also remains that the significant findings of Kahn and colleagues (2003) may be artefactual; the sample utilised was small and there are a number of methodological limitations, whilst there are no published replications of the findings. Although this analysis failed to successfully replicate the findings of Kahn and colleagues (2003), it merely highlights that further replication studies are

necessary, preferably using more similar samples to that of the original study, before conclusions can be drawn.

b). Other modifying GxE effects:

When covariates were included in the models, only three interaction terms remained significant. For the total number of hyperactive-impulsive symptoms, significant main effects of both the COMT Val¹⁵⁸Met genotype ($t=2.93$, $p=0.004$) and social class ($t=2.61$, $p=0.01$) were revealed (as described in chapter 5). In addition, a significant GxE effect was found between these two variables ($t= -2.34$, $p=0.02$). The interaction term additionally explains 2% of the variance in total number of hyperactive-impulsive symptoms when both main effects of COMT Val¹⁵⁸Met genotype and social class, as well as all significant covariates, are taken into account. Main genetic and environmental effects appear to be working in the expected direction, with the Val allele and lower social class conferring risk. However, surprisingly, the interaction term indicates that genotype is not significantly associated with number of hyperactive-impulsive symptoms in those from low social class families. This is not what would be expected, especially as those with the heterozygous Val/Met genotype have more symptoms than those with the Val/Val genotype. This possibly indicates that this is a chance finding. Thus caution is warranted in interpretation. These results can be seen in figure 7.3. This interaction may illustrate heterogeneity for hyperactive-impulsive symptoms, possibly implicating different pathways of risk dependent upon environmental circumstances.

Secondly, an interaction between the DRD3 Ser9Gly variant and social class was observed to influence total number of ADHD symptoms. A significant main effects of

social class ($t=2.11$, $p=0.04$) and a trend towards significance for main effects of the DRD3 Ser9Gly variant ($t=1.77$, $p=0.08$) were seen. The interaction suggests that where environmental risk is present by being of low social class, the DRD3 Ser9Gly variant has less of an influence (see figure 7.4). As no significant published findings have been reported for this variant previously, further investigation of this finding is warranted.

Interaction between COMT Val¹⁵⁸Met genotype and social class was also observed for the total number of conduct disorder symptoms, explaining 7% of the variance in this phenotypic measure. This finding was not explained by higher numbers of hyperactive-impulsive symptoms correlating with conduct disorder symptoms. As can be observed in figure 7.4, the effect of genotype appears to be much more prominent in the individuals from high social class families than for other social class families. Indeed, the main effect of the COMT Val¹⁵⁸Met genotype on conduct disorder symptoms ($t=4.67$, $p < 0.001$) appears to be observable only in the high social class group. Using ANOVA methods employed by Caspi and colleagues (Caspi et al., 2005; 2003; 2002), the effect of COMT Val¹⁵⁸Met genotype on conduct disorder symptoms for each of the social class groups was examined separately. A highly significant effect was observed within the high social class group ($F=5.78$, $p=0.007$), whilst COMT Val¹⁵⁸Met genotype did not significantly influence conduct disorder symptoms for either the medium ($F=0.55$, $p=0.58$) or low social class groups ($F=0.08$, $p=0.92$). This finding is extremely interesting, perhaps illustrating that where environmental risk is low (as for individuals from high social class families) other factors, such as genetic risk, may exert more of an influence. It is also interesting that the findings remain significant when birth weight, previously found to interact with

COMT Val¹⁵⁸Met genotype in this sample (Thapar et al., in press) is included as a covariate. This may indicate that there are a number of distal environmental risk factors that have main and interaction effects on conduct disorder symptoms in ADHD. Interestingly, a main effect of COMT Val¹⁵⁸Met genotype was found in this analysis, but not in the previous TRANSMIT analysis (See Chapter 3 for details). This is slightly surprising, especially as the sample has sufficient power to detect association with this variant using TRANSMIT analysis (see Table 3.7). The dissimilarity in findings is likely to be because the analyses are performed in quite different ways; TRANSMIT analysis investigates preferential transmission of a risk allele from parents to offspring, whilst the regression analysis undertaken in the investigation of GxE looks at possession of the risk genotype in the offspring, regardless of availability of parental genotypes or transmission of alleles.

Finally, bivariate correlations between the genetic variants and environmental factors for which interactions were found were not significant. This finding makes the possibility that these findings are due to rGE less likely. Overall, these interaction findings require replication in independent samples, but are intriguing.

3). Issues to be considered:

The above exploratory analyses testing for GxE, both as main effects on diagnosis and also as modifying effects on the ADHD phenotype, have revealed some interesting findings. Clearly these initial findings (both those indicating significant interactions and those which do not) should be considered preliminary, hypothesis generating given the possibility of both type one and type two errors and require independent replication. Identifying plausible mechanisms through which such interactions may

work is an important factor when looking for GxE effects (Moffitt et al., 2005), although Rutter and Pickles (1991) have commented that understanding of the exact pathways through which GxE effects work, may negate interest in the interactions themselves. As has been discussed previously (see chapter 6 for details) direct, causal pathways between environmental risk factors and ADHD have not yet been successfully demonstrated. Social class is undeniably a distal risk indicator for other mechanisms. This is not to dismiss the utility of social class as a risk indicator, or to diminish the GxE findings here; finding a distal indicator of risk may have public health implications, indicating at risk groups who need to be more closely monitored, whilst also informing future studies specifically aimed at identifying proximal environmental risk factors, as proximal risk factors will be significantly correlated with social class. Moreover, given the need for large sample sizes and replication, there is a strong argument that in the first stage of testing for GxE, the environmental measures should be readily and widely available and easily measured. However, it is necessary to note that significant GxE findings could be due to unmeasured, or unsuccessfully measured confounding variables such as family conflict or parental ADHD, although even these environmental measures can be problematic as they are likely to index genetic as well as environmental influence on ADHD.

The issue of insufficient power to detect GxE may be extremely relevant to these analyses. Power to detect GxE is less than that for detecting main effects (Chronbach, 1991), a problem exacerbated by the fact only small effects for each risk factor are likely in complex, psychiatric disorders such as ADHD (Rutter et al., 2001). Therefore, large samples and accurate measurement of both environmental and genetic risk factors, as well as the outcome measure, is essential. Accuracy of

measurement is improved here by the use of a standardised and widely reliable psychiatric interview (the CAPA) and the use of multiple informants to obtain diagnosis. Environmental variables are difficult to measure but maternal reports of birth weight and maternal smoking during pregnancy have been shown to be accurate, showing strong agreement with birth records. Moreover, the use of measured genotypes reduces correlated measurement errors and possible rGE, which can arise from individuals' knowledge of both genetic and environmental variables (as with family relatedness using in twin designs).

Given the need for larger sample sizes to detect GxE the fact that GxE effects are undetected, does not mean that they are not there (Rutter & Pickles, 1991). As asserted for the failed replication of Kahn and colleagues (2003) DAT1 and maternal smoking during pregnancy finding, further investigation is required before any possible interactions can be rejected. Furthermore, molecular genetic association studies of other genes and additional variants within these genes studied here are also required before any possible interactions can be dismissed.

In this analysis, independently significant covariates were added to the analysis of GxE. Although this acknowledges the high levels of correlation between environmental and phenotypic variables in an attempt to find independent interactions, this is a conservative approach and may be overly stringent. Researchers have argued that risk factors do not act independently and therefore should not be analysed as so (Rutter et al., 1997). Previous studies (e.g. Caspi et al., 2005, 2003, 2002) have either not considered covariates or have added them individually to the

model. The stringent approach undertaken in this thesis may be relaxed in future analysis.

To date, most studies of GxE in psychiatry (e.g. Kendler et al., 2005; Caspi et al., 2003) have been conducted on population based samples. From these exploratory analyses, it appears that investigation of GxE in ADHD using a clinical sample is feasible. Pseudocontrol conditional logistic regression analyses was successfully employed for this sample, but may be an overly stringent test of GxE. The presence of a control sample would enable main environmental effects to be accounted for and would have greater power, but such analyses are open to potential population stratification effects. Regression methods for looking at possible modifying effects on the ADHD phenotype also appear to have been useful.

CHAPTER 8

Conclusion

8.1 Summary of findings:

The findings within this thesis can be divided in to a number of distinct sections;

1). Main effects of genetic variants on the diagnosis of ADHD:

- A trend towards significant association was found for transmission of the 148bp putative risk allele of the DRD5 (CA)_n microsatellite variant ($\chi^2=2.83$, df, 1, $p=0.08$).
- No evidence for association with diagnosis of ADHD was found for any of the other variants investigated (DRD4 48bp VNTR, DAT1 480bp variant, DRD3 Ser9Gly variant, MAOA 30bp promoter variant and COMT Val¹⁵⁸Met variant).

2). Modifying effects of genetic variants on ADHD phenotype:

- A trend for association with lower number of hyperactive-impulsive symptoms was found for the DRD5 (CA)_n microsatellite variant (OR=0.71, 95% CI 0.48, 1.04, $p=0.07$).
- Low activity alleles of the MAOA 30bp promoter variant were significantly associated with conduct disorder symptoms (OR=1.53, 95% CI 1.00, 2.34, $p=0.05$). Trends for association with conduct disorder symptoms were found for the 7-repeat allele of the DRD4 48bp VNTR (OR=1.40 95%CI 0.95, 2.08 $p=0.09$), DAT1 480bp VNTR (OR=1.24, 95% CI 0.97, 1.59, $p=0.08$), and the DRD5 (CA)_n Microsatellite variant 5 allele (OR=0.68, 95% CI 0.43, 1.07, $p=0.09$).
- The MAOA 30bp promoter variant was found to be significantly associated with oppositional defiant disorder symptoms (OR=1.34 95% CI 1.07, 1.68, $p=0.01$).

3). Modifying effects of environmental factors on ADHD phenotype:

- Three environmental factors were investigated; maternal smoking during pregnancy, birth weight and social class.
- Maternal smoking during pregnancy was significantly associated with hyperactive-impulsive and conduct disorder symptoms
- Social class was significantly associated with hyperactive-impulsive, conduct disorder and total number of ADHD symptoms.

4). GxE for the diagnosis of ADHD:

- There was a trend towards significant interaction between the MAOA 30bp promoter variant (high activity alleles) and social class influencing diagnosis of ADHD ($\chi^2=5.74$, df 2, $p=0.06$).
- No other effects of GxE on diagnosis of ADHD were found in this sample.

5). Modifying effects of GxE on ADHD phenotype:

- This analysis failed to replicate the interaction between DAT1 genotype and maternal smoking during pregnancy on hyperactive-impulsive or oppositional defiant disorder symptoms found by Kahn and colleagues (2003).
- Three significant GxE effects were found. Interaction between COMT Val¹⁵⁸Met genotype and social class influenced total number of hyperactive-impulsive symptoms (r^2 change=0.02, $t=-2.34$, $p=0.02$) and conduct disorder symptoms (r^2 change=0.07, $t=4.67$, $p<0.001$), whilst interaction between DRD3 Ser9Gly variant and social class influenced total number of ADHD symptoms (r^2 change=0.02, $t=4.55$, $p=0.03$).

- Results for the conduct disorder symptoms were consistent with the Val/Val genotype compared to other genotypes increasing risk of symptoms in higher social class individuals.

8.2 Methodological strengths and limitations:

A number of methodological limitations must be considered when interpreting these findings. Firstly, statistical power to detect the small effect sizes expected for complex disorders (Rutter et al., 2001) may not have been sufficient. The sample size, although favourably comparable to those of other published molecular genetic studies of ADHD (see chapter 3), is small relative to that assumed to be required for other complex disorders and may have been insufficient to detect interaction effects which require greater statistical power than main effects (Chronbach, 1991) (see chapter 7). Furthermore, sample size was dependent upon the individuals who had been genotyped for specific variants. For LRTDT analyses, knowledge regarding parental transmission of alleles was necessary (see chapter 3), whilst for conditional logistic regression analysis, all parental genotypes were utilised to create pseudocontrols (see chapter 7). Such family-based statistical analyses were chosen as they are stringent in avoiding the potential problem of population stratification. In future, however, it may be preferable to use less stringent, non family-based approaches to maximise available sample sizes, especially when testing for interactions.

Secondly, as this sample consisted of clinically referred individuals with a diagnosis of ADHD, any biases inherent in the use of such samples will apply here. As mentioned previously (see chapter 3), within clinical samples variation in ADHD

symptom scores is reduced in comparison to those in the general population, making modifying effects of both genetic and environmental risk factors harder to identify. Conversely, clinical cases do have higher rates of and greater variation in comorbid antisocial behaviour symptoms. Furthermore, these findings can be viewed as being clinically relevant as the sample is representative of ADHD individuals utilising local child and adolescent mental health services.

Thirdly, the case only sample meant that main effects of environmental variables could not be investigated (see chapter 5). Thus it is not known to what extent modifying effects of environmental variables could have been due to main effects on ADHD. However, the aim of this study was not to identify main environmental effects on ADHD. For this other designs are needed (Rutter 2005).

Fourthly, as with any study, these findings are dependent upon accurate measurement of genetic, environmental and outcome variables as well as accounting for effects of any confounding factors which may also be involved. Finally, a single variant from each gene was analysed. This is not a strategy generally employed in current molecular genetic research where the necessity to analyse multiple variants and their haplotypes is recognised. However a selection of variants previously shown or hypothesised to be associated with ADHD were selected.

A clear strength of this study is the rigorous and robust clinical assessment undertaken to identify participants, using independent reports from both parents and teachers to obtain diagnosis of ADHD. The merits of such accuracy have been discussed and certainly enabled the dissection of heterogeneity within the ADHD phenotype which

has been demonstrated as beneficial. Similarly, the phenotypic manifestations of the disorder and the environmental variables to be investigated were selected in a stringent and hypothesis driven manner.

This investigation is relatively novel in a number of ways; although becoming more popular, investigation into the modifying effects of gene variants on specific aspects of the ADHD phenotype are in their infancy. Moreover, it seems that there have been no other studies into modifying effects of environmental risk variables on the ADHD phenotype, whilst there are virtually no studies of GxE in ADHD.

8.3 Discussion of findings:

Regardless of methodological limitations, these analyses have highlighted a number of important findings which require replication. Genetic heterogeneity of ADHD certainly appears to be present, with evidence in particular of the importance of comorbid antisocial behaviours indexing heterogeneity. This has also been suggested by clinical and genetic studies. It appears essential that researchers take such heterogeneity into account in a hypothesis driven manner when conducting molecular genetic studies of ADHD. Although this is starting to be recognised (e.g. Lowe et al., 2004; Lawson et al., 2003), the analyses within this thesis have further highlighted this fact whilst hopefully providing some direction for future investigation.

There also appears to be some evidence of heterogeneity depending upon exposure to environmental risk indicators. This is something not previously explored as the literature review illustrates. Especially in research investigating molecular genetic influences on ADHD, the possible role of environmental variables has been neglected.

Information regarding exposure to environmental factors is not routinely assessed or reported in genetic studies, despite the findings of this analysis which indicate that differences between samples in terms of environmental exposure could be a further source of inconsistent findings between groups. The impact of environmental risk factors and indicators on ADHD clearly requires further investigation – both the main effects of such factors on the disorder and their modifying effects within the ADHD phenotype. Accurate and careful measurement and replication are essential. Molecular genetic studies need to take account of the impact of environmental factors, although information regarding which factors should be considered has to be obtained using other study methods. However, this thesis indicates that such failures should be remedied and discussion should address how this can be achieved.

Furthermore, it seems that interactions between genetic and environmental factors may also be important in the aetiology of ADHD, both having effects on the diagnosis itself and also modifying effects on the clinical manifestation of the disorder. Failure to account for GxE effects could again be a source of non-replication of findings. Study of such GxE effects has been demonstrated here as being both feasible and successful, despite methodological limitations. As little research in ADHD has addressed possible interaction effects, this is again an area requiring further investigation.

Overall, the findings in this thesis suggest there may be a variety of different risk pathways into ADHD and that affect its clinical manifestation. For example, where environmental risk is low (e.g. being from a high social class family) genetic factors may play a more important role. Interactions may also work in the opposite direction.

8.4 Future directions:

A proportion of this thesis presents exploratory research which, to my knowledge, has previously been undertaken rarely, if at all. Therefore, replications are necessary before any conclusions can be accepted or suitably rejected. In future work, the emphasis should be on well characterised samples on which genetic, environmental and outcome variables are accurately measured whilst collaboration is necessary to expand sample size. The collection of control groups would also be advantageous. Including measurement of more proximal, causal environmental variables would be beneficial and may further inform regarding the aetiological pathways to the disorder. It would also be interesting to investigate other specific aspects of the aetiology of ADHD. For example, moves are being made towards identifying endophenotypes or intermediate phenotypes for ADHD. These are quantitative, heritable traits which index an individual's liability to develop or manifest a disease. Endophenotypes are thought to be more directly associated with aetiological factors than symptom presentation or diagnosis (Castellanos and Tannock, 2002). Identification and study of such endophenotypes for ADHD may therefore provide further insight into the main and modifying aetiological effects of genetic and environmental risk factors and their interaction. It may be advantageous to utilise some of the multiple animal models of ADHD to study GxE effects, considering the increased ability to manipulate both genetic and environmental variables using such designs (Plomin & Hershberger, 1991). Finally, in addition to looking at GxE, it may be interesting to investigate gene x gene interactions (epistatic effects) and see how these may influence the aetiology of ADHD and its phenotypic manifestations.

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Appendix i).

Diagnostic requirements for DSM-IV Attention Deficit Hyperactivity Disorder:

Inattention symptoms

- fails to sustain attention in tasks or play activities
- often fails to follow through on instructions from others
- often avoids tasks that require sustained mental effort
- often easily distracted
- often loses things that are necessary for tasks or activities
- appears not to listen to what is being said to him/her
- fails to pay attention to details, or makes careless mistakes
- often forgetful in daily activities
- often has difficulty organising tasks and activities

Hyperactivity-impulsivity symptoms

- often fidgets with hands or squirms in seat
- difficulty remaining seated when required
- runs about or climbs on things excessively in situations when it is inappropriate
- exhibits a persistent pattern of motor activity (always on the go)
- often noisy in playing or difficulty engaging quietly in leisure activities
- difficulty waiting turn in games or group situations
- often blurts out answers before questions have been completed
- often interrupts or intrudes on others
- often talks excessively

Additional criteria:

- Symptoms must be present in two or more settings (e.g. school, home etc)
- There must be clear evidence of clinically significant impairment in social academic or occupational functioning
- Some symptoms causing impairment must have been present before 7 years of age

Combined Type (314.01):

- Six or more symptoms are required from each symptom section.
- All other criteria must be met.

Predominantly Inattentive Type (314.00)

- Six or more symptoms are required from the Inattention symptom section.
- All other criteria must be met.

Predominantly Hyperactive-Impulsive Type (314.01)

- Six or more symptoms are required from the Hyperactivity-Impulsivity symptom section.
- All other criteria must be met.

Diagnostic requirements for ICD-10 Hyperkinetic disorder:

Inattention symptoms

- fails to sustain attention in tasks or play activities
- often fails to follow through on instructions from others
- often avoids tasks that require sustained mental effort
- often easily distracted
- often loses things that are necessary for tasks or activities
- appears not to listen to what is being said to him/her
- fails to pay attention to details, or makes careless mistakes
- often forgetful in daily activities
- often has difficulty organising tasks and activities

Hyperactivity symptoms

- often fidgets with hands or squirms in seat
- difficulty remaining seated when required
- runs about or climbs on things excessively in situations when it is inappropriate
- exhibits a persistent pattern of motor activity (always on the go)
- often noisy in playing or difficulty engaging quietly in leisure activities

Impulsivity symptoms

- difficulty waiting turn in games or group situations
- often blurts out answers before questions have been completed
- often interrupts or intrudes on others
- often talks excessively

Additional criteria:

- Onset of the disorder is no later than the age of 7 years
- The criteria should be met for more than a single situation
- The symptoms cause clinically significant distress or impairment in social, academic or occupational functioning
- The disorder does not meet the criteria for pervasive developmental disorders, manic episode, depressive episode or anxiety disorders

Hyperkinetic disorder (F90.0):

- Six or more symptoms required from the Inattentive symptoms section
- Three or more symptoms required from the Hyperactivity symptoms section
- One or more symptom required from the Impulsivity symptoms section
- All other criteria must be met.
- The general criteria for conduct disorders are not met

Hyperkinetic conduct disorder (F90.1):

- The general criteria for both hyperkinetic disorder (above) and conduct disorders must be met

Appendix ii).

Diagnostic requirements for ICD-10 and DSM-IV Oppositional Defiant Disorder and Conduct Disorder:

There is a repetitive and persistent pattern of behaviour in which basic rights of others or major age-appropriate societal norms or rules are violated lasting at least 6 months during which some of the following symptoms have been present (for DSM-IV criterion, behaviour pattern, but not symptoms have to have been present for 12 months)

The individual:

1. Has unusually frequent or severe temper tantrums for his or her developmental age
2. Often argues with adults
3. Often actively refuses adults' requests or defies rule
4. Often, apparently deliberately, does things that annoy other people
5. Often blames others for his or her own mistakes or misbehaviour
6. Is often "touchy" or easily annoyed by others
7. Is often angry or resentful
8. Is often spiteful or vindictive
9. Often lies or breaks promises to obtain goods or favours or to avoid obligations
10. Frequently initiates physical fights (this does not include fights with siblings)
- 11. Has used a weapon that can cause serious physical harm to others (e.g. bat, brick, broken bottle, gun, knife)**
12. Often stays out after dark despite parental prohibition (beginning before 13 yrs of age)
- 13. Exhibits physical cruelty to other people (e.g. ties up, cuts or burns a victim)**
14. Exhibits physical cruelty to animals
15. Deliberately destroys the property of others (other than by fire-setting)
- 16. Deliberately sets fires with a risk or intention of causing serious damage**

17. Steals objects of non-trivial value without confronting the victim, either within the home or outside

18. Is frequently truant from school, beginning before 13 yrs of age

19. Has run away from parental home at least twice or has run away once for more than a single night

20. Commits crime involving confrontation with the victim

21. Forces another person into sexual activity (NB: This symptom was not assessed in this sample)

22. Frequently bullies others

23. Breaks into someone else's house, building or car.

- **Criteria in bold need only to have occurred once for the criterion to be fulfilled**

Conduct disorder (F91.0, 312.8)

a). The general criteria for conduct disorder must be met

b). Three or more of symptoms 9-23 must have been present for at least 6 months

c). Diagnostic criteria for dissocial personality disorder, manic episode, depressive episode or pervasive developmental disorders are not met

d). The disturbance in behaviour causes clinically significant impairment in social, academic or occupational functioning

e). It is recommended that the age of onset be specified whereby

- Childhood onset is defined as one or more symptom before the age of 10 years
- Adolescent onset is defined as no conduct problems before the age of 10 years

Oppositional Defiant Disorder (F91.3, 313.81)

a). Four or more symptoms listed must be present, with no more than two symptoms from items 9-23

b). The symptoms must be maladaptive and inconsistent with the developmental level

c). Diagnostic criteria for dissocial personality disorder, manic episode, depressive episode or pervasive developmental disorders are not met

d). The disturbance in behaviour causes clinically significant impairment in social, academic or occupational functioning

e). At least four symptoms must have been present for more than 6 months.