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Examination of DRD4, DRD5 and SNAP-25 as candidate genes in Attention Deficit Hyperactivity Disorder

Naomi Temple Lowe

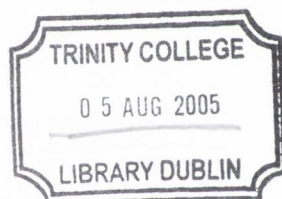


A thesis submitted to the University of Dublin for the degree of
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Declaration

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Summary

Attention deficit hyperactivity disorder (ADHD) is a common early onset childhood disorder. Symptoms of the disorder include inattentiveness, impulsiveness, distractibility and hyperactivity. Family, twin and adoption studies have estimated the heritability of ADHD to be 70-90% and it is believed that this genetic contribution to the disorder takes the form of multiple susceptibility genes of small individual effect. The dopaminergic neurotransmitter system has been implicated in the aetiology of ADHD by animal, pharmacological, neuroimaging and molecular studies and a number of susceptibility genes for the disorder have been identified. The aim of this thesis was to explore the molecular genetics of ADHD in relation to three dopaminergic related genes (DRD4, DRD5 and SNAP-25) using a sample of DSM IV diagnosed ADHD probands and their parents.

A mutation screening of the DRD5 gene was carried out using DHPLC, which resulted in the identification of six SNPs within the gene and promoter regions. Fine mapping association studies, coupled with TDT analyses, were executed on selected variants from each of the genes, with variants from the DRD4 and DRD5 genes displaying association with the Irish ADHD sample. Sub group analysis of the total DSM IV diagnosed sample was performed using TDT and logistic regression analyses. This resulted in the discovery of a number of sub phenotypes that displayed stronger association with the tested genetic variants than the total sample. In addition, a meta analysis was performed on a previously associated DRD5 microsatellite. The study definitively confirmed an association between the DRD5 gene and ADHD, and

suggested that the association was confined to the predominantly inattentive and combined subtypes.

The conclusions of the thesis are that DRD4 and DRD5 are true susceptibility genes for ADHD. In the case of the SNAP-25 gene the results are less compelling, with very little correlation between studies of this thesis and the literature in general. In addition, the thesis has highlighted the need to study more refined phenotypes and to increase collaboration between research groups.

This thesis is dedicated to my parents.

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Attribution of Work

The following work contained in this thesis is acknowledged as not being performed by the author.

1/ The genotyping of the 87610 and 87614 SNAP-25 SNPs for a subset of the sample used in this thesis, as outlined in Brophy et al, 2002. This work was performed by Dr Ziarh Hawi and Karen Brophy.

2/ The genotyping of the DRD4 VNTR for a subset of the sample used in this thesis, as outlined in Hawi et al, 2000. This work was performed by Dr Ziarh Hawi.

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Abbreviations

ADHD	Attention deficit hyperactivity disorder
AF	Attributable fraction
APS	Ammonium persulphate
bp	Base pair
CAPA	Child and Adolescent Psychiatric Assessment
CD	Conduct disorder
cDNA	Copy DNA
CI	Confidence interval
DAT1	Dopamine transporter
DBH	Dopamine beta hydroxylase
df	Degrees of freedom
DHPLC	Denaturing high performance liquid chromatography
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DRD1	Dopamine D1 receptor
DRD2	Dopamine D2 receptor
DRD3	Dopamine D3 receptor
DRD4	Dopamine D4 receptor
DRD5	Dopamine D5 receptor
DSM IV	Diagnostic and Statistical Manual, 4 th Edition
ETDT	Extended transmission disequilibrium test
F	Forward primer
FH+	Family history positive
FH-	Family history negative
FTP	File transfer programme
Grp	Group
HGMP	Human genome mapping project
HKD	Hyperkinetic disorder
H ₂ O	Water
LD	Linkage disequilibrium
LR	Logistic regression

M	Molar concentration
mA	MilliAmps
mg	Milligrams
ml	Millilitres
min	Minutes
n	Number
N	Number
No.	Number
ng	Nanograms
NT	Not transmitted
ODD	Oppositional Defiant Disorder
OR	Odds Ratio
PCR	Polymerase chain reaction
pmol	Picomoles
QTL	Quantitative trait loci
R	Reverse primer
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
RR	Relative risk
SAP	Shrimp Alkaline Phosphatase
SE	Standard Error
sec	Seconds
SNAP-25	Synaptosomal associated protein of 25 kiloDaltons
SNP	Single nucleotide polymorphism
SSCP	Single strand conformational polymorphism
T	Transmitted
TDT	Transmission disequilibrium test
TEAA	Triethylamine acetate
Temp	Temperature
U	Unit
UTR	Untranslated region
V	Volts
VNTR	Variable number tandem repeat

w/v	Weight per volume
α	Alpha
μCi	Microcuries
μl	Microlitres
χ^2	Chi Square
5-HT	Serotonin

Author Publications Relating to this Thesis

Bellgrove MA, Hawi Z, Lowe N, Kirley A, Robertson IH, Gill M (2005) DRD4 gene variants and sustained attention in attention deficit hyperactivity disorder (ADHD): Effects of associated alleles at the VNTR and -521 SNP. *Am J Med Genet* (in press)

Lowe N, Kirley A, Hawi Z, Sham P, Wickham H, Kratochvil CJ, Smith SD, et al. (2004) Joint analysis of the DRD5 marker concludes association with attention-deficit/hyperactivity disorder confined to the predominantly inattentive and combined subtypes. *Am J Hum Genet* 74:348-56

Lowe N, Kirley A, Mullins C, Fitzgerald M, Gill M, Hawi Z (2004) Multiple marker analysis at the promoter region of the DRD4 gene and ADHD: Evidence of linkage and association with the SNP -616. *Am J Med Genet* 131B:33-37

Kirley A, Lowe N, Mullins C, McCarron M, Daly G, Waldman I, Fitzgerald M, Gill M, Hawi Z (2004) Phenotype studies of the DRD4 gene polymorphisms in ADHD: Association with oppositional defiant disorder and positive family history. *Am J Med Genet* 131B:38-42

Hawi Z, Lowe N, Kirley A, Gruenhage F, Nothen M, Greenwood T, Kelsoe J, Fitzgerald M, Gill M (2003) Linkage disequilibrium mapping at DAT1, DRD5 and DBH narrows the search for ADHD susceptibility alleles at these loci. *Mol Psychiatry* 8:299-308

Kirley A, Lowe N, Hawi Z, Mullins C, Daly G, Waldman I, McCarron M, O'Donnell D, Fitzgerald M, Gill M (2003) Association of the 480 bp DAT1 allele with methylphenidate response in a sample of Irish children with ADHD. *Am J Med Genet* 121B:50-4

Hawi Z, Kirley A, Lowe N, Fitzgerald M, Gill M (2003) Recent genetic advances in ADHD and diagnostic and therapeutic prospects. *Expert Rev Neurotherapeutics* 3:453-464.



Chapter 1

Introduction

1.1 General Overview of Attention Deficit Hyperactivity Disorder

1.1.1 Clinical Features and Diagnosis of ADHD

Attention deficit hyperactivity disorder (ADHD) is an early onset childhood psychiatric disorder, the substantial recognition of which was relatively recent, when it appeared in the second edition of the DSM under the name of Hyperkinetic Reaction of Childhood. A brief overview of the history of ‘ADHD like disorders’, from first documentation as “Morbid Defect of Moral Control” to the present day, is provided in Table 1.1. ADHD manifests itself as a collection of symptoms of varying severity that fall broadly into the categories of inattentiveness and hyperactive/impulsive behaviour. Individual symptoms including distractibility, excessive fidgeting and lack of concentration are usually present in all areas of life including the home and school and may severely affect the life of the sufferer and their family.

Table 1.1 - A Brief Overview of the History of ADHD

Year	Event
1902	British doctor, Dr Still records cases of impulsiveness and deems it to be “Morbid Defect of Moral Control”
1922	Symptoms now associated with ADHD were documented and given a diagnosis of "Post-Encephalitic Behaviour Disorder."
1937	Dr. Charles Bradley introduced the use of stimulants to treat hyperactive children.
1956	Methylphenidate medication first introduced as a treatment for hyperactive children.
1960	Introduction of the name “Minimal Brain Dysfunction” to describe the hyperactive condition
1968	The disorder first enters the DSM (second edition) under the name “Hyperkinetic Reaction of Childhood”
1980	The disorder is renamed again for the DSM-III as Attention Deficit Disorder +/- Hyperactivity. ADHD and ADD were separate diagnosis.
1994	ADHD enters DSM-IV with the 3 subgroups used today of predominantly inattentive, predominantly hyperactive/impulsive and combined

To receive a diagnosis of ADHD under the DSM IV (American Psychiatric Association, 1994), an individual must exhibit six or more symptoms (from the nine listed) for one or both of the categories. Symptoms must be present in a minimum of two environments (eg home and school). Those who fulfil this criteria are then assigned a diagnosis of predominantly inattentive or predominantly hyperactive/impulsive ADHD depending on which category they satisfy. Alternatively if they score six or more from both sections they are diagnosed with combined ADHD. The complete DSM IV diagnostic criteria for ADHD are listed in Figure 1.1. The combined type is the most commonly diagnosed form, accounting

for 50-75% of all ADHD individuals, with the predominantly inattentive and predominantly hyperactive/impulsive subtypes averaging 20-30% and <15% respectively (Wilens et al, 2002).

Figure 1.1 DSM IV Criteria for ADHD (Derived from the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition)

DSM-IV Criteria for ADHD

I. Either A or B:

Six or more of the following symptoms of inattention have been present for at least 6 months to a point that is disruptive and inappropriate for developmental level:

A/ Inattention

- Often does not give close attention to details or makes careless mistakes in schoolwork, work, or other activities.
- Often has trouble keeping attention on tasks or play activities.
- Often does not seem to listen when spoken to directly.
- Often does not follow instructions and fails to finish schoolwork, chores, or duties in the workplace (not due to oppositional behaviour or failure to understand instructions).
- Often has trouble organizing activities.
- Often avoids, dislikes, or doesn't want to do things that take a lot of mental effort for a long period of time (such as schoolwork or homework).
- Often loses things needed for tasks and activities (e.g. toys, school assignments, pencils, books, or tools).
- Is often easily distracted.
- Is often forgetful in daily activities.

Six or more of the following symptoms of hyperactivity-impulsivity have been present for at least 6 months to an extent that is disruptive and inappropriate for developmental level:

B/ Hyperactivity

- Often fidgets with hands or feet or squirms in seat.
- Often gets up from seat when remaining in seat is expected.

- Often runs about or climbs when and where it is not appropriate (adolescents or adults may feel very restless).
- Often has trouble playing or enjoying leisure activities quietly.
- Is often "on the go" or often acts as if "driven by a motor".
- Often talks excessively.

Impulsivity

- Often blurts out answers before questions have been finished.
- Often has trouble waiting one's turn.
- Often interrupts or intrudes on others (e.g., butts into conversations or games).

Other Criteria for Diagnosis

Some symptoms that cause impairment were present before age 7 years.

Some impairment from the symptoms is present in two or more settings (e.g. at school/work and at home).

There must be clear evidence of significant impairment in social, school, or work functioning.

The symptoms do not happen only during the course of a Pervasive Developmental Disorder, Schizophrenia, or other Psychotic Disorder. The symptoms are not better accounted for by another mental disorder (e.g. Mood Disorder, Anxiety Disorder, Dissociative Disorder, or a Personality Disorder).

Based on these criteria, three types of ADHD are identified:

ADHD, *Combined Type*: if both criteria 1A and 1B are met for the past 6 months

ADHD, *Predominantly Inattentive Type*: if criterion 1A is met but criterion 1B is not met for the past six months

ADHD, *Predominantly Hyperactive-Impulsive Type*: if Criterion 1B is met but Criterion 1A is not met for the past six months.

American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision. Washington, DC, American Psychiatric Association, 2000.

In addition to the American DSM IV diagnosis of ADHD there is also a diagnostic classification under the International Statistical Classification of Diseases and

Related Health Problems (10th Revision), ICD-10, (World Health Organisation, 1992), known as Hyperkinetic Disorder (HKD). Although the most recent version of each of these instruments use the same 18 core symptoms for diagnosis of the disorder their operational criteria are slightly different. The main differences are as follows: 1) An ICD-10 diagnosis of HKD requires the presence of symptoms from all three sections of inattention, hyperactivity and impulsivity while the DSM IV diagnosis does not 2) ICD-10 diagnosis makes a special provision for a combined conduct category if a conduct disorder is also present which the DSM IV does not (Swanson et al, 1998a). The differences between the two approaches result in the ICD-10 diagnosis being a more stringent version of the DSM IV.

1.1.2 Category or Continuum

ADHD is usually thought of as a discrete category, however there is growing evidence to suggest that it may be one extreme of a continuum (Levy et al, 1997). Although ADHD is diagnosed categorically, measures of activity and attention are continuously distributed in the general population and studies have reported high correlation between quantitative measures of hyperactivity and attention, and the usual categorical ADHD diagnosis (Biederman et al, 1993; Chen et al, 1994; Maher et al, 1999). Gjone et al, (1996) applied a mathematical model to twin data to determine if the heritability of attention problems increased with their severity, implying that the clinical diagnosis of ADHD was not simply one end of the spectrum of a continuum but a distinct heritable disorder. They concluded that heritability did not change with increased severity of symptoms and therefore attention problems were a continuously distributed dimension of which the inattentive form of ADHD was an extreme.

1.1.3 Epidemiology of ADHD

The prevalence of diagnosed ADHD among children differs quite considerably across the world with rates varying from 1 to 10% (Swanson et al, 1998a; Faraone et al, 2003). There is a particularly noticeable difference between the prevalence rates quoted for North America and Europe with the Americans appearing to have a considerably higher prevalence of the disorder. While it is the case that using the more stringent ICD-10 the rate of diagnosis tends to be 1-2% while the DSM IV rate is 5-10% (Swanson et al, 1998a) there still appears to be considerable variation between Europe and America using the same diagnostic classification system (Faraone et al, 2003). Explanations for the apparent differences in the prevalence of the diagnosis of ADHD may be attributed to a number of factors. One of these factors may be differing diagnostic rating scales and instruments, used to gather clinical information, while a second is due to cultural differences, which may cause rater bias in two ways. Firstly if the raters themselves are culturally different then they may have a different understanding and interpretation of what constitutes the disorder and may use subtly different criteria to do this, despite using the same rating scales and diagnostic classification systems. Secondly the same individual may diagnose individuals from two distinct groups differently even if they present with the same conditions. This may be contributed to by variation in the parent or teacher interpretation of what constitutes “normal” behaviour for a child (Sonuga-Barke et al, 1993).

Children diagnosed with ADHD have been found to have very high rates of comorbidity with a range of other psychiatric diagnoses and/or learning disabilities, with 50-80% of ADHD diagnosed individuals also meeting diagnostic criteria for

other disorders (Tannock, 1998). The two most common co-existing conditions with ADHD are Oppositional Defiant Disorder (ODD) and Conduct Disorder (CD), which are reported to occur in 40-90% of cases (Jensen et al, 1997; Gilberg et al, 2004). ODD presents in about 50-60% of children with ADHD usually before the age of 9 or 10 and there is a commonly held view that ODD precedes CD (Gilberg et al, 2004). Conduct disorders are characterised according to ICD-10 criteria as demonstrating repetitive and persistent patterns of dissocial, aggressive or deviant conduct with major violations of age appropriate social expectations. Other conditions found comorbid with ADHD include Developmental Coordination Disorder (DCD), Anxiety Disorders, Depression, Bipolar Disorder (BP) and Autism Spectrum Disorders (ASD) (Gilberg et al, 2004). The importance of these comorbid disorders in relation to the phenotype of an ADHD individual is becoming more widely recognised. ADHD patients are now usually screened for comorbid disorders as part of the clinical work up. Divisions of study samples into ADHD with or without certain comorbid conditions is now more commonly observed in the literature (Holmes et al, 2002).

In addition to variation in rates of diagnosis and comorbid diagnoses, there is also a difference between the apparent prevalence of the disorder between males and females, with males being diagnosed three times more commonly than females, an observation which appears consistent across the world. Part of this sex difference may be due to referral bias related to disruptive symptoms, as boys tend to exhibit more hyperactive/impulsive symptoms than girls and greater comorbidity with ODD and CD (Biederman et al, 2002). The phenomenon may also be due to genetic and or environmental reasons that are sex specific.

1.1.4 Persistence of ADHD and Long Term Prognosis

Although ADHD is considered as a childhood onset disorder it frequently persists into adolescence and adulthood. Furthermore, the consequences of the childhood symptoms can have a severe effect on the adolescent and adult life of an ADHD individual even if the symptoms themselves diminish. Children diagnosed as predominantly inattentive tend to have difficulties in school and in completing homework that may result in long-term poor academic achievement. Those diagnosed with predominantly hyperactive/impulsive ADHD often do well at school but have difficulties at home and in situations of little structure unlike their inattentive counterparts (Wilens et al, 2002). The ADHD adolescent is at risk for school failure, emotional difficulties, poor peer relationships and trouble with the law, drugs and alcohol. (Hechtman et al 1986; Wilens et al, 2002; Barkley, 2002; Retz et al, 2004). The reported rates of persistence of ADHD into adulthood vary considerably across studies partially due to methodological differences. Studies report the persistence of the condition into adolescence in 50-75% of cases (Thorley et al, 1984; Klein et al, 1991) and into adulthood in 4-60% (Hechtman, 1983; Mannuzza et al, 1993). In general studies concur that persistence of ADHD into adolescence and adulthood includes symptoms of inattention, disorganisation, impulsivity, distractibility coupled with academic and occupational failure (Wilens et al, 2002).

1.1.5 Treatment of ADHD

Treatment of ADHD is predominantly with pharmacotherapy, with stimulant medication (eg Methylphenidate) being the most common form administered. Stimulant medication is effective in approximately 70% - 90% of cases (Amara and

Kuhar, 1993). In addition to the pharmacotherapeutic approach, parent and child education and counselling methods are used. Children are educated as to the nature of ADHD, how it is affecting their lives at the moment and how it may affect their future. Counselling methods include how to deal with this information, learning not to blame themselves, strategies for self-monitoring their attention, organizing school work, completing homework, dealing with parents and teachers, resolving conflict, and dealing with social situations. Parent focussed therapy aims to teach parents of ADHD children about the disorder and provide them with skills to help in their day-to-day management of affected children (Pierce and Reid, 2004).

1.1.6 ADHD and Environmental Factors

It is recognised that in addition to the role of genetics in the aetiology of ADHD (Chapter 1, Section 1.2.1), environmental factors also contribute to the development of the disorder. Proposed environmental factors include obstetric complications, lead exposure, parental substance abuse, maternal depression and chaotic family life. Complications in pregnancy, delivery and infancy, including antepartum haemorrhage and eclampsia, as well as chronic maternal smoking and illicit drug use have been reported to be associated with ADHD development (Milberger et al, 1997). Studies have shown that prenatal exposure to cocaine is a risk factor for sustained attention processing during the early childhood years (Bandstra et al, 2001), as is maternal smoking, with children of mothers who smoked during pregnancy displaying more signs of attention deficit and troublesome behaviour than non-cigarette exposed children (Batstra et al, 2003; Laucht et al, 2004). Furthermore, a study using Rutter's indices of adversity, which include severe mental discord, low social class, large family size, paternal criminality, maternal

mental illness and foster care placement, reported that the odds ratio for ADHD diagnosis increased according to the number of indices present (Biederman et al, 1995).

In addition to the traditional environmental studies referred to above, a new area of expertise is developing in the field of ADHD that examines the role of gene-environment interactions in relation to ADHD. These studies merge the two previously unique categories of environmental and genetic risk factors. Questions are asked pertaining to how an individual's genetic makeup may affect the likelihood of exposure to certain environmental factors, as well as how different genetic variants may contribute to a range of possible reactions to the same environmental factor. For example, a study into the effect of maternal prenatal smoking in conjunction with a DAT1 VNTR has shown that neither the presence of two copies of the high risk allele (480bp) or exposure to prenatal smoking alone display evidence of association with any of the examined traits. However, children homozygous for the 480bp allele that were also exposed to prenatal smoking did show an association with hyperactivity (Kahn et al, 2003).

1.2 Genetic Approaches To Understanding ADHD

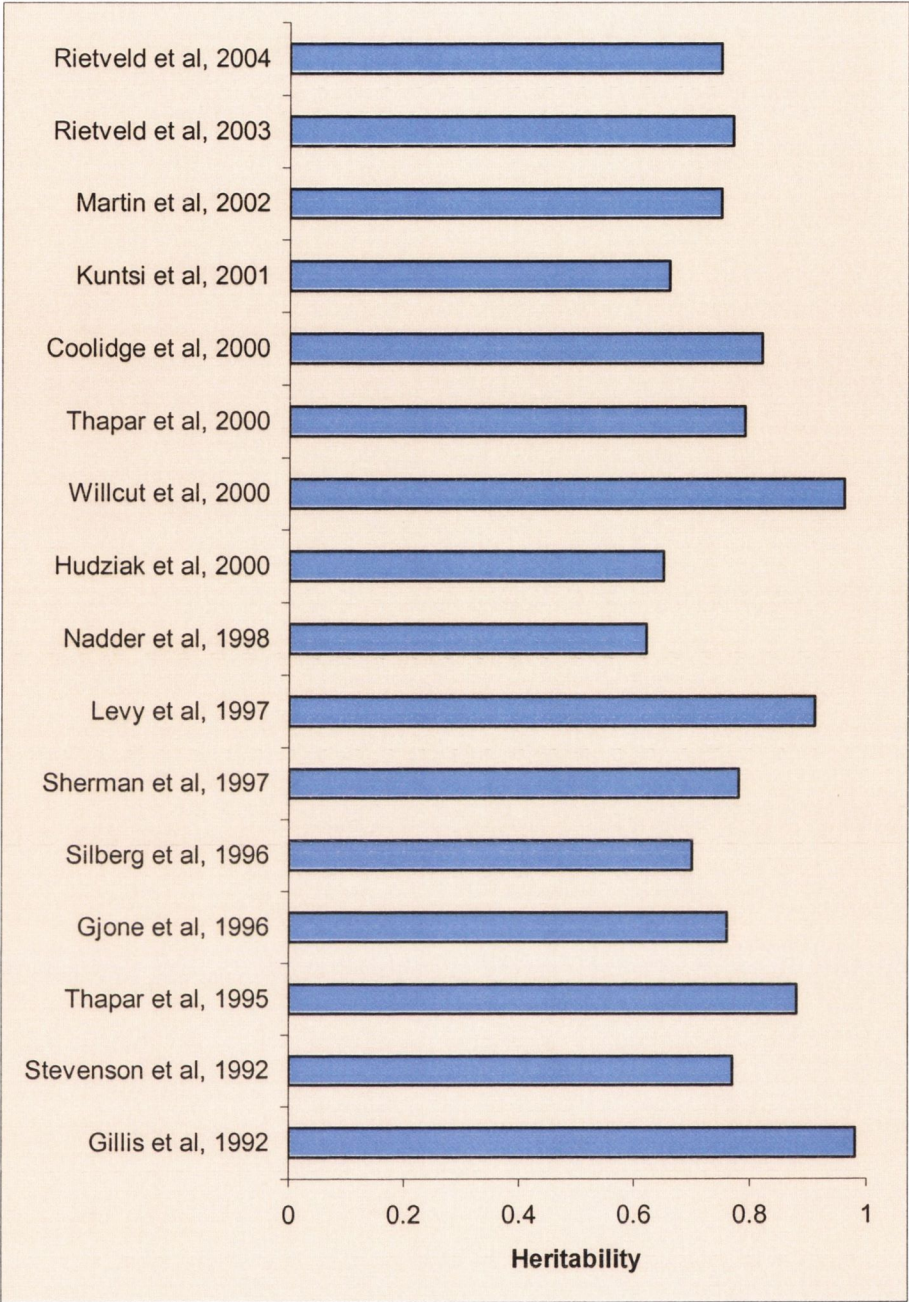
1.2.1 Family, Adoption and Twin Studies

While the exact aetiology of ADHD is still unknown it is readily accepted to have a significant genetic component as demonstrated by family, twin and adoption studies. Family studies, have demonstrated that there is an increased risk of ADHD in individuals related to an ADHD sufferer. Diagnostic concordance has been estimated at 32% in siblings of ADHD children and 15-20% and 25-30% in their mothers and fathers respectively (Biederman et al, 1992), with a decline in the diagnostic concordance of second degree relatives as might be expected with complex non Mendelian disorders (Faraone et al, 1994). Although the majority of studies to date have been conducted on Caucasian families there is evidence to suggest that the same pattern exists in other ethnicities (Samuel et al, 1999). Familiarity may be due to shared genes or shared environment and additional studies are required to separate out these factors.

There have been only a small number of adoptive studies in relation to ADHD to date. These studies have shown that adoptive relatives of ADHD individuals exposed to the same, shared environment are at a reduced risk of development of the disorder compared to biological relatives (Morrison and Stewart, 1973; Cantwell, 1975; Cadoret et al, 1991). A recent study reported that 6% of the adoptive parents of ADHD children had ADHD compared to 18% of the biological parents of ADHD sufferers (Sprich et al 2000).

Twin studies also help to separate familiarity into shared genes and shared environment (Thapar et al, 1999), and indeed, to quantify the variance due to each. All studies show higher concordance rates in monozygotic twins compared to dizygotic twins as would be expected under a genetic model. The data from twin studies is also used to measure the heritability (h^2), a measure of the degree a disease/disorder is affected by genetics, of the disorder, which in the case of ADHD is estimated to be 70-90% (see Figure 1.2), with the remaining 10-30% pertaining to environment factors.

Figure 1.2 - Heritability of ADHD as Calculated from Twin Studies



1.2.2 Genetic Linkage Studies

Linkage is the phenomenon whereby alleles at loci close together on the same chromosome will tend to be inherited together, due to the reduced likelihood of recombination occurring between them. This relationship between the likelihood of genetic recombination and the physical distance between two loci is the basis behind family linkage studies. Linkage studies examine the segregation of disease phenotype with variant genotype using either large family pedigrees or affected sib-pairs. This approach, when applied to Mendelian genetic disorders such as Huntington's disease has been very successful. However the situation becomes more complex for oligogenic or polygenetic disorders where the genetic contribution of the functional variants is much smaller and the disorder may only be partially due to shared genes. In these situations, linkage studies have less power to detect genes contributing to the disorder. This may explain why there is limited correlation between reported linkage regions in the three ADHD linkage studies reported to date (Bakker et al, 2003; Fisher et al, 2002; Ogdie et al, 2003).

1.2.3 Association Studies

For complex polygenetic disorders where the power of linkage studies may be compromised due to the relatively small contribution of each functional variant to the disorder, association studies of candidate genes may provide a more suitable alternative. This is because they possess greater power to detect contributing variants of small magnitude (but require DNA variants to be located much closer to the functional variant). Association studies are designed to determine if a particular allele of a particular polymorphism occurs more frequently in subjects with a specific phenotype of interest, (e.g. ADHD) than expected. In other words, is the

particular allele being examined associated with the phenotype? Mendel's second law (The Law of Independent Assortment) states that alleles of a given polymorphism will be inherited independently of each other, (ie randomly), resulting in a 1:2:1 ratio of AA, Aa and aa respectively. Association studies test for deviation from this expected pattern.

The original study design for testing association is the case control design. This method consists of a case sample for the given phenotype and a matched control sample. The control sample is matched as close as possible for age of onset, gender, ethnicity, and socio-economic background. The allelic frequencies of the cases and control samples are then statistically compared using a chi-square or similar analysis to determine if there is any statistical difference in allele frequency between the two groups. A statistical difference between the groups implies an association between the tested allele of the variant and the phenotype/disorder. However, if cases and controls are not suitably matched, population stratification may exist and could lead to false positives. In an attempt to avoid this potential problem, an alternative study design has been developed. This design, known as the family based or trio study design is based on families where the sample consists of an affected child and one or both biological parents. This approach tests for deviation from the expected transmission of alleles from parents to their affected offspring (i.e. the null hypothesis) and is the method used in this thesis. Both methods are used, depending on the population from which samples are drawn, the availability of matched controls, the age of onset of the disorder and consequently the availability of parents.

1.2.4 Linkage Disequilibrium Mapping and Haplotype Analysis

Linkage Disequilibrium (LD) is often more easily understood in terms of linkage equilibrium (LE). LE describes the situation in which the haplotype frequencies in a population have the same value that they would have if the alleles/variants at each locus were combined at random. LD is the opposite of this situation. In other words, LE occurs when $LD=0$. Linkage Disequilibrium can be defined as the non-random association between alleles at different loci. Complete LD (i.e. $LD=1$) means that the allele found at one of the loci exactly predicts the allele found at the other locus.

The phenomenon of LD is used in conjunction with genetic association studies to try and locate functional variants. This is achieved by examining the LD structure of polymorphisms surrounding the variant displaying association with a disorder. The theory behind the process is that if a variant associated with the phenotype/disorder is not itself the functional variant then it is likely to be in LD with the variant that is functional. Otherwise the non-functional variant would not display association with the phenotype.

Until relatively recently it was thought that LD across the genome was reasonably consistent. However it is now recognised that the genome consists of regions with high levels of LD interspersed with regions of high recombination and low LD known as recombination hotspots (Hey, 2004). This phenomenon results in the formation of what have become known as haplotype 'blocks'. Haplotype blocks refer to sites of closely located variants that are inherited together in blocks. Regions corresponding to blocks have a few common haplotypes, which account for a large proportion of chromosomes. Identification of haplotype blocks is a way of

examining the extent of LD in the genome, which may provide useful information for the planning of association studies (Gabriel et al, 2002). A multi centre project, known as the HapMap Project, is underway to map the genome for such blocks (www.hapmap.org). Awareness of this underlying haplotype structure may reduce the quantity of genotyping necessary over a region of interest, as one or a small number of variants might substantially represent the total genetic information contained within each block.

1.2.5 Mutation Screening

Screening genes for novel mutations is often a necessary precursory step to fine mapping and LD studies for genes with relatively little information available either in the literature or on the databases. Although the HGMP (www.hgmp.mrc.ac.uk) and the HapMap project (www.hapmap.org) are rapidly increasing available resources, there are still gaps in our knowledge, and with the HapMap aiming for an initial variant every 10kb, (to be followed with a 5kb map). There are still many smaller genes that will not be detected by this project at all.

Methods for screening genes include direct sequencing, which is highly effective but can be costly depending on the sample size to be screened. More often an initial procedure is utilised to determine regions with variation between individuals, which are then sequenced in the conventional manner. The most common procedure until relatively recently was single strand conformational polymorphism (SSCP) detection. To a large extent this has been superseded by denaturing high performance liquid chromatography (DHPLC). Both of these methods use the same chemical basis for the detection of heterozygote individuals. If the cooling of a

heterozygous sample from a denatured state is performed under controlled conditions it will result in the formation of homoduplexes (perfectly matching DNA sequences) and heteroduplexes (DNA fragments with a mismatch in the sequence resulting in a weakness in the double helix structure). The detection of the presence of heteroduplexes and homoduplexes in a sample, indicating the presence of a polymorphism, is determined by gel analysis in the case of SSCP or by chromatographic techniques in the case of DHPLC (further details of the methodology of DHPLC can be found in section 2.2.9).

1.3 Overview of Biological Pathways Implicated in ADHD

There are three main neurotransmitter systems implicated in the development of ADHD, to date. These are the serotonergic, dopaminergic, and noradrenergic systems. Of these the most extensively examined is the dopaminergic system, which includes the three genes studied in this thesis. All the systems are discussed in more detail below (Chapter 1, Section 1.3.1-1.3.3)

1.3.1 The Serotonergic System

Serotonin (5-hydroxytryptamine, 5-HT) is known to play a central role in many biological activities including the control of appetite, sleep, memory and learning, control of attention and locomotion, and muscle contraction (Nebigil et al, 2001). It exerts its effects through interaction with a variety of central nervous system targets in a complex pathway encompassing multiple receptors, enzymes and transporters including, the serotonin transporter (5-HTT), a member of a membrane transporter family that are Na^+ and Cl^- dependent and have 12 transmembrane domains, tryptophan hydroxylase (TPH), a rate limiting enzyme in the synthesis of serotonin and the monoamine oxidases (MAO-A and MAO-B) which catalyse the conversion of 5-HT to 5-hydroxyindoline acetic acid (5-HIAA) (Tang et al, 2001).

The demonstrated actions of serotonergic agents on animals with a compromised dopaminergic pathway suggest the close interaction of these two systems and implicate the serotonergic pathway in the aetiology of ADHD. The spontaneous locomotor activity exhibited by DAT1 deficient mice is attenuated by the administration of dopamine (Gainetdinov et al, 1999). However, in addition to

dopamine administration, serotonergic agents such as Fluoxetine (a selective serotonin re-uptake inhibitor) produce a calming effect that is attributed to an extracellular increase in the concentration of serotonin. Furthermore, DAT1-KO mice treated with 5-hydroxytryptophan or the dietary 5-HT precursor (L-tryptophan), display reduced hyperlocomotion (Gainetdinov et al, 1999).

Key candidate genes involved in genetic association studies in this system are listed below:

- 1) The serotonin transporter (5-HTT) (Seeger et al, 2001a; Manor et al, 2001; Kent et al, 2002; Zoroglu et al, 2002). The blockage of this transporter has been shown to attenuate the activity of the DAT1 knock out mouse (DAT-KO) but have no effect on the wild type (Gainetdinov et al, 1999).
- 2) The 5-HT1B receptor (Hawi et al, 2002; Quist et al, 2003) which has been implicated by pharmacological studies, using the agonist RU24969, which suggest that activation of the receptor in mice leads to increased anxiety and locomotion in wildtype animals, which is not observed in 5-HT1B KO mice (Brunner et al, 1999).
- 3) The 5-HT2A receptor (Quist et al, 2000; Levitan et al, 2002; Zoroglu et al, 2003), whose antagonism has been shown to reduce dopamine-induced hyperactivity in mice (Quist et al, 2000; O'Neill et al, 1999).
- 4) The MAO A and B genes, which encode for enzymes that have important functions in the metabolism of neuroactive amines in the central nervous system and peripheral tissues (Shih et al, 1999) have been examined (Jiang et al, 2001).

1.3.2 The Noradrenergic System

Noradrenaline is a member of the catecholamine sub-group of biogenic amines, which is catalysed by DBH resulting in the formation of dopamine (Zimmerman, 1993). Noradrenergic projections are particularly dense in the frontal cortex and cingulate gyrus, regions of the brain which are known to be involved in mood stabilisation and sleep regulation, as well as attention and alertness (Aston-Jones et al, 1999). Animals and humans with lesion in the pre-frontal cortex show poor attention regulation, disorganised impulsive behaviour and hyperactivity. There is also evidence that increased NE in the pre-frontal cortex, in response to stress, leads to cognitive difficulties which are similar to the symptoms of ADHD (Arnsten, 1999). In addition pharmacological studies have demonstrated the clinical usefulness of noradrenaline inhibitors (such as desipramine, nortriptyline and atomoxetine) in the treatment of ADHD (Popper, 2000; Davids et al, 2002). The mode of action of these antidepressants is to block the reuptake of dopamine and noradrenaline, consequently increasing the concentration of monoamines in the extraneuronal space (Biederman and Spencer, 1999).

To date genetic studies of the noradrenergic system genes in ADHD are few. DNA variants in the gene coding for the DBH enzyme were reported to be associated with ADHD although the gene was probably first examined for its role in the dopaminergic pathway. In addition, genetic studies have been carried out on the noradrenaline transporter protein (NET) (Barr et al, 2002; McEvoy et al, 2002), the adrenergic alpha2A receptor (ADRA2A) (Barr et al, 2001a; Xu et al, 2001) and the adrenergic alpha2C receptor (ADRA2C) (Comings et al, 1999; Barr et al, 2001a) and no findings have been replicated.

1.3.3 The Dopaminergic System

The dopaminergic system is known to have a direct influence on areas such as endocrine function, motor control, reward and cognition (Vallone et al, 2000), via the dopamine transporter (DAT1) and five receptors (DRD1-DRD5) which belong to the family of seven transmembrane domain G proteins (Vallone et al, 2000; Wong et al, 2000). Dopamine receptors transduce extracellular signals, in the form of dopamine, into several intracellular responses including effects on adenylyl cyclase, intracellular Ca^{2+} level, and K^{+} conductance. Disruption to the dopamine system has been implicated in ADHD as well as several additional psychiatric and behavioural disorders including Parkinsons' disease (Vila and Przedborski, 2004), autism (Jones et al, 2004) and Tourettes Syndrome (Diaz-Anzaldúa et al, 2004).

In genetic studies of ADHD, the dopaminergic neurotransmitter system has been the most extensively examined. Evidence to support dopaminergic dysfunction in ADHD derives from two main research areas; the neuropharmacology of stimulant medication (Amara and Kumar, 1993; Biederman and Spencer, 1999) and the behaviour and biochemistry of animal models (Russell et al, 1995; Giros et al, 1996; Jaber et al, 1999). The potential dopaminergic candidate genes examined to date are listed below:

- 1) DAT1, which is known to be the principal target for methylphenidate and other psychostimulant medication, used to alleviate the symptoms of ADHD (Seeman and Madras, 1998; Volkow et al, 1998; Daly et al, 1999).

- 2) The dopamine receptors, in particular DRD4 and DRD5 (Faraone et al, 2001; Lowe et al, 2004b), which are responsible for the pre and postsynaptic actions of dopamine.
- 3) Dopamine beta Hydroxylase (DBH) (Daly et al, 1999; Hawi et al, 2003b) which is a major enzyme for dopamine metabolism and catalyses the conversion of dopamine into noradrenaline.
- 4) SNAP-25 (Barr et al, 2000; Brophy et al, 2002), which is involved in synaptic vesicle fusion and neurotransmitter release.
- 5) Catechol-O-Methyltransferase (COMT) (Kirley et al, 2002; Hawi et al, 2003a) also involved in the degradation of dopamine via the transfer of a methyl group to the compound.
- 6) Tyrosine Hydroxylase (TH) (Kirley et al, 2002; Hawi et al, 2003a) a rate-limiting enzyme involved in the conversion of phenylalanine to dopamine.

To date the three genes with the most evidence as susceptibility loci for ADHD are DAT1, DRD4 and DRD5.

1.4 The ADHD Molecular Genetics Story to Date: DRD4, DRD5 and SNAP-25

1.4.1 DRD4

The DRD4 gene codes for a G-protein coupled receptor that is a member of the D2-like dopamine receptor family. It is one of the most extensively explored genes to date, along with the DAT1, in relation to ADHD and various comorbid conditions, including novelty seeking. Multiple polymorphisms of varying frequencies have been reported, however the most extensively examined variant in the field of ADHD is a VNTR of 48bp repeated up to seven times in the third intracellular loop of the protein (Vallone et al, 2000). The 7 repeat allele of the VNTR reportedly mediates a blunted response to dopamine (Asghari et al, 1995) (Chapter 4, Section 4.2.1 for further details). Several groups have reported positive association between the 7 repeat allele and ADHD (LaHoste et al, 1996; Rowe et al, 1998; Smalley et al, 1998; Swanson et al, 1998b; Faraone et al, 1999; Muglia et al, 2000; Sunohara et al, 2000; Tahir et al, 2000; Roman et al, 2001). Further support for the role of DRD4 in ADHD comes from a study by Holmes et al (2002), who examined 67 cases that fulfilled diagnostic criteria for ADHD and displayed conduct disorder symptoms and reported association with the 7 repeat allele of the gene. In addition, Manor et al (2002) observed an association between the 4 repeat allele and ADHD in a family-based design on a continuous performance test (TOVA). However, many other studies have failed to replicate these positive findings (Castellanos et al, 1998; Eisenberg et al, 2000; Hawi et al, 2000; Kotler et al, 2000; Mill et al, 2001; Todd et al, 2001; Mill et al, 2002b, Lowe et al, 2004a). A recent meta-analysis of DRD4 conducted by Faraone et al (2001) resulted in overall support for the association of

the 7 repeat allele of the DRD4 gene with ADHD for both the family based design studies (n=14) (OR= 1.4, p=0.02) and the case control (n=8) (OR=1.9, p=0.00000008). It should be noted that the significance level and the odds ratio were higher for the case control association study suggesting that either this study had greater power to detect association or the possible presence of population stratification in the case control samples. A further meta-analysis by Maher et al, (2002) also reported overall significance of association between ADHD and the 7 repeat allele of the DRD4 gene (n=13, OR=1.41).

In addition to the VNTR, other reported variants within and flanking the DRD4 gene include a 120bp insertion/deletion approximately 1.2kb 5' to the gene (Seaman et al, 1999), a 21bp and 12bp insertion/deletion located in exon 1 of the gene (Catalano et al, 1993; Nothen et al, 1994) and a G repeat in the first intron of the gene (Petronis et al, 1994). Reported SNPs are located at positions -1217, -1123, -809, -786, -616, -603, -602, -521, -376, -291, -128, -11, +31, and +581 (Cichon et al, 1995; Mitsuyasu et al, 1999; Seaman et al, 1994; Arinami et al, 2000). However, the frequency of many of these variants is <10% making them unsuitable for association studies in the average sample. Of these variants, only a few have been examined in relation to ADHD. McCracken et al (2000) reported association between ADHD and the long allele of the 120bp insertion/deletion ($\chi^2=5.4$, p=0.02). This finding has not been replicated to date. A study by Todd et al (2001) reported no bias in the transmission of either allele of the 120bp insertion/deletion, while Barr et al (2001b) found a trend toward association with the short allele of the variant. In addition, the -521 and -616 SNPs have been examined in two studies. Barr et al (2001b) reported a non significant over transmission of the C allele of –

521 and the T allele of the -616 SNP to ADHD cases while the Mill et al, (2003) reported no association with either allele.

1.4.2 DRD5

The DRD5 gene is located on chromosome 4 where it has been mapped to band 4p16.1. Like DRD4 it is also a G-protein coupled receptor, however in contrast to DRD4, which is a member of the D2-like dopamine receptor family, the DRD5 gene is a member of the D1-like family (Chapter 4, Section 4.3.1). Work on this gene in relation to ADHD has not been as extensive as for DRD4 or DAT1, although initial association studies were positive. This is probably due to the difficulty in working with this gene at a molecular level. The difficulty arises because of two D5 pseudogenes, which are to be found on chromosomes 1 and 2, and have 98% homology to each other and 95% homology to the true DRD5 gene on chromosome 4 (Grandy et al, 1991).

The genetic research into DRD5 and ADHD has mostly focused on a (CA)_n microsatellite variant located approximately 18.5kb 5' to the gene. This variant has been examined by many groups (not all individually published) and is the variant used for the meta analysis presented in Chapter 6 (Lowe et al, 2004b). Our own group made the first report of association with the 148bp allele (allele 10) of this variant and ADHD in 1999 (Daly et al, 1999). The finding has been confirmed by Tahir et al (2000) in a group of Turkish ADHD children who were methylphenidate responders and a trend for increased transmission of the same allele was also observed in a Canadian ADHD sample (Barr et al, 2000b) and in a UK sample (Payton et al, 2001) before the meta analysis was proposed in May 2000. Since then

a number of other studies have been published including Bakker et al (2004), Manor et al (2004), Mill et al (2004b) and Kustanovich et al (2004). All of these results and the unpublished individual results are discussed further in Chapter 6. In addition to this variant, three microsatellites located 5' of the gene, D4S1582 and D4S2928 (Hawi et al, 2003b) and a (TC)_n repeat (Mill et al, 2004b) and one microsatellite located 3' of the gene, D4S615 (Mill et al, 2004b) have been examined. Of these, significant over transmission to ADHD cases was only observed for allele 4 of D4S1582 ($\chi^2=4.34, p=0.037$). In addition, a SNP located at position 1481 was examined and an association between the C allele and ADHD was reported ($\chi^2=6.76, p=0.0093$) (Hawi et al, 2003b).

1.4.3 SNAP-25

The SNAP-25 gene encodes for a protein that is essential for synaptic vesicle fusion and neurotransmitter release. This gene has been implicated in the aetiology of ADHD based on the *Coloboma* mutant (Cm) mouse strain (Wilson, 2000). The hyperactive phenotype has been attributed to the deletion of the SNAP-25 gene and has been shown to be rescued by the transgene encoding SNAP-25. It is thought that mutations within the gene may affect the functions of synaptic vesicle fusion and neurotransmitter release leading to disruption of the neurotransmitter system.

To date there have been relatively few association studies on this gene. Barr et al (2000a) examined two 3' untranslated region polymorphisms [MnII (T-G) and DdeI (T-C)] of the SNAP-25 gene and observed biased transmission of the T/C haplotype. Consistent with this, Kustanovich et al (2003) also observed distortion in the transmission of the T/C haplotype in ADHD cases which was statistically

significant only when paternal transmissions alone were evaluated. We, (Brophy et al, 2002), examined these variants and obtained evidence of association between the T allele of the DdeI polymorphism and ADHD ($\chi^2= 6.55$, $p = 0.01$). Mill et al (2002a) identified a novel microsatellite repeat in SNAP-25 located between the 5' UTR and the first coding exon. Case-control analyses showed an over-representation of allele 5 of this variant in ADHD cases compared to ethnically matched controls. In addition, a mutation screen of the SNAP-25 gene (Mill et al, 2004a) revealed a number of novel mutations and further evidence of association between ADHD and the A allele of the -2015 SNP, the G allele of the 80609 SNP and the T allele of the 87610 SNP with ADHD. Collectively these observations provide strong evidence that the SNAP-25 gene may be implicated in the susceptibility to ADHD.

1.5 Additional Genetic and Biological Approaches

Other genetic and biological approaches to better understanding ADHD are discussed in this section. Although they are not all directly related to the work in this thesis, they provide a more complete picture of the work being carried out on ADHD. In addition, such studies may direct genetic analyses by suggesting appropriate candidate genes or underlying novel pathophysiological mechanisms.

1.5.1 Animal studies

Several animal models of ADHD have been proposed. One of the most widely characterized is the spontaneous hyperactive rat (SHR), while other models include the DAT1 knock-out (DAT1 KO) mouse and the *Coloboma* mouse (Cm) (Davids et al, 2003). These models provide valuable information about the pathophysiology of ADHD and have aided in the formulation of biological hypotheses for the cause of the disorder.

The SHR mouse is an animal model that shows many ADHD-like symptoms. Biochemical and physiological evidence suggest that mesocortical, mesolimbic, and nigrostriatal dopaminergic neurons of the SHR mouse release less dopamine in response to electrical stimulation than other mice. In addition autoreceptor mediated inhibition of noradrenaline release appears to be impaired in the SHR mouse suggesting that noradrenergic function may be poorly regulated (Russell, 2002).

Genetically engineered mice that lack the DAT1 gene (DAT1 KO) display features such as hyperactivity, cognitive impairment and impaired attention that are

characteristic of ADHD (Giros et al, 1996). Dopamine uptake is significantly reduced indicating that other monoamine transporters do not fully compensate for the loss of DAT1 function. Methylphenidate has been shown to have a calming effect on the knockout animals, in addition to dopamine itself (Gainetdinov et al, 1999). However the calming effect of this psychostimulant is mediated through the enhancement of serotonergic transmission probably through the interaction of methylphenidate with the serotonin transporter (Gainetdinov et al, 2000). It should be noted that an unexplained paradox exists between the animal and pharmacological studies of this gene. Blockage of DAT1 by medication is known to reduce hyperactivity in both the mouse and human however, knocking out the gene completely, appears to have the opposite effect resulting in increased hyperactivity of the animal. This paradox is likely to be due to the fact that DAT1 blockage by medication is a transient action on a neurotransmitter system which normally includes the transporter, while in the DAT1 knock out mouse the gene and therefore its functions are absent from birth, resulting in compensatory actions from other neurotransmitter systems.

The Cm mouse (Chapter 1, Section 1.4.3) is a result of a chromosomal deletion (located to mouse chromosome 2) produced by neutron irradiation. This mouse model exists only in the heterozygous form as possessing two copies of the deletion is non-viable (Wilson, 2000; Davids et al, 2003). This mouse mutant exhibits a behavioural phenotype similar to that of ADHD, characterized by spontaneous motor hyperactivity, head-bobbing, and ocular dystrophy. The phenotype of this model has been shown to be the result of the deletion of the SNAP-25 gene, necessary for vesicle exocytosis of neurotransmitters including dopamine. In

addition, the hyperactivity of the animal has been shown to be ameliorated by low doses of the psychostimulant D-amphetamine and can be genetically rescued by a transgene encoding SNAP-25 located within the deletion (Davids et al, 2003).

1.5.2 Pharmacology

In the case of a medicinally treated disorder like ADHD, pharmacological studies assume two roles. The first of these is the classic role of pharmacology in the exploration of the aetiology of the disorder through the administration of agonists and antagonists to the biological systems hypothesised to play a role in susceptibility. The most commonly administered medications in the case of ADHD are methylphenidate and D-amphetamine, which are known to exert their actions through DAT1, implicating the dopaminergic system in the aetiology of the disorder. In addition to the role of dopamine, the administration of fluoxetine (a selective serotonin re-uptake inhibitor) has also been found to attenuate the hyperactivity phenotype of the DAT1-KO mouse. This action is mediated by increased extracellular concentration of serotonin (5-HT) due to the blockade of the serotonin transporter and has no obvious changes in the extracellular dopamine level (Gainetdinov et al, 1999). Furthermore, stimulant drugs (such as D-amphetamine), also used in the treatment of ADHD, are known to block the reuptake and promote the release of noradrenaline as well as dopamine into the presynaptic cleft (Solanto, 1998) implicating, in addition to the dopaminergic and serotonin systems, the dysregulation of the noradrenaline neurotransmission system in the aetiology of ADHD.

The second function of pharmacological studies in the case of ADHD is to further examine the effects of existing medication in an attempt to better understand its mode of action and/or to determine whether or not viable tests can be conducted to determine if a given patient is more suited to one form of medical treatment than another, possibly due to their genetic makeup (Tahir et al, 2000; Seeger et al, 2001b; Kirley et al, 2003). This type of pharmacogenetic study is relative new with most studies to date examining a VNTR located in the 3' UTR of the DAT1 gene. Reported results are conflicting, with Kirley et al (2003) reporting that ADHD individuals possessing the 10-repeat allele respond well to methylphenidate treatment while Winsberg and Comings (1999) and Cheon et al (2005) have reported that individuals homozygous for the 10-repeat allele show poor medication response.

1.5.3 Neuroimaging

Structural and functional imaging studies of individuals with ADHD are both costly and difficult for the patient and as such the number of cases are usually small and the studies themselves few. The methods used include computerised tomography (CT) and magnetic resonance imaging (MRI) in the case of structural imaging studies and positron-emission tomography (PET) and functional magnetic resonance imaging (fMRI) for the functional imaging studies. To date the most consistent findings have implemented abnormalities in the frontal cortex of the brain (Faraone and Biederman, 1998a). However, it should be noted that the relatively small data sets used in these imaging studies raises issues in relation to the representativeness and statistical power of these experiments (Faraone and Biederman, 1998).

1.5.4 Neuropsychological Function Studies

Cognitive function studies tend to compare test and control samples to each other for a range of measures although they can also be performed on individuals before and after medication exposure. Neuropsychological tests are used to assess areas such as focused attention, reaction time and difficulties in inhibitory control through a multitude of psychological tests including, for example, measures of response to a stop signal (Schachar and Logan, 1990), the continuous performance test (CPT) (Losier et al, 1996), aversion to delay tests (Sonuga-Barke et al, 1992; Solanto et al, 2001), and the Stoop Color-Word Naming test (Barkley et al, 1992). In addition, more recently, these tests are being coupled with the genetic findings of individuals in association analyses (Bellgrove et al, in press).

1.5.5 Combined/Meta Analyses

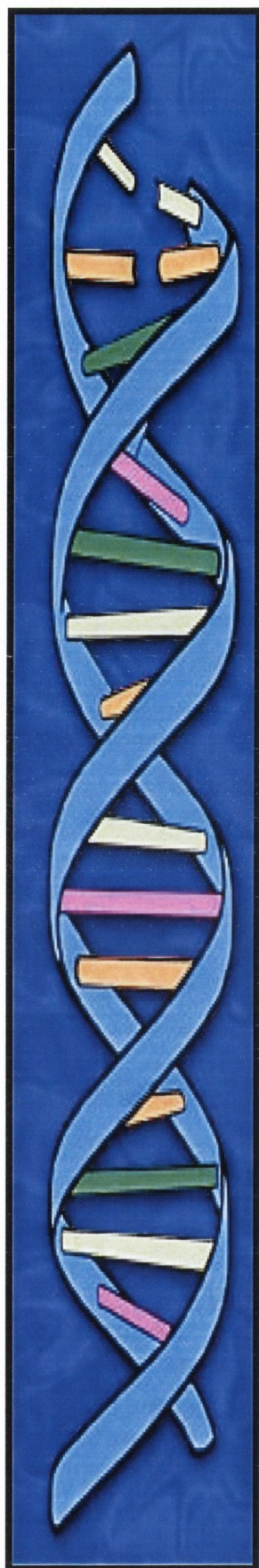
The collection of ADHD samples is both expensive and time consuming. It is therefore not surprising that the samples collected are usually of a much smaller size than other non-psychiatric medical conditions, where diagnostic methods are more straightforward. The issue of small sample sizes, in conjunction with the fact that ADHD is a polygenetic disorder reduces the power of ADHD studies to detect and replicate association findings between genetic variants and the disorder. In addition, these relatively small sample sizes are not particularly well suited to sub analysis of the broad ADHD phenotype. To date, a number of ADHD meta analyses have been reported in the literature (eg Faraone et al, 2001; Maher et al, 2002). This form of analyses involves the combining of multiple ADHD studies of a particular genetic variant to increase the sample size and therefore the power of the study. Usually these studies include homogeneity testing before individual samples are included in

the analyses, as well as selection bias and sensitivity testing. This form of analysis has been used to examine the role of the DRD5 microsatellite variant, DRD5.PCR1, in this thesis. Full details of the results and a more complete description of the study design are available in Chapter 6.

1.6 Aims of the Thesis

The aims of this thesis are outlined below:

- 1) To conduct a mutation screen of the DRD5 gene to examine the genetic variation within the promoter and coding sequence of the gene, with an aim to discovering novel variants for use in an association study.
- 2) To execute a fine mapping association study of the DRD4, DRD5 and SNAP-25 genes in the Irish population, coupled with LD and haplotype analysis.
- 3) To analyse the DRD4, DRD5 and SNAP-25 genotype data in relation to a number of subcategories, including sex of transmitting parent, family history of ADHD, medication response to methylphenidate and various comorbid diagnoses.
- 4) To perform a meta analysis on the 148bp allele of the microsatellite variant (DRD5.PCR1) of the DRD5 gene to definitively determine its hypothesised role in the aetiology of ADHD.
- 5) To review the results of the study in relation to the current literature, its strengths and weaknesses and to discuss potential future directions of the work.



Chapter 2

Materials and Methods

2.1 The Sample

2.1.1 The Clinical Sample

Ethics approval for the study was obtained from the South Western Area Health Board ethics committee. The families were recruited from ADHD support groups throughout Ireland (The Irish National Council of ADHD Support Groups) or were referred by Prof Michael Fitzgerald, Consultant Child and Adolescent Psychiatrist. The sample used for the studies in this thesis, consisted of 178 school aged children, diagnosed with ADHD according to DSM IV criteria, and either or both of their parents. DNA for both parents and the affected child was available for 171 of these families (trios), while the remaining 8 families were comprised of mother-child duos. Of the 178 probands, 22 (12.3%) were female and 157 (87.7%) male.

2.1.2 Clinical Diagnosis

To establish a DSM IV diagnosis of ADHD one or both parents of each child were interviewed using the Child and Adolescent Psychiatric Assessment (CAPA) (Angold et al, 1995), which also enabled assessment for common comorbid disorders including Oppositional Defiant Disorder (ODD) and Conduct Disorder (CD). Information about ADHD symptoms at school was obtained from the child's teacher, using the Teacher Telephone Interview (Holmes et al, unpublished data), to fulfil DSM IV criteria for symptom pervasiveness. The child's response to medication was ascertained using the Conners' Parent Rating Scale-Revised: Long Version (Conners et al, 1998) which retrospectively rates a child's response on and off medication as well as by a simpler three point rating scale retrospective parent questionnaire of "very good", "mediocre" or "no response". In addition, parents also

completed the Wender Utah Rating Scale (WURS) (Ward et al, 1993) on themselves to retrospectively determine the presence and extent of ADHD symptoms during their childhood. A positive family history of ADHD was defined by at least one parent scoring 36 or greater on the 25 item subscale of the WURS.

2.1.3 The Control Sample

In certain experiments a control sample was used. This sample consisted of random DNA's collected as blood samples from the Irish Blood Transfusion Service. The samples were not screened and the individuals underwent no questionnaire. No personal information including age or gender was obtained.

2.2 Biological Techniques

All relevant buffers and solutions are described in full in Appendix A.

2.2.1 DNA Extraction from Blood

Approximately 6ml of blood was collected from each individual in the study for DNA extraction, with an additional 6ml obtained where possible to be stored at –70°C as a backup sample. For those people unable or unwilling to provide a blood sample a cheek swab was obtained instead (Chapter 2, Section 2.2.2).

Six millilitres of whole blood was added to a 50ml falcon tube, with 6.5ml sterile water, and 12.5ml Lysis Buffer. The samples was placed on ice for 30 minutes with occasional inversion and then centrifuged at 3500 rpm for 15 minutes. The supernatant was poured off to a volume of 4ml and the sample tubes refilled to a volume of 25ml with Lysis Buffer. The samples was returned to the ice for 10 minutes and centrifuged as before. The total supernatant was then removed and the pellets resuspended in 2ml Suspension Buffer. The contents was then transferred to a 15ml falcon tube containing 150µl 10% (w/v) SDS and 60µl Proteinase K (10mg/ml) and incubated at 37°C overnight. An equal volume of buffer saturated phenol was added to the samples, which were thoroughly mixed, and centrifuged at 3800 rpm for 10 minutes. The aqueous layer was removed and extracted twice more using 2ml of a 1:1 phenol chloroform solution and pure chloroform respectively. Precipitation of the DNA was achieved by the addition of 50µl 3M Sodium Acetate (pH 5) and 6ml ice cold 100% ethanol. The DNA was removed to labelled eppendorfs using pasteur pipettes, where it was washed three times with 70%

ethanol. The pellets were then air dried and resuspended in 250µl TE Buffer before quantification.

2.2.2 DNA Extraction from Cheek Swabs

DNA was extracted from cheek swabs within two weeks of collection. Prior to extraction, samples were stored at 4°C. To extract the DNA, two brush heads were placed in an eppendorf containing 500µl of 50mM NaOH and vortexed for 60 seconds. The tubes were then heated to 95°C for 10 minutes before 50µl of 1M Tris (pH 8) was added and the samples vortexed for another 30 seconds. 200µl of Instagene Matrix (Biorad Labs, 2000 Alfred Nobel Dr., CA, USA) was added and the samples incubated for a further 30 minutes at 56°C. They were then vortexed for 10 seconds and then heated for an additional 8 minutes at 100°C. The brush heads were then removed from the tubes, which were centrifuged at 6000rpm for 3 minutes. The supernatant containing the DNA was then transferred to a labelled tube and stored at 4°C when not in use.

2.2.3 Quantification of DNA

The stock DNA solutions were analysed by spectrophotometry at appropriate dilutions to determine concentration of DNA (260 nm) and RNA (280 nm). The DNA concentration (µg/ml) was calculated using the formula:

$$[\text{DNA}] = \text{OD}_{260} \times 50 \times \text{Dilution Factor}$$

where OD_{260} is the absorbance of the sample at 260nm, and the dilution factor represents the dilution from stock of the test solution. The ratio of $\text{OD}_{260}:\text{OD}_{280}$ was used as a control for the presence of RNA. If this value exceeded 1.7 the sample

was not deemed to be of sufficient purity, and was re-extracted from the back-up blood vial.

2.2.4 Non Radiolabelled Polymerase Chain Reaction (PCR)

For non radiolabelled PCR, 60ng of DNA was added to a PCR master mix containing 2.5µl of (X10) MgCl₂ PCR Buffer, 4µl non radiolabelled dNTP mix (200 µM dATP, dCTP, dGTP, dTTP), 20 pM of F and R primers and 1U Taq Polymerase and made up to a final volume of 25µl. All PCR reactions were performed on a MJ Research DNA Engine and consisted of an initial denaturing step of 4 mins at 94°C typically followed by 30 cycles of 30 sec at 94°C, 30 sec at optimum annealing temperature and 30 sec at 72°C. This was followed in all cases by a final extension step of 10 mins at 72°C. Details of variations to these conditions, primer sequences and of optimum annealing temperatures are provided in the materials and methods sections of relevant Chapters and in Appendix B.

2.2.5 Radiolabelled PCR

This protocol is consistent with the non radiolabelled PCR except for the dNTP mix, which consisted of 250 µM dATP, dGTP, dTTP and 25µM dCTP and the addition of 0.05µCi of αP³² dCTP (P³² half life = ~ 14 days) (Goodier and Pritchard, 1996).

2.2.6 Nested PCR

Nested PCR involves the amplification of one PCR product from another, instead of from genomic DNA or cDNA, i.e. an initial PCR is performed using standard primers and the PCR product is then used as the template for a second PCR which uses a set of primers that bind to sequences within the boundaries of the first set of

primers. The procedure was performed by similar methods to the non-radiolabelled PCR except that the number of cycles of the first PCR was reduced to 10. From this first product 1µl was then used as the template for the second nested PCR, which was performed according to the standard non radiolabelled PCR.

2.2.7 Gel Electrophoresis and Visualisation

2.2.7.1 Agarose Gels

Non-radiolabelled PCRs were visualised on 2% (w/v) agarose gels containing 0.02mg/ml ethidium bromide. The gels were run in (X1) TAE buffer at 100V. The gels were then viewed on an ultra violet illuminator and photographed using a Polaroid camera. PCR samples were mixed with 1µl loading dye prior to being loaded on to the gel along with appropriate size standards.

2.2.7.2 Non Denaturing Polyacrylamide Gels

Non-denaturing polyacrylamide gels were used to analyse some of the RFLPs. The 6% (w/v) gel was run at 100V after samples mixed with 1µl loading dye had been loaded on to the gel. The gels were stained with 0.02mg/ml ethidium bromide before being visualised on an ultra violet illuminator and photographed using a Polaroid camera.

2.2.7.3 Denaturing Polyacrylamide Gels for Use with the ABI PRISM 377 DNA Sequencer

These gels were used to visualise fluorescently labelled PCR fragments in conjunction with the ABI PRISM 377 DNA Sequencer. A 6% (w/v) gel was used for genotyping microsatellites while a 12% (w/v) gel was used for SNPs. The denaturing polyacrylamide gel was preheated to optimum temperature on the

sequencer before the samples mixed with 1µl formamide loading dye were applied. The gel was then electrophoresised for sufficient time to allow the samples to run off the end of the gel where their fluorescent label was read by laser and recorded on to an attached computer.

2.2.7.4 Denaturing Polyacrylamide Gels for Radiolabelled PCR

These gels were used to visualise radiolabelled PCRs. After an initial 1 hour pre run of the 6% (w/v) denaturing polyacrylamide gel to allow the gel to reach optimum temperature, a 1:1 ratio of formamide loading dye and sample was loaded on to the gel which was run at a maximum constant current of 40mA and voltage of 2000V. When the samples had run far enough (determined by the size of the fragment and the required separation) the gel was removed from the rig and transferred to Whatman paper before being dried in a speed GelTMSG 200 drier at 80°C for 1 hour. The dried gel was then exposed to AGFA X-ray film and developed for visualisation on a white light box.

2.2.8 Genotyping

Two independent investigators called all genotypes and any inconsistent results were repeated. Such samples and those that failed to amplify were repeated three times before being recorded as unsuccessfully genotyped. All primers and variations to standard conditions can be found in Appendix B.

2.2.8.1 Restriction Fragment Length Polymorphisms (RFLPs)

Aliquots of PCR product were digested using the appropriate restriction enzyme and buffer supplied by New England Biolabs (New England Biolabs, Beverly, MA, USA). Details of the enzymes used and conditions of the digest can be found in

Appendix B. The appropriate enzymes in each case were either determined from the literature or obtained by electronically digesting the PCR product sequence using the web based restriction analysis program WEBCUTTER 2.0 (www.firstmarket.com/cutter/cut2.html).

2.2.8.2 SNaPshot

The SNaPshot method of primer extension (Applied Biosystems, Foster City, CA, USA) employs the principle of extending an unlabelled oligonucleotide primer in the presence of fluorescently labelled ddNTPs. Each of the 4 ddNTPs is tagged by a different fluorescent dye (Red=dTTP, blue=dGTP, green=dATP and black/yellow=dCTP). Hence, when the primer extension products are run on an ABI DNA Sequencer or ABI Genetic Analyser, the specific allele products can be differentiated from each other on the basis of which fluorescent dye they carry. The initial step of the SNaPshot primer extension reaction involves the treatment of the amplified PCR product with Shrimp Alkaline Phosphatase (SAP) (USB Corp., Cleveland, Ohio, USA) and Exo I restriction enzyme (New England Biolabs, Beverly, MA, USA) to remove the excess dNTP's and the forward and reverse primers respectively (1U SAP, 1U Exo I and 0.5µl SAP dilution buffer per 5 µl PCR product). Conditions for this treatment step are 37°C for 30 mins followed by 80°C for 15 mins. The 10µl primer extension reaction is then set up consisting of 1.5µl treated PCR product, 0.4µl extension primer [5pmol], 1µl SNaPshot buffer, 1.5µl SNaPshot Ready Reaction Mix and 5.6µl H₂O. The ABI Prism SNaPshot ddNTP Primer Extension Kit (Applied Biosystems, Foster City, CA, USA) provides the SNaPshot Ready Reaction Mix that contains AmpliTaq DNA polymerase and fluorescently labelled ddNTPs. The thermocycler conditions for the extension reaction consist of 50 cycles of 95°C for 5 sec, 43°C for 5 sec and 60°C for 5 sec

preceded by a 2 min denaturing step. The extension reaction then undergoes another treatment step, containing only SAP at the same conditions given above (0.5 units SAP and 0.5 µl SAP dilution buffer per 5µl DNA) before being analysed on an ABI 377 or 3100 DNA Sequencer.

2.2.8.3 Microsatellites

Microsatellite markers were either genotyped using the radiolabelled techniques described in sections 2.2.5 and 2.2.7.4 or using standard PCR methods described in section 2.2.4 with the addition of fluorescently labelled primers. The fluorescently tagged PCR product was then visualised using an ABI 377 DNA Sequencer (Chapter 2, Section 2.2.7.3) or an ABI 3100 DNA Sequencer.

2.2.8.4 Variable Number Tandem Repeats (VNTR)

VNTR polymorphisms were amplified according to the PCR conditions in section 2.2.4 and then loaded on non-denaturing polyacrylamide gels and electrophoresed according to section 2.2.7.2.

2.2.9 Denaturing High Performance Liquid Chromatography (DHPLC)

DHPLC is a highly sensitive and specific method for mutation detection. The process uses ion-pair reverse-phase high-performance liquid chromatography under partially denaturing conditions to differentially retain double stranded homoduplex (standard complimentary DNA double helices) and heteroduplex (double-stranded DNA molecules with non-complementary strands that originate from different duplex DNA molecules) molecules, which are formed when PCR products are

heated to 95°C for 5 mins and then cooled at a rate of 0.5°C per minute to a temperature of 40°C.

The principle behind DHPLC is that of ion pairing. For this study DHPLC was performed on a WAVE DNA Fragment Analysis System (Transgenomic Inc, Omaha, NE, USA) containing a DNASep column. The column is composed of a matrix of non-porous styrene-divinylbenzene beads, which are electrically neutral and hydrophobic. As double stranded DNA molecules are negatively charged they cannot adsorb to the column's matrix without a 'bridging' molecule or ion-pairing reagent. This molecule is known as triethylammonium acetate (TEAA), which consists of positively charged ammonium ions (which interact with the negatively charged DNA) and alkyl chains (which interact with the hydrophobic surface of the column matrix) and is present in the buffer passed through the column before the DNA is injected. The DNA molecules are then injected onto the column where they bind to the matrix via the already bound TEAA molecules.

The column is maintained at a temperature, selected by the DNA sequence, that results in the DNA being partially denatured (~ 70%). Due to the weakness in the heteroduplex molecules they will be more denatured than the homoduplex samples at this given temperature and as such will have a less negative charge and bind less tightly to the column. Therefore, when the DNA molecules are eluted from the column, by a change in the buffer gradient (decrease in TEAA concentration and increase in acetonitrile concentration), the less tightly bound heteroduplex molecules will elute sooner than the homoduplex molecules. The eluted molecules are detected using a UV detector set at 260 nm and the data is recorded

automatically in the form of chromatogram images, by the WAVEMAKER software (Transgenomic Inc, Omaha, NE, USA)

The temperatures used for the analysis of each amplicon were determined using the DNA sequence and WAVEMAKER software (Transgenomic Inc, Omaha, NE, USA). PCR products were eluted from the column using a linear acetonitrile gradient in a 0.1M triethylamine acetate buffer (TEAA), pH 7 at a constant flow rate of 0.9 ml/min. The gradient was achieved by the mixing of Buffer A (0.1 M TEAA (pH 7), 0.1 mM Na₄EDTA) and Buffer B (25% acetonitrile in 0.1M TEAA (pH 7)). The combining of buffers to create the gradient was done automatically once the column temperature was given to the software. At the end of each analytical run the column was washed with a solution of 50% acetonitrile in 0.1M TEAA (pH 7). Results were automatically recorded in the form of chromatogram images, by the WAVEMAKER software (Transgenomic Inc, Omaha, NE, USA), which were then visually analysed to determine difference between the traces indicative of the presence of a sequence variation. Individual samples with differing traces for the same amplified region were sequenced using both the forward and reverse primers of the original PCR to identify the sequence variation.

2.2.10 DNA Sequencing

Sequencing was either performed in house, by the methods described below, or sent out for commercial sequencing to MWG Biotech (MWG Biotech, Ebersberg, Germany), after the post PCR cleanup step.

2.2.10.1 Post PCR Cleanup

The post PCR cleanup step was performed to remove the original PCR primers and unlabelled dNTPs to ensure they did not interfere with the subsequent sequencing reaction. The cleanup process was performed using the QIAquick PCR Purification Kit (Qiagen Ltd, Crawley, West Sussex, UK).

2.2.10.2 Sequencing Reaction

Sequencing of the amplified DNA was performed using the ABI BigDye Terminator (v3.0) Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The process is similar in nature to that of a PCR except fluorescently labelled ddNTPs are present in the reaction mix that randomly terminate the amplification process and the mix contains proof reading polymerase. The reaction mix contains 8 µl Dye Terminator reaction mix, 3-8 µl PCR product (post cleanup) and 3.2 pmol primer. The mix was made up to a final volume of 20 µl with sterile H₂O. The cycling conditions for the sequencing reaction were 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 mins. All sequencing reactions were performed in the forward and reverse directions to confirm results.

2.2.10.3 Post Sequencing Reaction Cleanup

The post sequencing reaction cleanup was performed using the DyeEx 2.0 Spin Kit (Qiagen Ltd, Crawley, West Sussex, UK). This process removes the excess unincorporated fluorescently labelled ddNTPs, which would interfere with the analysis of the sequencing products.

2.2.10.4 Sequencing Analysis

Sequencing reactions were run on the ABI 3100 DNA Sequencer and the results were visualised and analysed using SeqScape (v.2) and Sequencing Analysis 5.1 (Applied Biosystems, Foster City, CA, USA).

2.3 Statistics

2.3.1 Hardy Weinberg Equilibrium

The sample was tested for Hardy Weinberg Equilibrium for all genotyped markers. The analyses were performed using only the parental genotypes. The Hardy Weinberg equation states that

$$p^2 + 2pq + q^2 = 1$$

where p=the frequency of allele A and q=the frequency of allele a. This equilibrium this can be tested for using the χ^2 statistic according to the equation

$$\chi^2 = (O-E)^2 / E$$

where O= the observed frequency of the allele and E=the expected frequency of the genotype. A significant χ^2 is indicative of genotypic error if the assumptions of the test are true. Hardy Weinberg Equilibrium assumes: 1) an infinite population size, 2) discrete generations, 3) random mating, 4) no selection, 5) no migration, 6) no mutation, 7) equal initial genotype frequencies in the two sexes.

2.3.2 The Transmission Disequilibrium Test (TDT)

The Transmission Disequilibrium Test (TDT) (Spielman et al, 1993) is a family based analysis that tests for the presence of linkage and association between a disorder/disease and a particular allele. The test uses the frequency of the transmitted and non transmitted alleles from the parents of the affected child as the raw data, instead of the more traditional presence or absence of the allele as in case control studies. This approach to the analysis is designed to avoid population

stratification effects. The TDT χ^2 statistic is calculated using the McNemar Equation of

$$\chi^2 = (b-c)^2 / (b+c)$$

Where b=the number of high risk alleles transmitted from heterozygous parents and c=the number of high risk alleles not transmitted from heterozygous parents. A corresponding p-value was calculated using SymtWin.exe software.

2.3.3 The Extended Transmission Disequilibrium Test (ETDT)

The Extended Transmission Disequilibrium Test (Sham and Curtis 1995) is a variation of the standard TDT, which allows for the analysis of multi allelic markers. Using logistic regression methods, it also tests for linkage and association between a disease/trait and a set of alleles. The ETDT package is available from the Human Genome Mapping Project website at www.hgmp.mrc.ac.uk/Menu/etdt.html. The structure of the input files required and details of how to use this program, are available in Appendix C.

2.3.4 Linkage Disequilibrium Analysis

Linkage disequilibrium between markers (measured as D') was calculated from the genotypic data of the parents only using the GOLD programme, available online at www.well.ox.ac.uk/asthma/GOLD. Details of how to use this program, and the structure of the input files required are shown in Appendix C.

2.3.5 Haplotype Analysis

Haplotype analysis was performed using the TRANSMIT software available for free from the HGMP website at www.hgmp.mrc.ac.uk/Menu/transmit.html. Specific

details of the necessary input files and operational commands are available in Appendix C.

2.3.6 Logistic Regression

Logistic regression analysis tests the prediction of a discrete outcome such as group membership from a set of continuous and or discrete variables for example age or gender (Tabachnick and Fidell, 2001). The logistic regression equation states that

$$\ln (p/1-p) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \dots \beta_x x_x$$

where x =independent variable, p =the probability of the presence of the characteristic of interest (eg genotype), $(p/1-p)$ =the odds ratio for the independent variable x and β =the regression coefficient. This method of analysis can be utilized for multiple purposes. It is used in this thesis to test for significant differences between two different statistical models (heterogeneity testing) and to examine several clinical variables as potential predictors of allele transmission. These variables include the sex of the affected child, the sex of the transmitting parent, the presence or absence of a family history of ADHD, a good or bad response to medication and the presence or absence of ODD and CD. All logistic regression analyses were executed using the Statistical Package for the Social Sciences (SPSS) (version 11.0).

2.3.6.1 Heterogeneity Testing

For this analysis, the Goodness-of-Fit of two different logistic regression models were compared to each other using the -2Log-likelihood values. The first model assumed heterogeneity (ie the regression coefficients for each variable were not

assumed to be equal to each other) according to the equation below and was weighted according to the transmissions and non transmissions of each variable.

$$\ln (p/1-p)= \beta_1x_1+ \beta_2x_2+..... \beta_{14}x_{14} \quad (\text{i.e. } \beta_1 \neq \beta_2 \neq \beta_3 \neq \beta_{14})$$

The second model assumed homogeneity (ie that all the variables had the same regression coefficient) with the logistic regression equation

$$\ln (p/1-p)= \beta_0+ \beta_x x_x \quad (\text{i.e. } \beta_1 = \beta_2 = \beta_3 = \beta_{14})$$

The two models were compared to each other in relation to how well the data fit the chosen statistical model using the -2Log-likelihood values and a χ^2 statistic was produced according to the formula

$$\chi^2 = (-2\text{Loglikelihood}_{\text{individual}}) - (-2\text{Loglikelihood}_{\text{combined}})$$

The degrees of freedom for the χ^2 statistic is calculated by the difference between the individual degrees of freedom for each model. A significant χ^2 value indicates a significant difference between the two models suggesting the presence of heterogeneity between samples. The structure of the input file used is provided in Appendix C.

2.3.6.2 Additional Variable Analyses

These analyses were designed to determine if a given variable was a predictor of the tested allele. Data files were set up according to Appendix C. Input files consisted of a column indicating transmission of the high risk allele (1) or non transmission of the high risk allele (0), a second column with the variable of interest (e.g. gender, medication response) also coded as ones and zeros and a third column indicating the number of transmissions or non transmissions of the allele in question. The analyses were weighted according to the transmissions and non transmissions before the programme was run in SPSS. The results obtained included a χ^2 statistic, p-value,

degrees of freedom and an odds ratio with confidence interval. A significant result indicated that the tested variable was a predictor of the transmission of the allele in question.

2.3.7 Testing for Selection Bias

Bias in the meta analysis was tested for using the funnel plot (Egger et al, 1997), which tests for publication and participation bias. The funnel plot is constructed by plotting the size of each sample against the odds ratio (OR) for the data. The theory behind the method is that the larger samples will have ORs closer to the combined OR, which, in the absence of heterogeneity between the samples (see 2.3.5.1), should also be the true OR of the combined sample. The smaller samples scatter themselves either side of the true OR value and when plotted they visually construct the shape of an inverted funnel. The presence of bias in the sample would result in a shape not resembling and the inverted funnel. For example if the study excluded all of the negative findings then one side of the funnel diagram would be missing.

In addition, to the funnel plot, the standard normal deviate of the OR against the precision of the OR was plotted according to Egger et al, (1997) using STATA 6.0 (Stata Ref Manual. 1999) (analyses kindly performed by Prof Steve Faraone, Harvard Medical School Department of Psychiatry at the Massachusetts General Hospital, USA). This analysis works on the same basis as the funnel plot, in that the precision of the OR increases with sample size. Egger showed that in the absence of bias the regression would run through the origin resulting in data points closer to the line. This method produces an R^2 (measure of the fit of the regression to the data) and a value for the intercept of the line, along with a standard error and 95% CI.

2.3.8 Sensitivity Testing

Sensitivity analysis was performed to ensure that no individual group's data were biasing the results. The method used was the straightforward removal of each sample in turn from the overall combined sample and a recalculation of the statistics in the absence of that group's data. Any significant deviation from the total joint analysis implied that the removed sample was biasing the overall result of the data.

It should be noted that none of the results in this thesis have been corrected for multiple testing and that all p-values presented are 2-tailed p-values.



Chapter 3

Mutation Detection Analysis of the DRD5 Gene and Promoter Region using Denaturing High Performance Liquid Chromatography (DHPLC)

3.1 Introduction

Searching disorders associated genes, for additional novel variants is a valuable method when trying to move from an initial association, to a functional variant. It may also be necessary to find additional variants for fine mapping if there is little information on your gene of interest in the databases or literature. The simplest method for the detection of novel mutations is sequencing of the DNA region, however, this method can prove expensive and time consuming depending on the size of the region and number of individuals to be screened. More commonly, especially in smaller laboratories not equipped for high throughput sequencing an initial screening method, such as SSCP or DHPLC is used to determine which specific regions need to be sequenced.

Although today the electronic databases contain extensive information about the DRD5 gene, due predominantly to the HGMP, when this work was begun several years ago the information was far less complete. Previous ADHD association work carried out on the gene at that time was primarily directed at the microsatellite DRD5.PCR1 located 18.5kb from the transcription start site (Daly et al, 1999), as the field was still focusing strongly on single variant associations rather than the multiple variant analyses including LD mapping and haplotype studies. Based on our original single variant association with this gene (Daly et al, 1999) it was decided to screen the DRD5 gene and promoter region for additional polymorphisms using DHPLC. Due to the position and nature of the microsatellite variant it was not believed likely that it was functional itself. Instead, it was hypothesised that the associated variant was in LD with a functional variant located in or closer to the gene, which may be discovered during the screening process.

3.2 Materials and Methods

3.2.1 Sample Selection

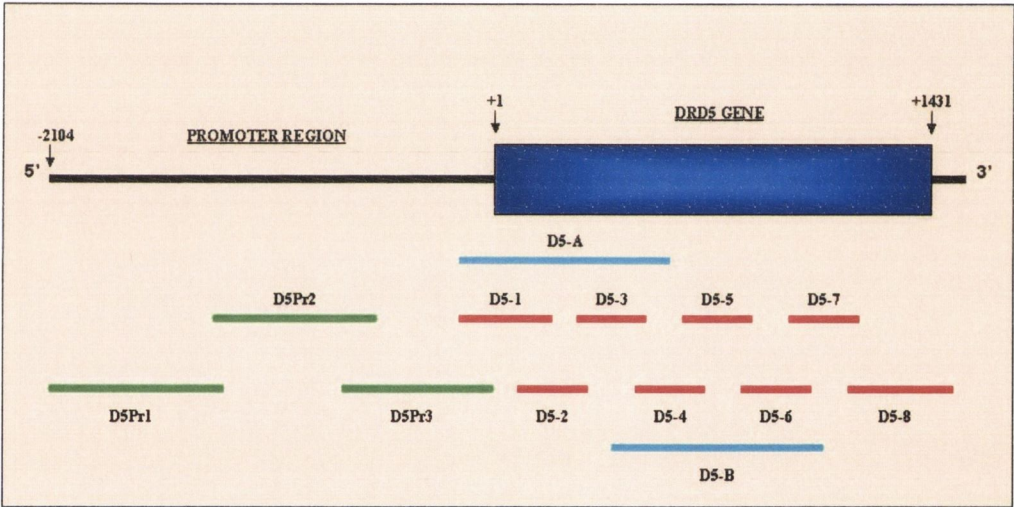
The individuals to be tested using DHPLC were carefully chosen from the total ADHD sample described in section 2.1.1 or from our control population (section 2.1.3). The testing of 30 chromosomes (15 individuals) is the optimum number to detect mutations of 10% or greater frequency in a population (Todd and Lobos, 2002). For this study the DNA of 45 individuals (3 sets of 15) were screened. The first 15 of these were selected from the ADHD sample as being homozygous for the high risk allele of the associated microsatellite and had inherited this allele from heterozygous parents possessing the associated allele. The second set consisted of cases that did not possess any copies of the high risk allele, with their parents again being heterozygous for the associated allele. The third set of 15 was taken randomly from our control population. The first set of 15 was selected as being the ADHD children most likely to possess risk variants in LD with the associated DRD5.PCR1 microsatellite. The second group was selected to screen for variants in ADHD children that were not in LD with the microsatellite variant and the third set was chosen as a control sample.

3.2.2 Amplicon Design, PCR and Heteroduplexing

Figure 3.1 displays the selection of amplicons used to screen the DRD5 gene and promoter region. These were designed to be overlapping to ensure the entire gene was fully screened, as the DHPLC method is not well suited to analysing the first and last 10-20 bps of amplicons. The design of the amplicons was complicated by the need to avoid pseudogene (Chapter 4, Section 4.3.1) amplification and was

therefore performed manually, with the aid of OLIGO 3.0 to visualise the sequence. The approximate 2kb region 5' of the gene was amplified in 3 sections referred to as D5Pr1, D5Pr2 and D5Pr3. These sections were then digested with specific restriction enzymes to check for the presence of pseudogene amplification before being screened using DHPLC (see Table 3.1). The gene itself proved more difficult to amplify without pseudogene contamination. Amplicons D5-7 and D5-8 were successfully amplified without the presence of pseudogenes however amplicons D5-1 through D5-6 required amplification via nested PCR, from two larger pseudogene free amplicons (D5-A and D5-B).

Figure 3.1 – Positions of the DHPLC Amplicons of the DRD5 Gene and Promoter Region



Red and green lines indicate the positions of the gene, promoter amplicons. Blue lines mark the positions of D5-A and D5-B, the amplicons used for the nested PCRs.

The absence of pseudogenes was tested for using restriction enzymes specific to the DRD5 gene or the pseudogenes (Table 3.1). This method involved sourcing an enzyme that would cut either the real gene or the pseudogenes but not both. The PCR product was then digested with the chosen enzyme to determine if pseudogenes were present as well as the actual DRD5 gene. In the case of enzymes

chosen that only cut the pseudogenes, another PCR product that did contain the restriction site was included in the experiment as a positive control. In addition, the sequencing results from DHPLC sections suggesting the presence of a SNP were compared to the NCBI database using BLAST (www.ncbi.nlm.nih.gov/BLAST) to ensure that the closest match was to the true gene sequence on chromosome 4.

Table 3.1 – Restriction Enzymes used to Test DHPLC Amplicons for the Presence of Pseudogenes

Amplicon	Restriction Enzyme	Cut Gene or Pseudogene	Temp (°C)	BSA Added	No. of Units of Enzyme	Duration of Digest (Hrs)
D5Pr1	Msc I	Gene	37	No	3U	4
D5Pr2	Msc I	Pseudogene	37	No	3U	4
D5Pr3	AlwN I	Gene	37	No	3U	4
D5-A	Dde I	Gene	37	No	3U	6
D5-B	EcoN I	Pseudogene	37	No	3U	4
D5-7	Sau96 I	Pseudogene	37	No	4U	6
D5-8	Xmn I	Gene	37	Yes	4U	O/N

All enzymes were supplied by New England Biolabs, Beverly, MA, USA. O/N indicates overnight digestion.

Further details of the amplified sections can be found in Table 3.2 with complete details of the primers and conditions of the PCRs in Appendix B. Following PCR of the samples according to section 2.2 an additional step of heating to 95°C for 5mins followed by controlled cooling of the samples at a rate of 1.5°C a minute was performed to encourage the formation of heteroduplexes.

Section D5-5 was never successfully amplified without the presence of pseudogenes using regular PCR, or without the presence of non-specific amplification using

nested PCR. It was therefore not analysed using DHPLC but was sequenced (Chapter 2, Section 2.2.10) using D5-B as the template and D5-4F and D5-6R as the sequencing primers.

Table 3.2 – Details of the DHPLC Amplicons and their PCR Conditions

Amplicon Name	Amplicon Size (bp)	Beginning Position	End Position	Nested PCR	DHPLC Temps (°C)
D5Pr1	798	-2014	-1306	No	60, 62
D5Pr2	671	-1324	-653	No	58, 60, 61
D5Pr3	683	-715	-32	No	64, 67, 69
D5-1	374	-114	260	Yes - from D5-A	62, 67
D5-2	240	150	390	Yes - from D5-A	63, 66, 68
D5-3	219	313	532	Yes - from D5-A	59, 61, 65
D5-4	227	514	741	Yes - from D5-B	62, 64, 66
D5-5	228	717	927	Sequenced	-----
D5-6	209	904	1113	Yes - from D5-B	59, 63, 65
D5-7	232	1058	1291	No	60, 62, 64
D5-8	416	1269	1685	No	56, 60, 63

All bp positions are given in relation to the transcription start site (+1).

3.2.3 DHPLC

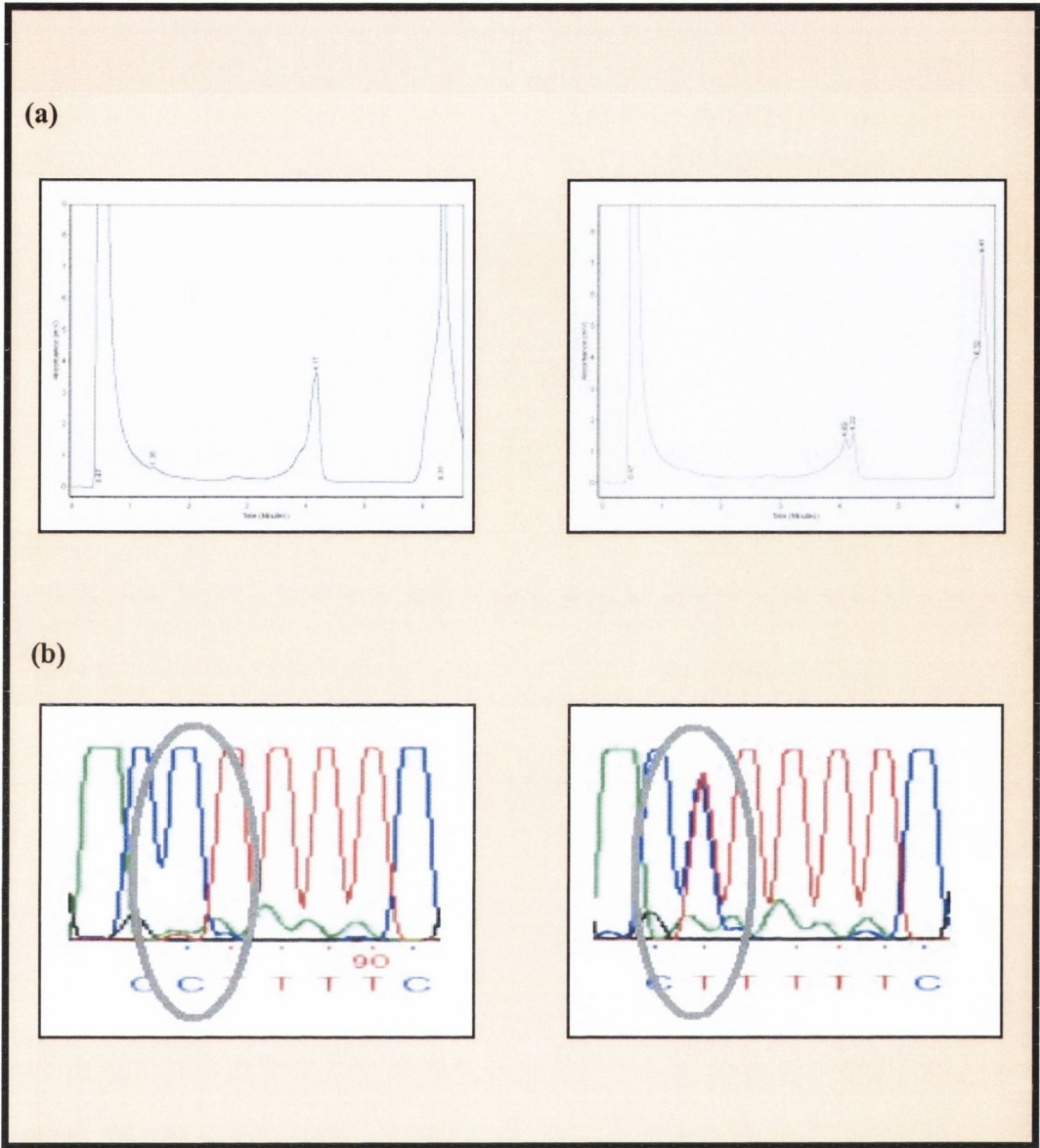
DHPLC was performed using a Transgenomic WAVE DNA Fragment Analysis System (Transgenomic Inc, Omaha, NE, USA) and the software WAVEMAKER 4.0 (Chapter 2, Section 2.2.9). The software was used to determine the optimum temperatures at which to run the samples to ensure complete screening of each section (see Table 3.2 for details).

3.3 Results

DHPLC analysis resulted in the identification of 6 SNPs in the screening of the DRD5 gene and 5' promoter region. The differing traces produced by the WAVEMAKER software indicating the presence of a SNP, are shown in part (a) of Figures 3.2-3.7.

Individuals with different traces for the same amplified section of the gene, produced by the DHPLC software, were sequenced according to section 2.2.10 resulting in the discovery of SNPs in the coding region of the DRD5 gene at positions 263 (section D5-2) (Figure 3.2 (b)) and 978 (section D5-6) (Figure 3.3(b)), and in the 3' untranslated region at position 1481 (section D5-8) (Figure 3.4(b)). For the promoter region SNPs were detected in sections D5Pr1, D5Pr2 and D5Pr3 at positions -1785 (Figure 3.5(b)), -972 (Figure 3.6(b)) and -294 (Figure 3.7(b)) respectively. The location of the SNPs is represented diagrammatically in Figure 3.8. The sequencing of all samples for section D5-5 failed to detect any sequence variation between individuals.

Figure 3.2 – DHPLC Chromatographs and Sequencing Results for D5-2



Part (a) of the figure shows the two DHPLC traces. The right hand trace indicates the presence of a mutation, which is not present in the left hand trace.

Part (b) of the figure shows the sequencing results for the traces in (a). The right hand sequence shows the presence of the mutation, which is absent in the left hand trace.

Figure 3.3 – DHPLC Chromatographs and Sequencing Results for D5-6

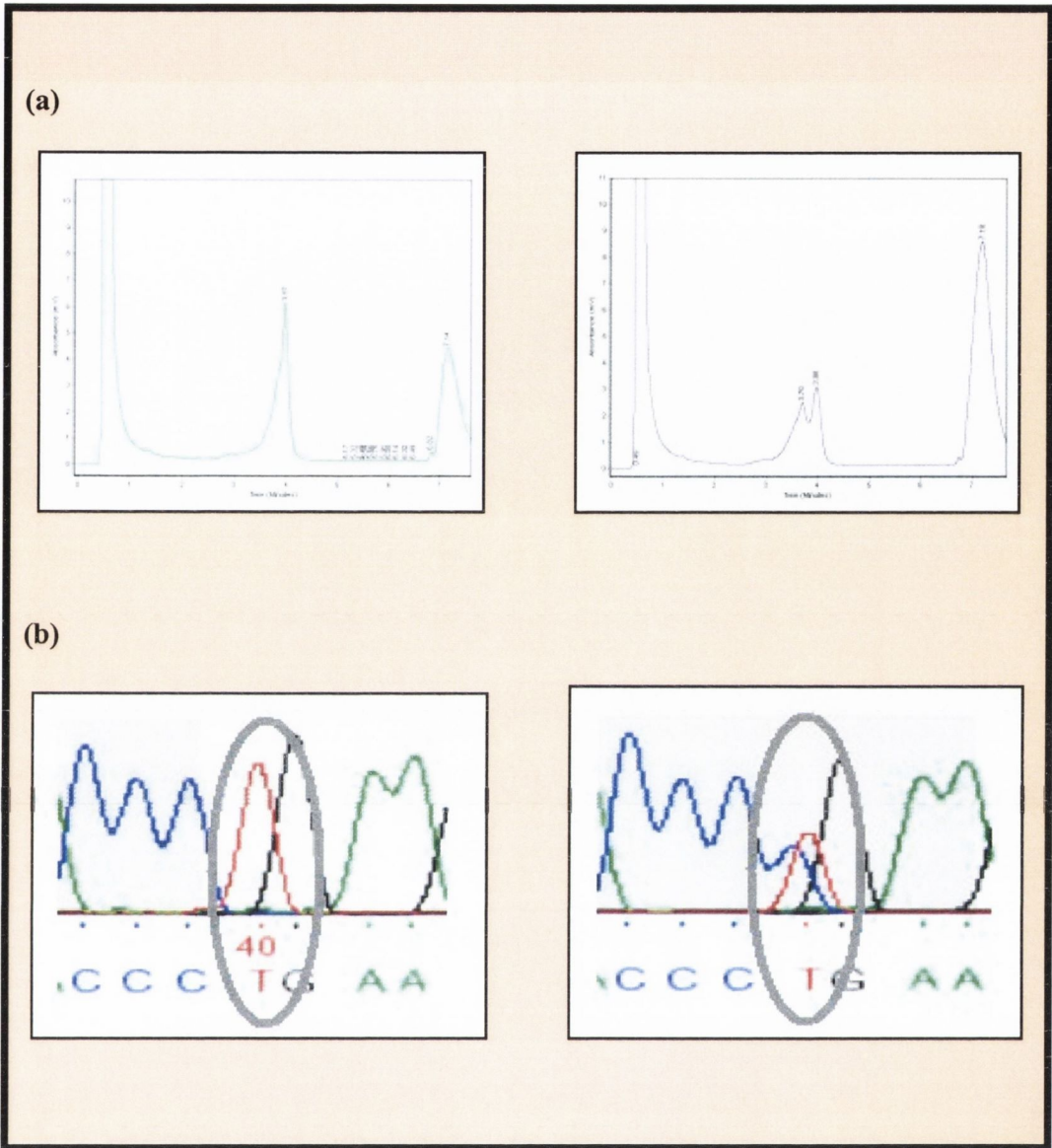


Figure 3.4 – DHPLC Chromatographs and Sequencing Results for D5-8

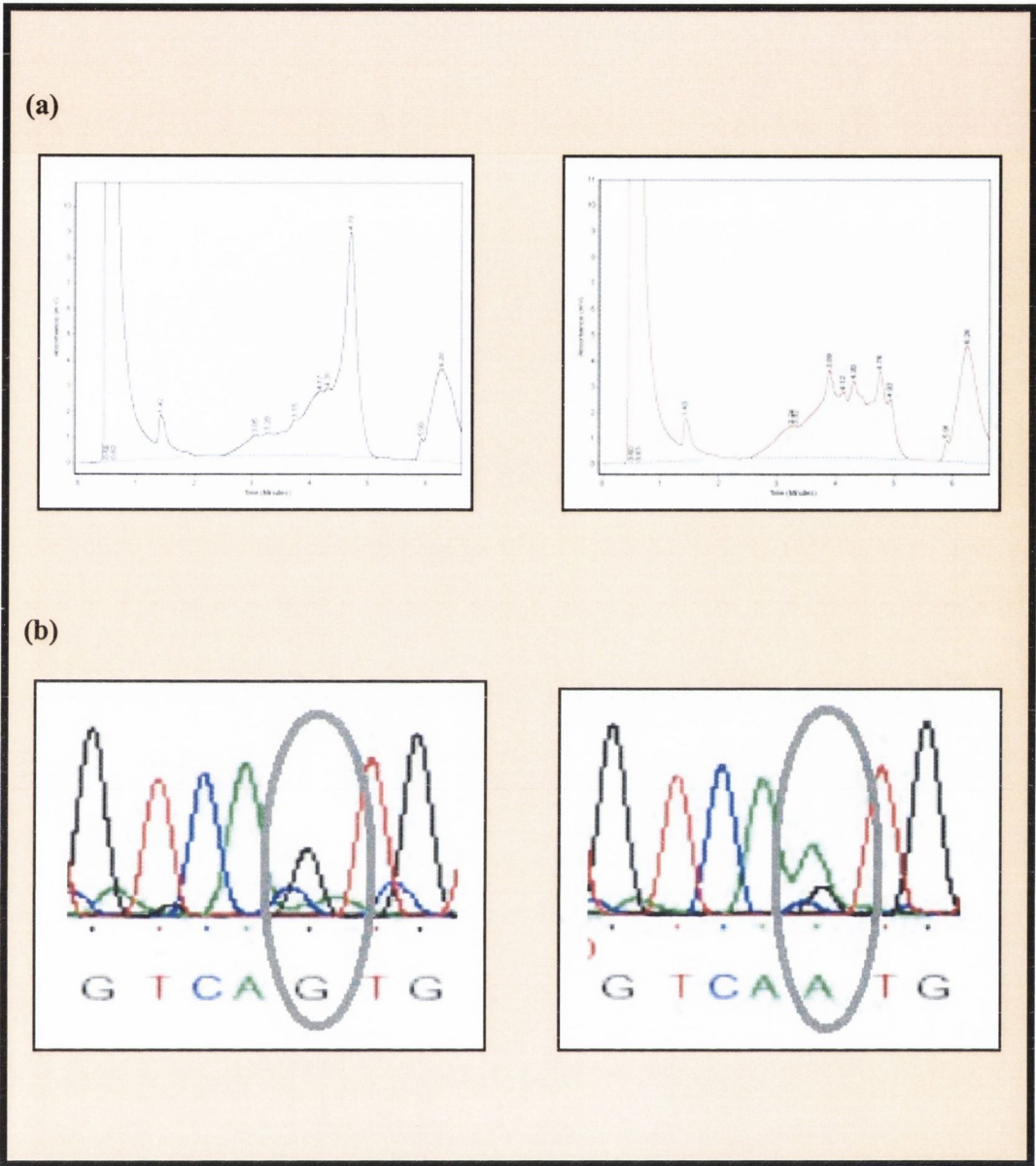


Figure 3.5 – DHPLC Chromatographs and Sequencing Results for D5Pr1

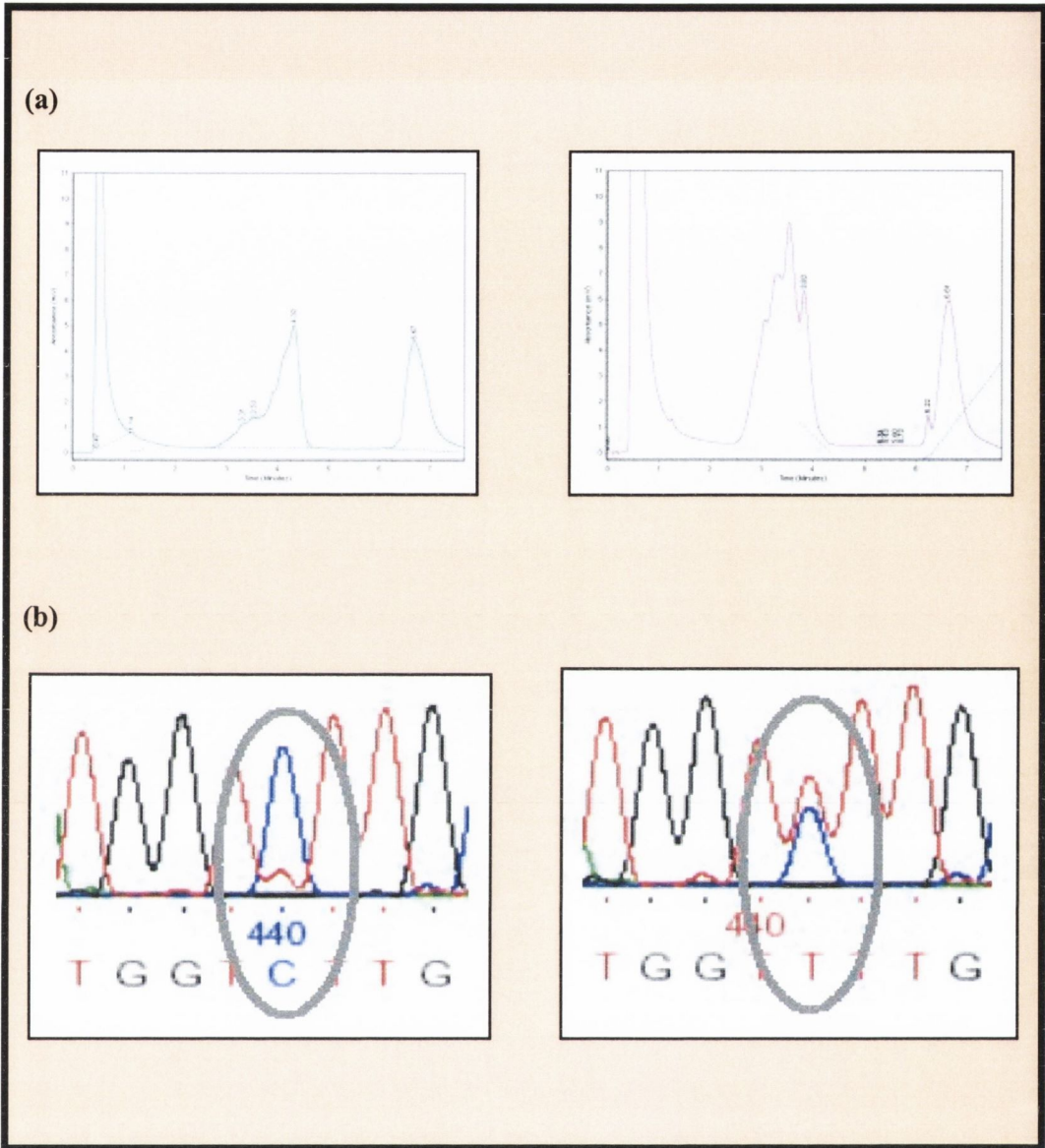
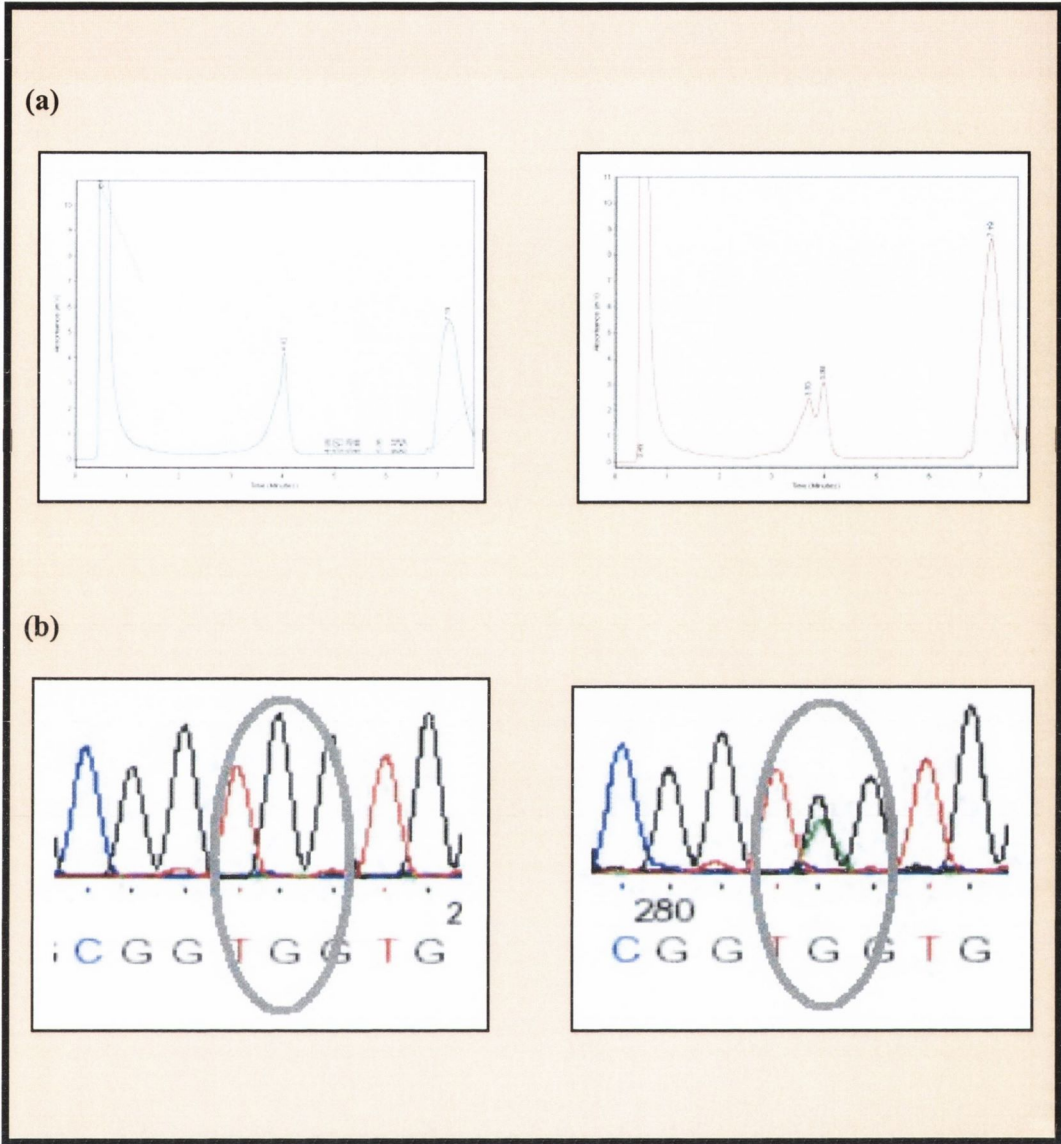


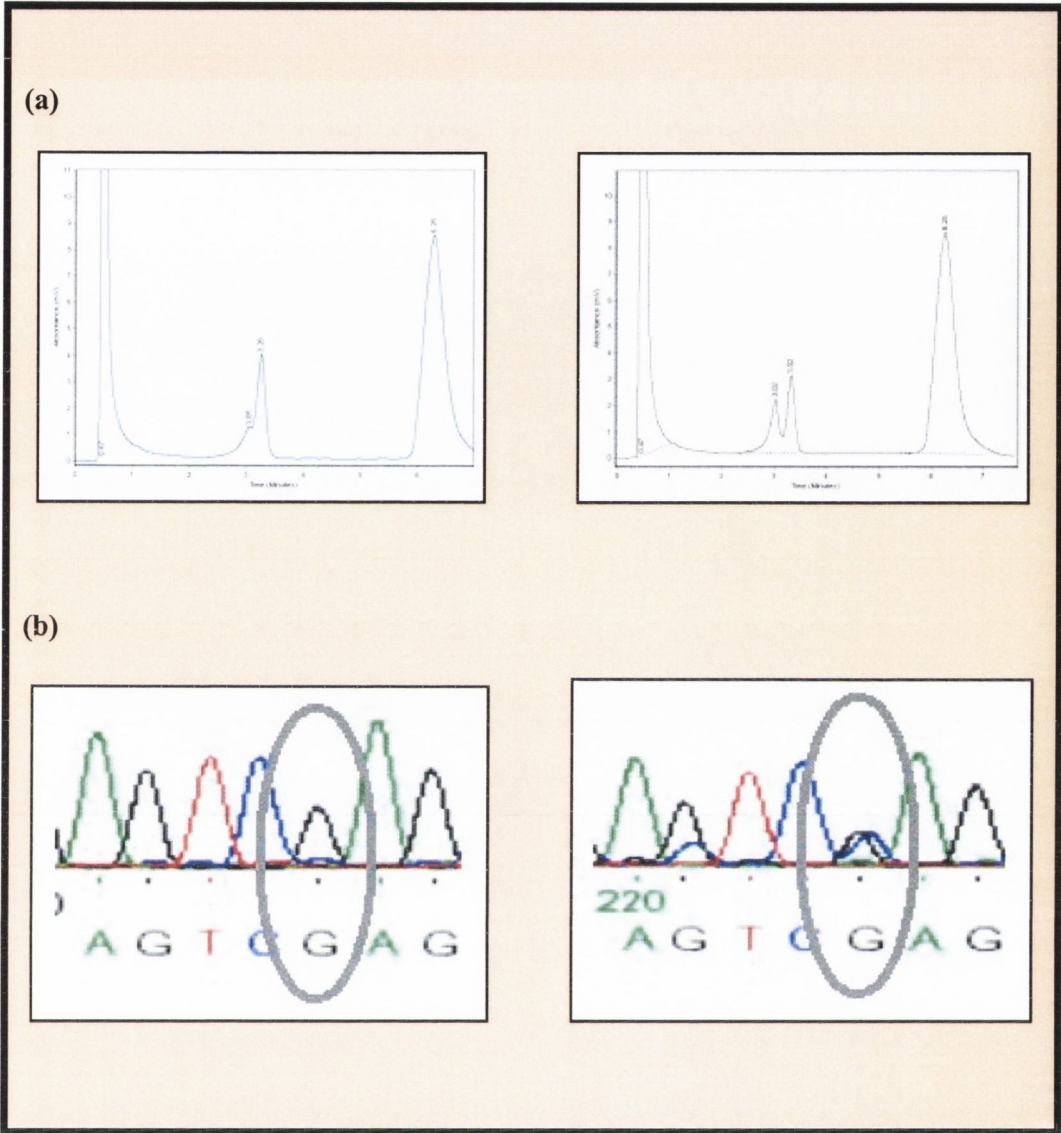
Figure 3.6 – DHPLC Chromatographs and Sequencing Results for D5Pr2



Part (a) of the figure shows the two DHPLC traces. The right hand trace indicates the presence of a mutation, which is not present in the left hand trace.

Part (b) of the figure shows the sequencing results for the traces in (a). The right hand sequence shows the presence of the mutation, which is absent in the left hand trace.

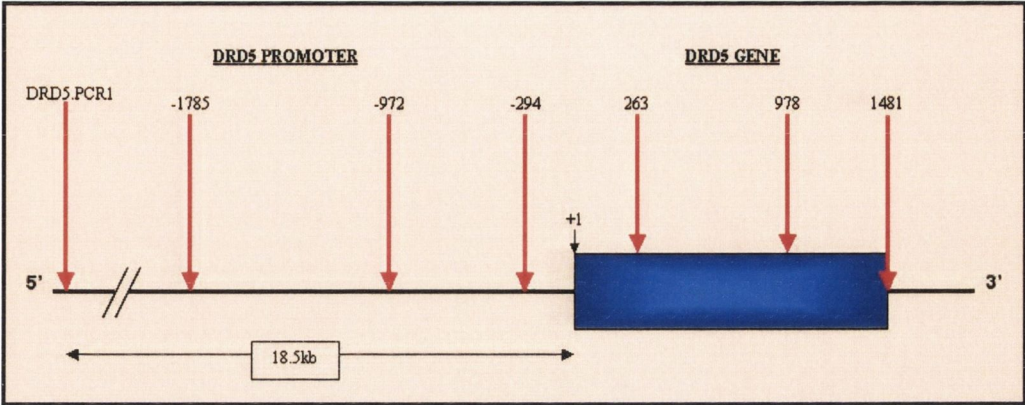
Figure 3.7 – DHPLC Chromatographs and Sequencing Results for D5Pr3



Part (a) of the figure shows the two DHPLC traces. The right hand trace indicates the presence of a mutation, which is not present in the left hand trace.

Part (b) of the figure shows the sequencing results for the traces in (a). The right hand sequence shows the presence of the mutation, which is absent in the left hand trace.

Figure 3.8 – Location of DRD5 Variants detected by DHPLC



The blue rectangles represent the exon of the gene. +1 indicates the transcription start site. Red arrows indicate the location of detected variants.

The frequencies of the detection of heterozygous samples (indicating the presence of a mutation) are shown in Table 3.3. However it should be noted that only heterozygous samples are detected using DHPLC methods and therefore these results are not quantifiable. Of the 6 SNPs, two were detected at a frequency of 1 in 45 individuals screened, which was below the threshold to be included in the DRD5 association analysis (Chapter 4, Section 4.3), two were borderline at 3 in 45 individuals and two were well within the inclusion threshold at 19 in 45 and 21 in 45 respectively. The two SNPs at the borderline threshold of 3 in 45 individuals were genotyped in 100 parental samples to get a more accurate assessment of their frequency. In the case of both of the SNPs their frequency was less than 10% and so they were not included in the DRD5 association study either.

Table 3.3 – Frequency of Heteroduplexes Detected in DHPLC Analysis

Amplicon	SNP	Freq in 1st set of 15 individuals	Freq in 2nd set of 15 individuals	Freq in 3rd set of 15 individuals	Freq in total 45 individuals
D5-2	263	0	0	1	1
D5-6	978	5	7	9	21
D5-8	1481	6	9	4	19
D5Pr1	-1785	1	0	0	1
D5Pr2	-972	1	2	0	3
D5Pr3	-294	0	3	0	3

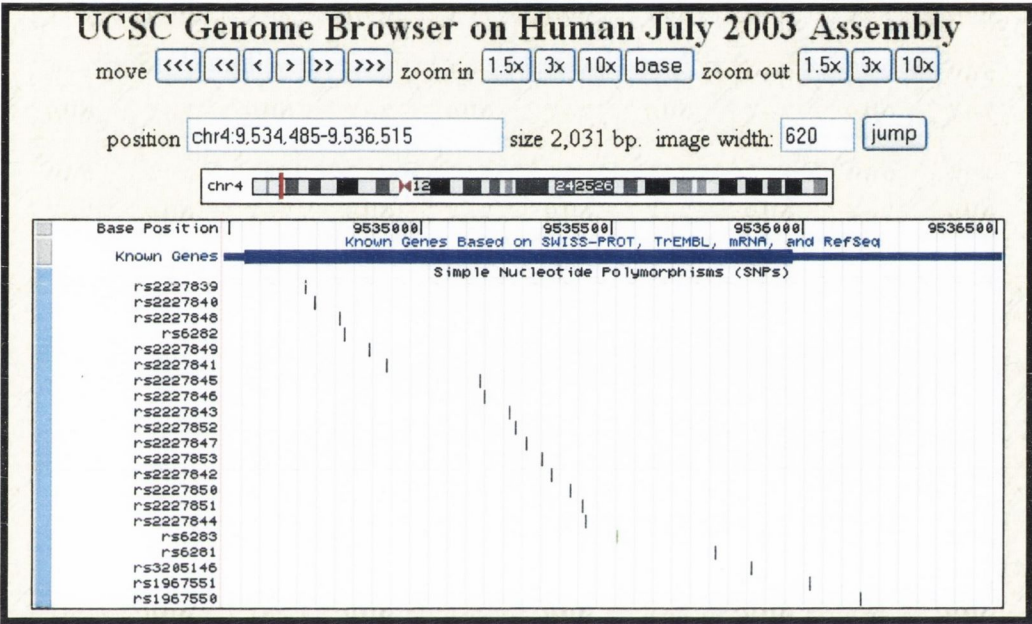
SNPs are numbered in relation to the transcription start site (+1)

These findings of this study are comparable to those reported in the general literature. A screening of 78 schizophrenia patients using SSCP methods by Sobell et al (1995) reported nine variants, including the 263, 978 and 1481 SNPs detected here. The additional 6 were found at a frequency of less than 1%, which this screen was not designed to detect. In addition, a further SSCP study consisting of 4 affected individuals from a family with psychosis, schizophrenia and schizoaffective disorder and 36 unrelated schizophrenics by Asherson et al (1998) also reported the 1481 SNP but failed to detect any others in the gene or promoter region.

Although only 3 mutations were detected in the DRD5 gene, 21 SNPs are reported on the Human Genome Browser (<http://genome.ucsc.edu/>) (see Figure 3.9) with 11 of them having a frequency over 10%. The fact that we only detected 3 was a cause for concern so I designed SNaPshot assays, using the PCR primers from the DHPLC amplicons, for the additional SNPs that had a reported frequency of greater

than 10% (see Appendix B for primers and conditions) to ensure the DHPLC analysis had not missed anything. The additional SNPs were examined in 64 people from our Irish ADHD individuals and their parents with no evidence of the polymorphisms detected.

Figure 3.9 – Image from Human Genome Browser Depicting Known SNPs of the DRD5 Gene



This image was obtained from the Human Genome Browser and shows the region of chromosome 4 containing the DRD5 gene. The short horizontal lines with rs numbers to the left hand side indicate the known SNPs in this chromosomal region.

3.4 Conclusions

The method of DHPLC is a widely used method for the discovery of DNA mutations, with its accuracy for detection of sequence variants being equated to 100% under optimum conditions (Spiegelman et al, 2000). Although the DHPLC system has multiple applications it is most commonly used to detect the presence of SNPs in DNA sequences of interest, usually exons and promoter regions of candidate genes as a preceding step to sequencing.

In this study the screening of the promoter and coding region of the DRD5 gene resulted in the detection of 6 SNPs. Three of these were located in the coding region of the gene and were already reported in the literature or present on the databases Ensembl and NCBI (www.ensembl.org and www.ncbi.nlm.nih.gov). Three additional unreported SNPs were also located in the promoter region of the gene. Of these variants, 2 of the 6 were deemed to have sufficiently high frequency (ie >1:30 chromosomes) to warrant their genotyping for LD and fine mapping analysis (Chapter 4, Section 4.3).

The findings of the screening process in the 3'untranslated and coding regions of the gene are consistent with reports from the literature. Sobell et al (1995) reported the presence of the 3 non promoter SNPs, as well as a number of others with a frequency of less than 1%. In addition, the 1481 SNP was also reported by Asherson et al, (1998) but this study failed to detect the 978 and 263 variants. The promoter region has received less interest to date with only one other study performing SSCP on the region and detecting no variation (Barr et al, 2000).

I was concerned at the relatively small number of SNPs detected compared to the number present on the databases and in the literature. To examine the situation further, assays were designed using the SNaPshot method of primer extension and pooled DNA samples for each of the undetected SNPs with a reported frequency of greater than 10%. These assays failed to detect any individuals with the reported mutant allele in our Irish sample. This result coupled with the lack of detection of these reported mutations during the DHPLC screening questioned the validity of the information in the databases. Comparison of the sequences of the true DRD5 gene and its pseudogenes at the location of the reported SNPs revealed that over 3 quarters of the SNPs had alleles that corresponded exactly with the sequence change between the DRD5 gene and the pseudogenes at that position. Due to the high sequence similarity between the pseudogenes and the actual DRD5 gene (98%) and the resulting difficulty in amplifying only the chromosome 4 sequence, this finding casts doubts on the probability that all of these SNPs are true sequence variants of the DRD5 gene. It should be noted however, that the 978 SNPs alleles are the same as the differences between the actual gene and the pseudogenes confirming that this phenomenon does occur, however the reported frequency of this event coupled with the inability to detect these additional reported SNPs using two different methods in our sample does suggest that not all of the reported sequence variations in the databases are true DRD5 SNPs.

In conclusion, this study has identified the presence of 6 SNPs in the DRD5 gene and approximately 2100bp up stream of the transcription start site. Of these detected variants, 2 have sufficient frequency to be genotyped and used in a fine mapping

study of the DRD5 gene (chapter 4). In addition, this screening process has raised questions as to the accuracy of the reported SNPs in this region due to the high homology of the sequence with other chromosomes and the lack of detection of many of them using two methods of analysis in the Irish population.



Chapter 4

Fine Mapping of DRD5, DRD4 and SNAP25 in an Irish ADHD Population. (Genotyping of Gene Variants Coupled with Association, Linkage and Haplotype Analyses)

4.1 General Introduction

Initial reported association between a variant and a disorder/disease should be followed up by replication studies as well as further investigation and analysis of variants in the surrounding region (known as fine mapping). Replication should be attempted in an independent sample, whereas the additional mapping and analysis of the surrounding variants should initially be performed in the sample that reported the original association.

Fine mapping of a region involves the selection, genotyping and analysis of additional variants in the area immediately surrounding the first associated variant in an attempt to narrow down or define a smaller more precise region of association where a potentially functional variant may lie. Techniques used include single variant testing, haplotype analysis and the establishment of the LD relationship (Chapter 2, Section 2.3). Additional variants are usually selected from previously reported studies (often from different disciplines) where assays have already been optimised, and from genetic databases such as HGMP (www.hgmp.mrc.ac.uk), NCBI (www.ncbi.nlm.nih.gov) and Ensembl (www.ensembl.org).

A fine mapping approach has been adopted here to further examine the initial reported findings of association with the DRD5, DRD4, and SNAP-25 genes in the Irish population.

4.2 DRD4

4.2.1 Introduction

The dopamine D4 receptor, which mediates the postsynaptic action of dopamine, is a member of the G protein coupled receptor family. Along with the DRD3 receptor it is classified as a D2-like receptor due to its similarity in sequence, binding affinities and secondary messenger system (inhibition of adenylyl cyclase activity) with the DRD2 receptor. The DRD4 gene has been extensively examined at a molecular level in relation to ADHD and the trait of novelty seeking.

One of the most replicated associations between ADHD and variants in dopaminergic system genes is the 7 repeat allele of the variable number tandem repeat (VNTR) polymorphism of the DRD4 gene (Hawi et al, 2003a). Asghari et al (1995) reported a reduction in the dopamine stimulated inhibition of cAMP levels in Chinese hamster ovary (CHO-K1) cell lines expressing the 7-repeat allele of the VNTR as opposed to the 4 or 2-repeat alleles. In addition to the sustained interest with the VNTR, recent sequence analysis at the 5' end of the DRD4 gene has identified several new polymorphisms in this region (Wong et al, 2000) (Chapter 1, Section 1.4.1). This study focuses on the polymorphisms from this 5' region with a hypothesis for functionality, as well as the VNTR.

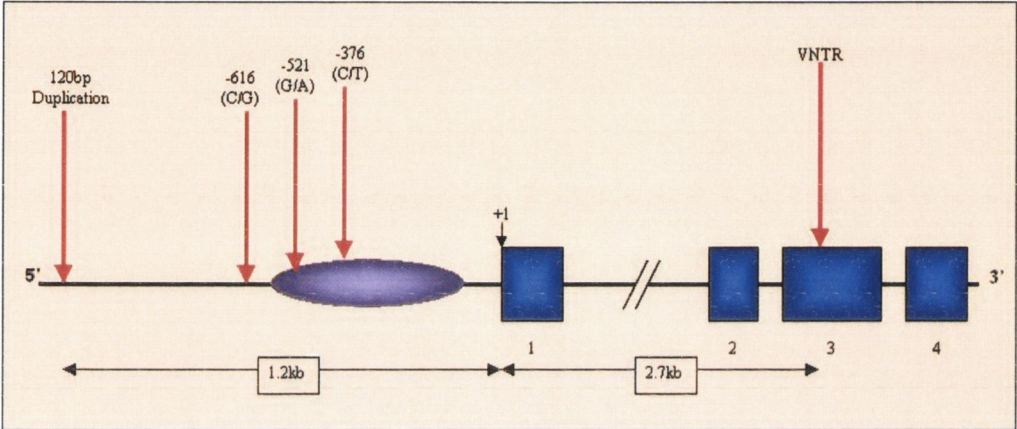
The analysed variants include the -616, -521 and -376 SNPs, a 120bp duplication located 1.2kb up stream of the gene and the VNTR in exon 3. The 120bp duplication is a repeat known to contain several transcription factor binding sites

(Seaman et al, 1999). It is postulated that the long allele (240bp) may have a different effect on the transcription of the gene than its short counterpart due to it possessing twice as many TF binding sites (Barr et al, 2001b), although this has not yet been explored at a functional level. The -616 SNP, is a C to G substitution resulting in the introduction of an AP-2 transcription factor binding site in the promoter region of the DRD4 gene. Structural and functional analysis of DNA binding and transcription activity of the AP-2 protein has shown that these transcription factors can affect activation and suppression of gene transcription (Williams and Tjian, 1991) and it is thought that this binding site introduction may affect the regulation of transcription of the DRD4 gene. The -521 variant is located in a region of the promoter (-591 to -123) known to mediate the transcription of the gene (Wong et al, 2000). Functional analyses of human retinoblastoma Y-79 cells transfected with chloramphenicol acetylase (CAT) vectors driven by inserted promoter regions containing the C and T versions of the -521 SNP have shown that the T or A allele reduces transcription levels of the gene by up to 40% compared to the C or G allele (Okuyama et al, 1999). The -376 SNP is also located in this transcription mediating region and therefore I hypothesised that it may also affect regulation of the gene.

4.2.2 Gene Structure and Location of Analysed Variants

The DRD4 gene is located on chromosome 11 at 11p15.5. Sequence analysis shows that the DRD4 gene comprises 4 exons and has a total transcript size of 1360bp (Accession Number = NM_000797). Figure 4.1 displays the structure of the DRD4 gene and indicates the location of the analysed variants.

Figure 4.1 - Structure of the DRD4 Gene Showing the Location of Analysed Variants



The blue rectangles represent the exons of the gene. The purple oval indicates the presence of a promoter regulatory region. +1 indicates the transcription start site.

4.2.3 Materials and Methods

All variants were genotyped for the sample described earlier in Chapter 2. The 120bp duplication was genotyped using standard PCR and electrophoresised on ethidium bromide agarose gels. The three SNPs were genotyped using the SNaPshot method of primer extension and analysed using an ABI PRISM 377 DNA Sequencer. The VNTR was amplified and visualised on nondenaturing polyacrylamide gels stained with ethidium bromide. Complete details of methods, primer sequences and PCR conditions can be found in Chapter 2 and Appendix B. Analyses were performed using the TDT, ETDT, GOLD and TRANSMIT. Full details of the statistics and software packages can be found in Chapter 2 and Appendix C.

4.2.4 Results

Tabulated below are the results for all 5 variants, (none of which displayed any significant deviation from Hardy Weinberg Equilibrium), analysed using the TDT or ETDT (Table 4.1). Of the five variants tested only one, the -616 SNP, displayed significant association with ADHD ($\chi^2=7.45$, $p=0.008$), with an OR value of 1.63. The -521 SNP showed non significant preferential transmission of the C allele to affected cases ($\chi^2=2.14$, $p=0.166$) and the 7-repeat allele of the VNTR was also shown to be over transmitted to ADHD children ($\chi^2=1.94$, $p=0.198$). Although the VNTR result was not statistically significant the excess transmission of the 7-repeat allele is in keeping with the published literature.

Table 4.1 - TDT Results of Individual Variant Analysis for the Genotyped DRD4 Polymorphisms

Variant	Allele Frequency	T	NT	χ^2	p-value	OR	95%CI
120bp (Long)	83%	48	46	0.04	0.918	1.04	0.70-1.56
-616 (C allele)	70%	80	49	7.45	0.008	1.63	1.16-2.38
-521 (A allele)	60%	94	75	2.14	0.166	1.25	0.96-1.77
-376 (C allele)	95%	16	12	0.14	0.572	1.33	0.63-2.82
VNTR (7 rpt)	19%	50	37	1.94	0.198	1.35	0.88-2.07

SNPs are numbered in relation to the transcription start site (+1).

LD analysis between the examined variants (measured as a D' value) is presented in Table 4.2 and Figure 4.2. Highly significant LD was observed between the variants, -616 and -376, -521 and -376 and -376 and the VNTR. However, little or no LD was present between any of the other tested variants. These results appeared unusual, as the physical distance between the variants is small. The apparent highly

significant LD relationship between the -376 and the other variants may be caused by the low allele frequency (5%) and age of this polymorphism. If the variant is younger in it's origin than those surrounding it, it will appear to be in LD with surrounding variants due to the lack recombination events that it will have undergone relative to the other variants. In conjunction with this if the variant is rare as is the case here then the chance of recombination occurring on the chromosome with the mutant allele is further reduced, thus slowing the time taken to effect LD relationships.

Figure 4.2 – LD Values for DRD4 Variants

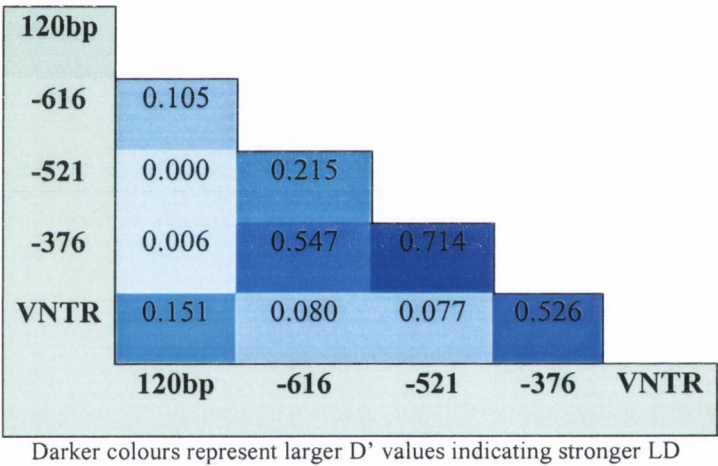


Table 4.2 - LD Analysis between DRD4 Variants

Variant 1	Variant 2	χ^2	p-value	D'
120bp	-616	0.49	0.48371	0.105
120bp	-521	0.00	0.99956	0.000
120bp	-376	0.01	0.91568	0.006
120bp	VNTR	12.86	0.00496	0.151
-616	-521	12.22	0.00047	0.215
-616	-376	7.20	0.00730	0.547
-616	VNTR	5.23	0.15592	0.080
-521	-376	11.57	0.00067	0.714
-521	VNTR	3.23	0.35785	0.077
-376	VNTR	31.63	0.00000	0.526

Two variant haplotype analysis using TRANSMIT (Table 4.3) revealed a marginal significance between a haplotype constructed of the long allele of the 120bp duplication and allele C of the -616 SNP and ADHD ($\chi^2=4.26$, $p=0.04$). A non-significant increase in the transmission of the -616 and -521 haplotype ($\chi^2=2.9$, $p=0.086$), the -521 and -376 haplotype ($\chi^2=3.4$, $p=0.06$) and the -376 and VNTR haplotype ($\chi^2=2.6$, $p=0.11$) to ADHD individuals was also observed. These findings support the TDT results in continuing to suggest a role for the C allele of the -616 SNP, the A allele of the -521 and the 7 repeat allele of the VNTR as well as implicating the long allele of the insertion/deletion and the C allele of the -376 SNP. Multiple variant analysis (Table 4.4) also concurred with these findings, with the only haplotypes of interest involving these alleles. In addition the analysis showed association with the non transmission of two haplotypes (120bp(long)/-616

(G) and -616(G)/-521(G)). This may suggest a protective role for these two haplotypes.

Table 4.3 – Two Variant Haplotype Analysis for DRD4 using TRANSMIT

Haplotype	Observed	Expected	χ^2 (1df)	p-value
120 (short) / -616 (C)	39.081	37.044	0.2813	0.595850
120 (long) / -616 (C)	188.69	175.89	4.2596	0.039029
120 (short) / -616 (G)	19.641	22.275	0.7688	0.380589
120 (long) / -616 (G)	100.59	112.79	4.8431	0.027757
-616 (C) / -521 (G)	72.115	67.524	0.9964	0.318183
-616 (G) / -521 (G)	54.078	70.201	10.710	0.001066
-616 (C) / -521 (A)	149.74	139.38	2.9440	0.086197
-616 (G) / -521 (A)	60.065	58.891	0.0715	0.789165
-521 (G) / -376 (T)	115.25	123.64	1.7568	0.185024
-521 (G) / -376 (C)	13.728	15.471	0.4949	0.481750
-521 (A) / -376 (T)	209.21	197.02	3.4193	0.064438
-521 (A) / -376 (C)	1.8173	3.8696	2.7486	0.097340
-376 (T) / VNTR (7 rpt)	70.993	63.426	2.5841	0.107942
-376 (C) / VNTR (7 rpt)	0.73108	1.6998	1.4946	0.221504

Haplotypes of significance are highlighted in bold type.

Table 4.4 – Selected Multi Variant Haplotype Analysis for DRD4 using TRANSMIT

Haplotype	Observed	Expected	$\chi^2(1df)$	p-value
120 (long)/-616(C)/-521(A)	132.52	121.8	3.4127	0.064697
-616(C)/-521(A)/VNTR (7 rpt)	27.217	22.494	2.5699	0.108915
120(long)/-616(C)/- 521(A)/VNTR(7rpt)	25.298	20.911	2.2657	0.132266

4.2.5 Conclusions

The five DRD4 variants examined all had hypothesised or previously reported functional significance. The 120bp duplication located 1.2kb upstream of the transcription start site has been hypothesised to affect the level of transcription of the gene (Seaman et al, 1999) as the repeat is known to contain several transcription factor (TF) binding sites and it is postulated that the long allele (240bp), possessing twice as many TF binding sites, may have a different effect on the transcription of the gene than its short counterpart. The C to G substitution at the -616 SNP results in the introduction of an AP-2 TF binding site in the promoter region of the DRD4 gene. Structural and functional analysis of DNA binding and transcription activity of the AP-2 protein has shown that these TF's can affect activation and suppression of gene transcription (Williams and Tjian, 1991). The -521 and -376 SNP are both located in a region of the promoter mediating the transcription of the DRD4 gene (Wong et al, 2000), with functional analysis of the -521 variant showing that the A-allele reduces transcription levels of the DRD4 gene by up to 40% compared to the G allele (Okuyama et al, 1999) (Chapter 4, Section 4.2.1). The 7-repeat allele of the VNTR has been reported as having a reduced response to dopamine compared to the 2 or 4 repeat alleles (Asghari et al, 1995). However, this finding has been disputed by Jovanovic et al (1999) who failed to observe a direct relationship between the number of repeats and changes in pharmacological or functional activity of the protein.

In this study we observed no evidence of the biased transmission of the long allele of the 120bp duplication reported by McCracken et al (2000) or the short allele as

observed by Barr et al (2001b). Our study concurs with Todd et al (2001) and Mill et al, (2003) in reporting no association between ADHD and the 120bp duplication.

Significant association with ADHD was however observed with the C allele of the -616 SNP ($\chi^2=7.45$, $p=0.008$). In support of this Barr et al (2001b), also observed a trend towards increased transmission of the same allele (referred to in their paper as the C allele of Ava II) to ADHD cases in a Canadian sample. One possible explanation for the failure of the Barr et al study to attain significance may be the lack of sufficient power to detect a gene of small or moderate effect especially since their sample consisted of only 82 trios. Mill et al (2003) also reported failure to detect a significant association between this variant and ADHD. It is suggested that this variant be further explored at a functional level due to its positive association with ADHD and its biological hypothesis for affecting transcription.

A non significant excess transmission of the A allele of the -521 SNP was observed, ($\chi^2=2.14$, $p=0.17$). This trend was also observed in the Canadian study by Barr et al (2001b) ($\chi^2=0.818$, $p=0.366$). TDT analysis on our sample combined with the Canadian sample (Barr et al, 2001b) enhanced this trend although it still did not attain statistical significance ($\chi^2=2.9$, $p=0.098$). Mill et al (2003) reported no finding of association with this locus.

TDT analysis did not reveal any distortion in the transmission of the -376 SNPs alleles to ADHD cases in this study. In addition, the SNP has been shown to be less polymorphic than the others examined, and as a result less informative in a sample of this size, with a T allele frequency of only 5% in the Irish population.

In addition, no significant association between ADHD and the 7-repeat allele of the VNTR was seen, although there was excess transmission of the allele ($\chi^2=1.94$, $p=0.198$) as reported by other groups. This variant has been examined more than any other DRD4 variant, with both positive and negative association studies being reported (Hawi et al, 2003a). However, a meta analysis of the variant conducted on 21 studies reported overall association between the 7 repeat allele and ADHD (Faraone et al, 2001). In addition, a study by Holmes et al, (2002) on 2 UK and 1 Irish samples (Hawi et al, 2000; Holmes et al, 2000; Mill et al, 2001) reported association between the high risk allele and ADHD with comorbid conduct problems. The samples used in this analysis had not yielded significant association when previously analysed under an unrefined ADHD phenotype. This finding strengthens the role of the DRD4 gene is a susceptibility gene for ADHD and suggests that the use of more heritable subtypes of the ADHD phenotype, such as ADHD with comorbid conduct problems may yield greater success in molecular genetic studies (Faraone et al, 2000; Holmes et al, 2002).

LD analysis performed using GOLD, revealed significant but weak LD between the 120bp duplication and the VNTR ($D'=0.151$), but no evidence of LD between either the -616 or -521 promoter SNP's and the VNTR (Table 4.2). In addition, another weak but significant LD relationship was observed between the -616 and -521 SNP's ($D'=0.22$). As already discussed (Chapter 4, Section 4.2.4), the differences in the allele frequency between the -376 SNP and the rest of the examined variants, may have resulted in the unexpected patterns of LD between this variant and the others tested. The absence of LD between the rest of the variants of the promoter

region of DRD4 and the VNTR suggests that this is a region of high recombination. In this context, the association of the VNTR with ADHD observed in several studies may be independent of the association of the C allele of the -616 variant since no LD was detected between the two loci.

Using the program TRANSMIT, we performed haplotype analyses between the examined variants. A weak but significant association ($\chi^2=4.26$, $p=0.039$) was observed between ADHD and a haplotype composed of the long allele of the 120bp duplication and the C allele of the -616 SNP. Barr et al (2001b) reported no significant distortion in the transmission of any haplotype consisting of alleles from the variants 5' to the DRD4 gene. However, the addition of the VNTR into their analysis resulted in a significant association with ADHD of a haplotype composed of the long allele of the 120bp duplication, the C allele of the -616 variant, the A allele of the -521 and the 7 repeat of the VNTR. This association was not detected in our sample. In addition, Mill et al (2003) reported association between their ADHD cases and a haplotype comprised of the long allele of the 120bp duplication, and the C allele of the -616 and -521 SNPs ($\chi^2=4.68$, $p=0.03$).

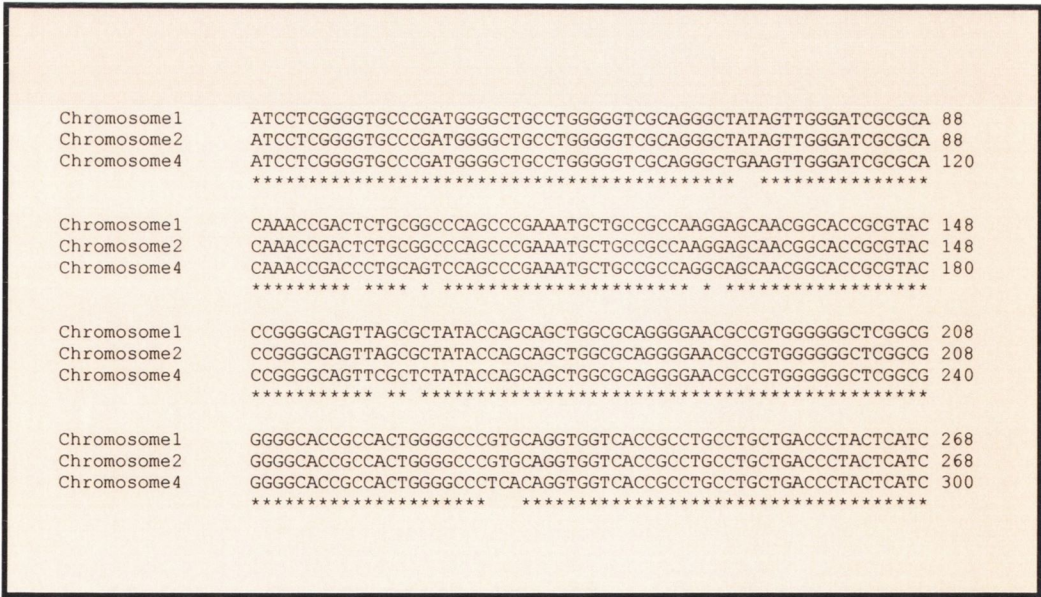
In conclusion, the results of the current investigation suggests that the association between the -616 SNP and ADHD is independent of those reported with the VNTR. However, this finding needs to be further examined, preferably in samples that already show significant association with the VNTR. It is also suggested that the associated -616 SNP be functionally tested as it has a biological hypothesis for altering transcription.

4.3 DRD5

4.3.1 Introduction

The DRD5 receptor is a member of the family of G-protein coupled receptors. It is classified as a D1-like dopamine receptor, along with the DRD1 receptor. This classification is based on the D1-like receptors activation of adenylyl cyclase (its secondary messenger system), as supposed to the D2-like receptors suppression of this enzyme, as well as its different ligand binding properties. The gene has been localised to chromosomal position 4p16.1 (Eubanks et al, 1992), with two known pseudogenes located on chromosome 1 and 2 (Nguyen et al, 1991; Grandy et al, 1992). Due to the high homology of the sequences of the real gene and its pseudogenes (95%) (Grandy et al, 1991) (See Figure 4.3) the DRD5 gene is far more complex to work with than other ADHD candidate genes. This may have contributed to it being less extensively examined than other ADHD candidate genes.

Figure 4.3 – Sequence Alignment of a Section of the DRD5 Gene from Chromosome 4 and its Two Pseudogenes from Chromosomes 1 and 2.



Our laboratory first reported the first association between this gene and ADHD in 1999 (Daly et al, 1999) in the form of a (CA)_n microsatellite marker (DRD5.PCR1) located 18.5kb from the transcription start site. Although this variant has been extensively examined by other groups working in the field, few additional DRD5 variants have received the same attention. A meta analysis of this variant in 14 ADHD samples has concluded that the DRD5 gene is a true susceptibility locus for ADHD (Lowe et al, 2004b) (see Chapter 6).

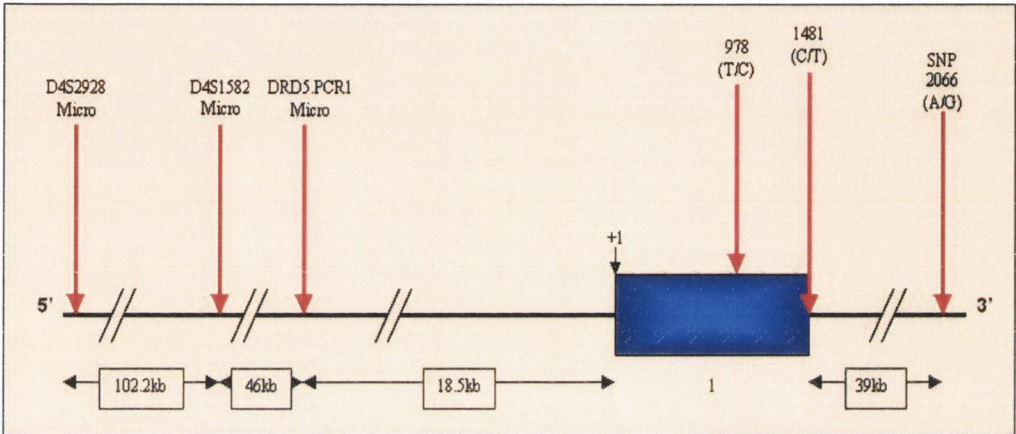
As the microsatellite marker itself has no known functional effect, it is possible that the true functional variant lies in or around the gene and is in LD with the (CA)_n repeat. Other DRD5 variants examined to date are located either 5' or 3' to the gene and tend to be in regions that do not have high sequence similarity with the pseudogenes, as these areas are easier to work with (Hawi et al, 2003b; Mill et al, 2004b). It was our aim to screen the DRD5 gene using DHPLC (see Chapter 3) to find SNP variants within the coding and promoter region of the DRD5 gene, suitable for fine mapping association studies in our Irish ADHD sample. The screening process revealed the presence of 3 SNPs in the promoter region of the gene and 3 in the gene itself (see Chapter 3, Figure 3.8), although only two, the 978 (which exists in the literature but has never been examined in relation to ADHD) (Feng et al, 1998) and the 1481 (which had already been studied in relation to ADHD) (Hawi et al, 2003b) were present at a frequency of over 10% and as such were deemed suitable for this study.

In addition to these two SNPs, the (CA)_n polymorphism (DRD5.PCR1) as well as two additional microsatellites D4S1582 and D4S2928 and a 3' SNP (rs2066), located outside of the mutation screening region were genotyped in the Irish ADHD sample.

4.3.2 Gene Structure and Location of Analysed Variants

Located on chromosome 4 the DRD5 gene consists of a single exon of transcript length 2030bp (Accession Number=NM_000798). Figure 4.4 displays the structure of the DRD5 gene and indicates the location of the analysed variants.

Figure 4.4 - Structure of the DRD5 Gene Showing the Location of Analysed Variants



The blue rectangle represents the exon of the gene. +1 indicates the transcription start site

4.3.3 Materials and Methods

The microsatellite marker (DRD5.PCR1) was genotyped using radiolabelling methods whereby αP^{32} dCTP was incorporated into the PCR product. The varying lengths of PCRs were then visualised after exposure to X-ray film. The other two microsatellites were typed using fluorescently tagged primers and analysed using an ABI 377 DNA Sequencer. The SNPs were all analysed using the SNaPshot methodology and visualised on an ABI 3100 DNA Sequencer. For further information of the methods used and details of the primers see Chapter 2 and Appendix B respectively. Analyses were performed using the TDT, ETDT, GOLD and TRANSMIT. Full details of the statistics and software packages can be found in Chapter 2 and Appendix C.

4.3.4 Results

The variants tested were examined for Hardy Weinberg Equilibrium with no significant deviation from the expected distribution being observed. Table 4.5 shows the results of individual variant association analysis performed using the TDT or ETDT methods. The results show that two of the variants display association with ADHD. These were the 148bp allele (allele 10) of DRD5.PCR1 ($\chi^2=11.1$, $p=0.0009$) and the C allele of the 1481 SNP ($\chi^2=4.96$, $p=0.03$). A trend towards association was observed with the D4S2928 and 978 variants and ADHD cases.

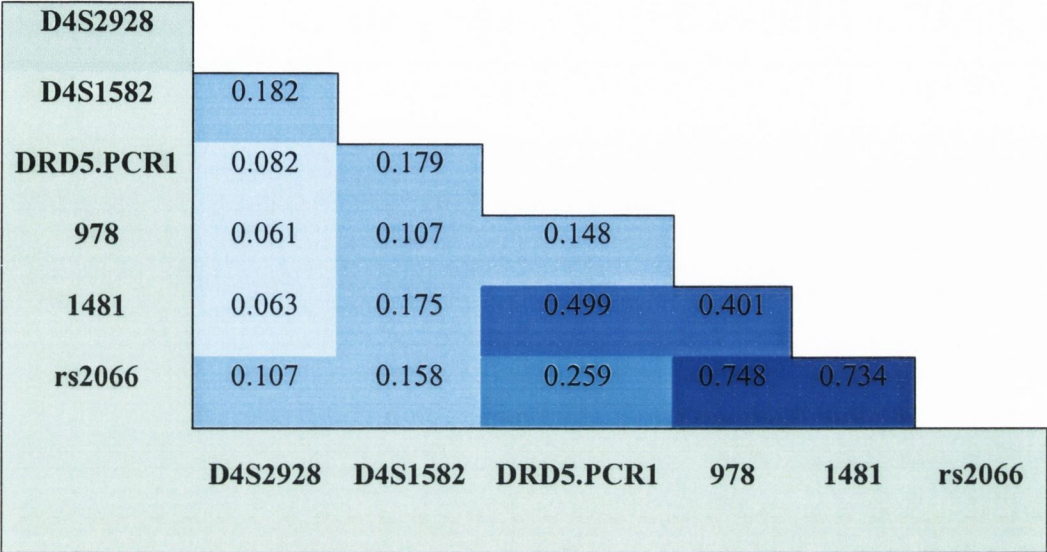
Table 4.5 - TDT Results of Individual Variant Analysis for the Genotyped DRD5 Polymorphisms

Variant	Allele Frequency	T	NT	χ^2	P-value	OR	95%CI
D4S2928 (allele3)	54%	89	70	2.27	0.1532	1.3	0.93-1.74
D4S1582 (allele2)	35%	79	65	1.36	0.2786	1.2	0.88-1.69
DRD5.PCR1 (allele10)	45%	114	69	11.1	0.0009	1.6	1.07-2.29
978 (T allele)	63%	86	65	2.92	0.1033	1.3	0.96-1.83
1481(C allele)	63%	99	70	4.96	0.0309	1.4	1.04-1.92
rs2066 (A allele)	11%	40	31	1.14	0.3425	1.3	0.81-2.06

SNPs 978 and 1481 are numbered in relation to the transcription start site (+1)

LD analysis, shown in Table 4.6 and Figure 4.5 revealed a high level of LD at the 3' end of the gene between the 978 and 1481 SNPs and rs2066 ($D'=0.748$ and 0.734 respectively). LD to a lesser degree was also observed between DRD5.PCR1 and the 1481 and rs2066 SNPs ($D'=0.499$).

Figure 4.5 – LD Values for DRD5 Variants



Darker colours represent larger D' values indicating stronger LD

Table 4.6 - LD Analysis between DRD5 Variants

Variant 1	Variant 2	χ^2	p-value	D'
D4S2928	D4S1582	19.82	0.07066	0.182
D4S2928	DRD5.PCR1	6.96	0.72899	0.082
D4S2928	978	2.30	0.31637	0.061
D4S2928	1481	2.69	0.26086	0.063
D4S2928	rs2066	1.11	0.57529	0.107
D4S1582	DRD5.PCR1	31.73	0.38002	0.107
D4S1582	978	5.88	0.43622	0.107
D4S1582	1481	15.21	0.01869	0.175
D4S1582	rs2066	6.39	0.38106	0.158
DRD5.PCR1	978	9.42	0.09336	0.148
DRD5.PCR1	1481	111.39	0.00000	0.499
DRD5.PCR1	rs2066	17.62	0.00347	0.259
978	1481	51.86	0.00000	0.401
978	rs2066	40.81	0.00000	0.748
1481	rs2066	43.39	0.00000	0.734

Haplotype analyses for all two-variant haplotypes are presented in Table 4.7. Haplotypes of interest have been highlighted in bold. The haplotypes attaining significant levels of association with ADHD consist of D4S1582 (allele 3) and DRD5.PCR1 (allele 10), D4S1582 (allele 7) and DRD5.PCR1 (allele 10), DRD5.PCR1 (allele 10) and 978 (allele T), 978 (allele T) and 1481 (allele T) and the 1481 (allele T) and rs2066 (allele A) haplotype. Significant multi-variant haplotypes are displayed in Table 4.8. As with the two-variant haplotypes allele 10

of the DRD5.PCR1 microsatellite features commonly in the significant results along with the T alleles of the 978 and 1481 SNPs.

Table 4.7 – Two Variant Haplotype Analysis for DRD5 using TRANSMIT

Haplotype	Observed	Expected	χ^2 (1df)	p-value
D4S2928(allele3)/D4S1582(allele2)	79.329	71.88	2.1803	0.139787
D4S2928(allele3)/D4S1582(allele7)	43.897	38.92	1.5802	0.208732
D4S2928(allele4)/D4S1582(allele2)	34.401	33.772	0.03125	0.859684
D4S2928(allele4)/D4S1582(allele7)	37.088	33.415	0.98131	0.321876
D4S1582(allele3)/DRD5.PCR1 (allele10)	63.418	52.547	6.3482	0.011750
D4S1582(allele3)/DRD5.PCR1 (allele5)	11.398	14.134	1.5494	0.213224
D4S1582(allele7)/DRD5.PCR1 (allele10)	50.093	39.173	7.4215	0.006445
D4S1582(allele7)/DRD5.PCR1 (allele5)	7.5366	9.7062	1.1763	0.278111
DRD5.PCR1(allele10)/978(T)	120.55	98.452	13.274	0.000269
DRD5.PCR1(allele10)/978(C)	53.446	53.444	2.73e-07	0.999583
DRD5.PCR1(allele5)/978(T)	17.807	22.777	2.2644	0.132377
DRD5.PCR1(allele5)/978(C)	19.693	21.703	0.46614	0.494768
978(T)/1481(C)	187.54	169.84	7.022	0.008051
978(T)/1481(T)	40.813	48.986	3.4608	0.062840
978(C)/1481(C)	78.622	84.628	1.2086	0.271610
978(C)/1481(T)	45.027	48.546	0.69557	0.404276
1481(T)/rs2066(A)	85.795	97.216	3.3993	0.065224
1481(T)/rs2066(G)	32.832	34.892	0.25707	0.612140
1481(C)/rs2066(A)	225.2	210.06	5.2027	0.02255
1481(C)/rs2066(G)	4.1682	5.8321	1.0042	0.316296

Table 4.8 – Selected Multi Variant Haplotype Analysis for DRD5 using TRANSMIT

Haplotype	Observed	Expected	χ^2 (1df)	p-value
D4S2928(allele3)/D4S1582(allele2)/ DRD5.PCR1(allele10)	37.238	29.928	4.9939	0.025437
D4S2928(allele3)/D4S1582(allele7)/ DRD5.PCR1(allele10)	24.524	19.638	3.2368	0.072001
D4S2928(allele4)/D4S1582(allele7)/ DRD5.PCR1(allele10)	19.903	13.793	6.9935	0.008181
D4S1582(allele2)/ DRD5.PCR1(allele10)/ 978(T)	34.26	28.377	3.1396	0.076413
D4S1582(allele7)/ DRD5.PCR1(allele10)/ 978(T)	39.311	28.36	10.225	0.001385
D4S1582(allele2)/ DRD5.PCR1(allele10)/ 978(C)	27.375	23.04	2.0854	0.148713
DRD5.PCR1(allele10)/978(T)/ 1481(C)	118.32	95.927	13.481	0.000241
978(T)/1481(C)/rs2066(A)	181.92	162.62	8.3311	0.003897

Haplotypes of interest are highlighted in bold type.

4.3.5 Conclusions

The purpose of this study was to map the extent of the association across the DRD5 gene locus and to attempt to find a possible functional variant hypothesised to be in LD with the DRD5.PCR1 microsatellite. The variants chosen for the analysis were the original microsatellite marker (DRD5.PCR1) and two additional 5' microsatellites already examined in relation to ADHD (D4S2928 and D4S1582) (Hawi et al, 2003b). In addition, the two exonic SNPs that were detected during the mutation screen of the gene (978 and 1481) and one 3' SNP (rs2066) (chosen to examine the LD beyond the 3' UTR) were also included.

Individual TDT analysis of these variants revealed a lack of significant association between ADHD and the two most 5' microsatellites although there was excess transmission of one allele in each case. As expected the microsatellite marker located closest to the gene and the focus of the meta analysis (see Chapter 6) showed association with ADHD ($\chi^2=11.1$, $p=0.0009$). Of the 3 SNPs tested only one attained a level of significance exceeding the threshold of $p<0.05$. This was the 1481 SNP ($\chi^2=4.96$, $p=0.0309$).

LD analysis of the variants, displayed low levels of LD at the 5' end of the gene, high levels of LD among the 3 SNPs tested at the 3' end and moderate LD between the SNPs and the DRD5.PCR1 microsatellite. The strongest LD was seen at the 3' end of the gene between the 978 and 1481 SNPs and rs2066, which are located closet together. There was no LD observed between the three 5' microsatellites although there is a much greater distance between them for than the 3' SNPs.

Haplotype analysis, using TRANSMIT, revealed the presence of multiple common haplotypes displaying association with ADHD. These included overlapping haplotypes spread across the gene, with the most significant involving allele 10 of DRD5.PCR1 and the T allele of both the 978 and 1481 SNPs ($\chi^2=13.481$, $p=0.0002$). The most significant haplotypes are comprised of the DRD5.PCR1 microsatellite and SNPs from the 3' end of the gene. This is consistent with the LD analysis, which showed the highest levels of LD at that end of the gene. However, significant haplotypes were also found across areas displaying low levels of LD (ie the 5' microsatellites). These haplotypes all contain the independently significant microsatellite DRD5.PCR1. This suggests that some associated haplotypes are probably due to this variant. This would be possible if the DRD5.PCR1 variant was considerably younger than the other variants. In this situation, as the associated allele is very common (~40%), it may result in significant haplotypes although the LD between variants is low. This is due to the fact that less recombination events will have effected the relative new variant (DRD5.PCR1) compared to the other older microsatellites.

The results of this study are similar to that of Hawi et al, (2003b) in which a subset of this ADHD sample was used (37% overlap between the two samples). Since that study the sample has been extended, re-diagnosed using the CAPA, and individuals no longer willing to participate have been removed. This has resulted in a slight drop in the significance levels of the association with the DRD5.PCR1 microsatellite and the 1481 SNP as well as their haplotype and a loss of the significant association with the D4S1582 microsatellite. As the sample used in this thesis is more stringently diagnosed and is of a larger size it is likely that these

results are more accurate than those of the paper of Hawi et al, (2003b). The overall results for both studies are the same except for the D4S1582 variant. The association with this variant may have been a false positive in the original study or alternatively the sample from that study may simply represent a slightly different clinical phenotype for which the variant was associated.

In conclusion, this study supports a role for the DRD5 gene in the development of ADHD. Association has been demonstrated between Irish ADHD children and both the DRD5.PCR1 microsatellite and the 1481 SNP. Although there is no direct evidence that either of these variants are themselves functional this cannot be concluded without expression studies. Significant haplotypes have been found encompassing variants at the 5' end of the gene, which have also been shown to be in strong LD. Replication of this study by an independent sample, is suggested to confirm the results.

4.4 SNAP-25

4.4.1 Introduction

SNAP-25 is a presynaptic plasma membrane protein that is specifically and highly expressed in nerve cells (Sollner et al, 1993). The gene encodes a protein component, which interacts with the membrane associated protein syntaxin, and the vesical associated membrane protein (VAMP). This complex interacts with membrane proteins known as synaptotagmins to make up a core complex, essential for docking and holding synaptic vesicles at the presynaptic membrane in preparation for Ca^{2+} -triggered neurotransmitter exocytosis (Sollner et al, 1993). Interest in the SNAP-25 gene in relation to ADHD stems from the various behavioural problems displayed by the Coloboma mouse mutant, including ~5 fold increased activity compared to control littermates (Giros et al, 1996). The hyperactive mouse model is a semi dominant mutation (cm/+) which has been shown to be the result of ~2cM deletion encompassing genes including the SNAP-25 (Hess et al, 1994; Xue et al 1999).

In addition to the animal model studies, recent genetic association studies also support a role for the SNAP-25 gene in ADHD susceptibility. Initial analysis conducted by Barr et al (2000) on two SNPs (87610 and 87614) located at the 3'untranslated region of the gene showed no significant association between either variant and ADHD, although an excess transmission of the C allele of 87614 was observed along with the over transmission of a haplotype comprising of the T allele of the 87610 SNP and the C allele of the 87614 SNP. This haplotype was also found to be over-transmitted to ADHD cases in an independent study by Kustanovich et al

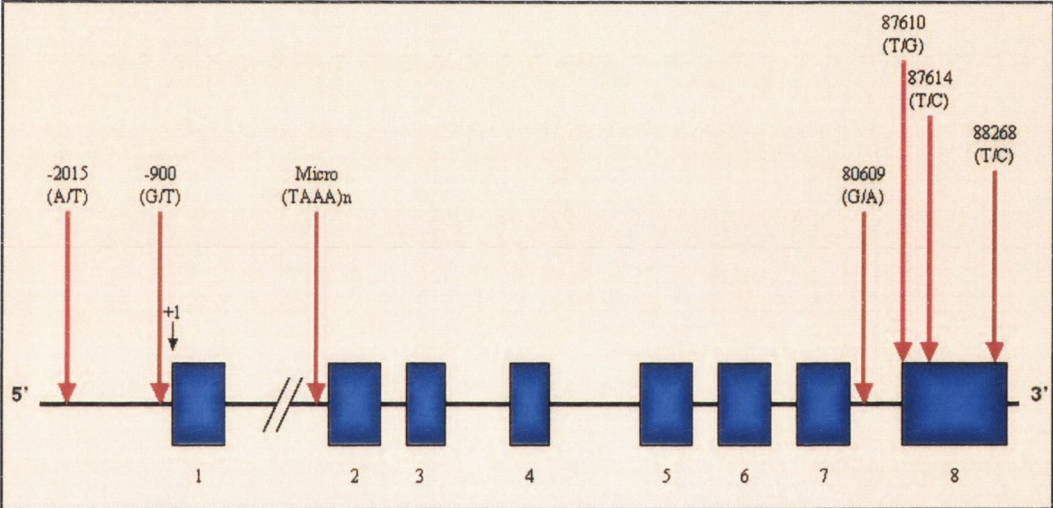
(2003), although this result was not statistically significant. In addition, Brophy et al (2002) reported over transmission of the other allele (allele T) of the 87614 SNP to ADHD cases from the Irish population (an earlier analysis of a subset of the sample used in this thesis). Two other studies by Mill et al. (2002 and 2004), which included a mutation screen of the gene using DHPLC, have examined an additional 6 variants. These studies have shown association between ADHD and a microsatellite marker (located to the first intron of the gene) and the 80609 and – 2015 SNPs using the TDT.

In this study, 7 variants from the SNAP-25 gene and surrounding regions are genotyped in our Irish ADHD sample and analysed using TDT, haplotype and LD methods in an attempt to further examine the role of SNAP-25 in ADHD and narrow the region of association.

4.4.2 Gene Structure and Location of Analysed Variants

The SNAP-25 gene maps to human 20p11.2 (Maglott et al, 1996). Figure 4.6 displays the structure of the SNAP-25 gene and indicates the location of the analysed variants. Sequence analysis shows that the SNAP-25 gene has two possible transcripts comprising of either 7 or 8 exons with a total cDNA size of 1841bp or 2053bp respectively (Accession Numbers = NM_003081 and NM_130811). In the case of the 7 exon transcript it is exon 5 that is missing from the sequence.

Figure 4.6 - Structure of the SNAP-25 Gene Showing the Location of Analysed Polymorphisms



The blue rectangles represent the exons of the gene. +1 indicates the transcription start site

4.4.3 Materials and Methods

All seven variants were genotyped for the sample previously described in Chapter 2. The microsatellite marker was PCR amplified using fluorescently labelled primers and the product was analysed using the ABI 377 DNA Sequencer. The -2015, -900, 80609 and 88268 SNPs were genotyped using the SNaPshot primer extension method. Details of amplification primers and conditions as well as extension primers can be found in Appendix B. The 87610 and 87614 SNPs were amplified using the primers and conditions outlined in Appendix B and then digested with restriction enzymes MnlI and DdeI respectively. Digested fragments were then electrophoresed on 6% polyacrylamide gels and stained with ethidium bromide. Analyses were performed using the TDT, ETDT, GOLD and TRANSMIT. Full details of the statistics and software packages can be found in Chapter 2 and Appendix C.

4.4.4 Results

The following Tables (4.9-4.12) show the complete results of TDT, haplotype and LD analysis for the seven SNAP-25 variants tested in the Irish population sample. The variants were tested for Hardy Weinberg Equilibrium and no deviation from the expected equation was observed.

Table 4.9 shows the results of the variants when analysed individually using the TDT or ETDT. None of the variants display individual significant association with ADHD, however several, including the -2015, 87610, 87614 and 88268 SNPs shown a non significant over transmission of one of their alleles.

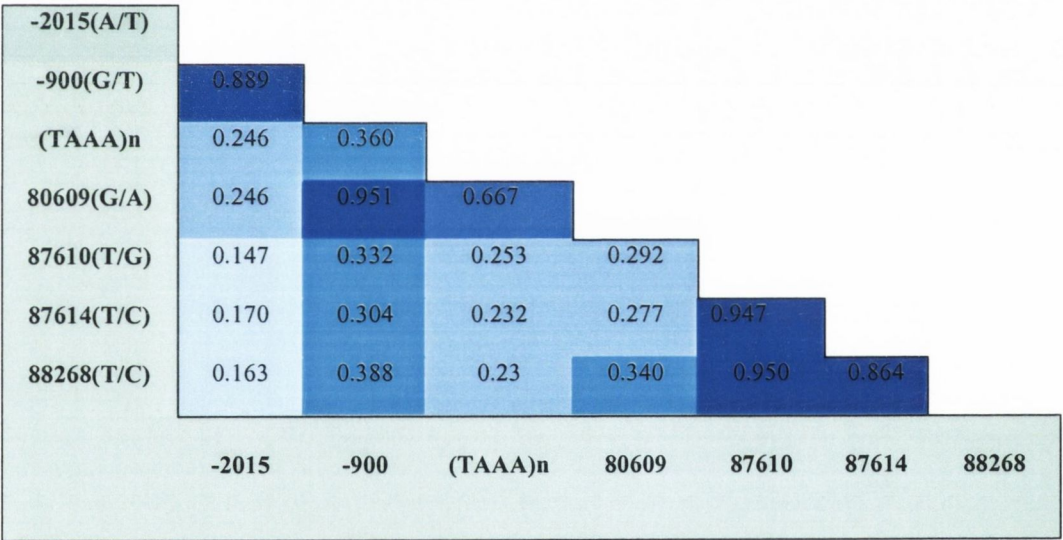
Table 4.9 - TDT Results of Individual Variant Analysis for the Genotyped SNAP-25 gene

Variant	Allele Frequency	T	NT	χ^2	p-value	OR	95%CI
-2015 (T)	55%	87	68	2.6	0.13	1.3	0.93-1.76
-900 (G)	89%	38	33	0.4	0.64	1.2	0.72-1.84
(TAAA)n (allele4)	18%	46	38	1.2	0.45	1.2	0.79-1.86
(TAAA)n (allele5)	64%	87	76	0.4	1.14	1.1	0.84-1.56
80609 (A)	15%	41	39	0.05	0.91	1.1	0.68-1.63
87610 (G)	38%	90	68	3.1	0.095	1.3	0.97-1.81
87614 (T)	81%	70	52	2.7	0.12	1.4	0.94-1.93
88268 (T)	39%	97	75	2.8	0.1	1.3	0.96-1.75

SNPs are numbered in relation to the transcription start site (+1)

LD analysis showed strong LD between the two SNPs located 5' to the gene (-2015 and -900) and also at the other end of the gene between the three SNPs 87610, 87614 and 88268. These results are displayed in Table 4.10 and Figure 4.7 as D' values with associated p-values. In addition, there is also some evidence (although the D' values are smaller) of LD between the microsatellite marker and 80609 and between -900 and 80609. Taking the results, as an overall picture however there appears to be two areas of high LD located either end of the gene separated by a region of lower LD. This region of low LD may be attributed to the lack of analysed variants in this region.

Figure 4.7 – LD Values for SNAP-25 Variants



Darker colours represent larger D' values indicating stronger LD

Table 4.10 - LD Analysis between SNAP-25 Variants

Variant 1	Variant 2	Chi Sq	p-value	D'
-2015	-900	33.43	0.00000	0.889
-2015	Micro	13.70	0.00334	0.246
-2015	80609	3.19	0.07408	0.226
-2015	87610	4.57	0.03258	0.147
-2015	87614	1.96	0.16156	0.170
-2015	88268	5.97	0.01455	0.163
-900	Micro	19.68	0.00020	0.360
-900	80609	4.63	0.03148	0.951
-900	87610	2.15	0.14262	0.332
-900	87614	0.87	0.35218	0.304
-900	88268	3.19	0.07417	0.388
Micro	80609	182.93	0.00000	0.667
Micro	87610	7.91	0.04799	0.253
Micro	87614	19.41	0.00023	0.232
Micro	88268	6.80	0.07867	0.204
80609	87610	2.95	0.08614	0.292
80609	87614	14.53	0.00014	0.277
80609	88268	4.28	0.03848	0.340
87610	87614	38.36	0.00000	0.947
87610	88268	437.88	0.00000	0.950
87614	88268	35.41	0.00000	0.864

Two-variant haplotype analysis did not reveal any significant haplotypes among the genotype variants (results are shown in Table 4.11). Multi-variant analysis (Table 4.12) did display the presence of two four-variant haplotypes and one five-variant haplotype that reached nominal statistical significance. Of these the greatest level of association with ADHD was the five-variant haplotype composed of the -900 SNP (allele G), the microsatellite (allele 5) and the 80609 (allele G), 87610 (allele G) and 87614 (allele T) SNPs ($\chi^2=5.53$, $p=0.019$).

Table 4.11 – Two Variant Haplotype Analysis for SNAP-25 using TRANSMIT

Haplotype	Observed	Expected	χ^2 (1df)	p-value
-2015 (T)/-900 (G)	154.63	146.78	1.5832	0.208300
-2015 (A)/-900 (G)	147.87	155.51	1.5262	0.216684
-2015 (T)/-900 (T)	37.384	37.106	0.0043577	0.947368
-2015 (T)/-900 (T)	0.11579	0.599	0.9322	0.334292
-900(G)/Micro(allele4)	44.05	40.598	0.78262	0.376341
-900(T)/Micro(allele4)	18.248	17.604	0.050956	0.821408
-900(G)/Micro(allele5)	200.65	196.25	0.48	0.488422
-900(T)/Micro(allele4)	16.701	17.525	0.084249	0.771620
Micro(allele4)/80609(G)	67.135	62.732	0.86078	0.353521
Micro(allele5)/80609(G)	210.52	206	0.47741	0.489598
Micro(allele5)/80609(A)	14.577	14.947	0.019371	0.889308
80609(G)/87610(T)	173.17	181.93	1.8015	0.179531
80609(A)/87610(T)	38.995	38.057	0.054339	0.815678
80609(G)/87610(G)	122.83	116.32	1.1385	0.285970
80609(A)/87610(G)	15.005	13.689	0.3021	0.582569
87610(T)/87614(T)	150.28	151.03	0.01437	0.904582
87610(A)/87614(T)	133.22	124.85	1.7471	0.186242
87610(T)/87614(C)	60.263	66.411	1.314	0.251672
87610(A)/87614(C)	2.2365	3.7143	1.7568	0.185024
87614(T)/88268(T)	145.41	148.76	0.27116	0.602555
87614(C)/88268(T)	60.07	66.788	1.5734	0.209714
87614(T)/88268(C)	139.09	127.96	2.8454	0.091636
87614(C)/88268(C)	1.4302	2.4938	1.1426	0.285104

Haplotypes of interest are highlighted in bold

Table 4.12 – Selected Multi Variant Haplotype Analysis for SNAP-25 using TRANSMIT

Haplotype	Observed	Expected	χ^2 (1df)	p-value
-2015 (T)/-900 (G)/Micro(allele5)	90.937	82.997	2.2155	0.136631
Micro(allele5)/80609(G)/87610(G)	93.067	83.318	3.4573	0.062973
80609(G)/87610(G)/87614(T)	118.73	111.34	1.504	0.220057
87610(G)/87614(T)/88268(C)	129.63	119.1	2.6859	0.101240
-2015 (T)/-900(G)/Micro(allele5)/ 80609(G)	82.484	76.803	1.2168	0.269990
900(G)/Micro(allele5)/80609(G)/ 87610(G)	87.558	76.716	4.6405	0.031226
Micro(5allele)/80609(G)/ 87610(G)/ 87614(T)	90.276	79.1	4.7424	0.029428
-900 (G)/Micro(allele5)/ 80609(G)/ 87610(G)/ 87614(T)	85.944	74.125	5.5281	0.018713

Haplotypes of particular interest are highlighted in bold

4.4.5 Conclusions

A total of seven variants were examined throughout the SNAP-25 gene and promoter region. Of these, none displayed independent association with ADHD when tested with the TDT, although 4 of the SNPs (-2015, 80609, 87610 and 87614) did show non-significant preferential transmission of one allele over the other. However, three overlapping haplotypes (shown in bold type in Table 4.12) showed significant association with ADHD. LD analysis indicated two areas of high LD located at either end of the gene with a region of lower LD between them. This may imply the presence of greater recombination in this middle area. However it is more likely that the LD pattern observed is due to a lack of tested variants in the middle region.

To date only a small number of studies have investigated the relationship between ADHD and the SNAP-25 gene. Barr et al (2000) examined the 87610 and 87614 SNPs located at the 3' un-translated region of SNAP-25 gene in a Canadian sample of 97 ADHD trios, with significant over-transmission of a haplotype consisting of the T allele of 87610 and the C allele of 87614 to ADHD cases reported ($\chi^2 4.738$, $p=0.03$). Although, the two variants are in close proximity to each other and in high LD according to the study in this thesis ($D'=0.947$), we failed to replicate this finding, in fact observing more instances of the haplotype being not transmitted than transmitted. (ie the opposite to the Barr et al study). In addition to these findings Kustanovich et al (2003) reported a non significant increase in the transmission of the T allele of the 87610 SNP to the ADHD cases, which increased beyond the significance level of $p<0.05$ when the data were examined by paternal transmission alone. We too observed an increase in our transmission values for this variant when

analysed for paternal transmission alone (Chapter 5, Section 5.3.1) however our observation was with the other allele.

Mill et al (2002, 2004) have published two reports investigating the relation between SNAP-25 and ADHD. The first of these studies reports evidence of association between their ADHD cases and allele 5 of the (TAAA)_n microsatellite of intron 1 in a case control and family based analyses (Mill et al, 2002). Although the study in this thesis did not replicate the association, it does display non significant increased transmission of the same allele to our ADHD cases.

The second paper (Mill et al, 2004) consisted of a mutation screen of the gene and association analysis of the discovered variants. They reported significant associations between allele A of -2015 ($\chi^2=4.55, p=0.03$) and allele G of 80609 ($\chi^2=4.7, p=0.03$) and ADHD. This study observed a trend toward association with the T allele of the -2015 SNP, the opposite direction to that found by Mill et al, and no evidence of distortion in the transmission of the alleles at 80609. In contrast, we noted an insignificant increase of allele T of the variant 88268, which was not associated with ADHD in Mill et al 2004.

It is hard to reconcile all the inconsistencies of the studies on this gene to date. Particularly as the associated or over transmitted alleles are not consistent throughout. Although it may be premature to exclude SNAP-25 from having a role in the aetiology of ADHD altogether, I am not convinced at this time that there is sufficient evidence to conclude that it is a susceptibility gene for ADHD. The variants examined to date mainly cluster at either end of the gene leaving no

investigation of the region between exon 2 and 7. This area will need to be extensively examined before of the role, if any, of SNAP-25 in the aetiology of ADHD can be determined. This will most probably mean returning to the gene and exploring the large expanse of intronic sequence as the exons of the gene have already been screened (Mill et al, 2004) and the variants tested.



Chapter 5

Phenotype : Genotype Analysis of DRD4, DRD5 and SNAP-25

5.1 Introduction

The use of operational diagnostic criteria for ADHD as described by DSM IV in 1994 has resulted in a considerably improved reliability of the diagnosis. This has been advantageous to the development of genetic studies of the disorder, as the clinical samples being tested are more homogeneous. Despite this, ADHD genetics research still sees a marked variability in reported findings. Accepted reasons for this variability include small samples sizes (lacking in power) and false positive findings but it is also acknowledged that the DSM IV may still be too broad or that it may represent a clinically heterogeneous group of conditions.

Clinical studies have shown that ADHD comorbid with conduct disorder (CD) is a clinically more severe condition and has a worse outcome than ADHD alone (Barkley, 1990; Jensen et al, 1997; Kuhne et al, 1997). Family studies have suggested that ADHD with comorbid conduct disorder may represent a more familial subtype (Faraone et al, 1998b). Furthermore, a twin study by Thapar et al (2001) suggested that children with ADHD and comorbid CD might represent a more genetically loaded, as well as clinically severe, group than children with only ADHD. This group confirmed that 1 Irish and 2 UK samples together showed association between ADHD and the 7-repeat DRD4 allele only when the ‘affected’ diagnosis was restricted to ADHD individuals with comorbid CD symptoms (Holmes et al, 2002). Kirley et al, (2004) also reported association between the 7-repeat allele and ADHD individuals comorbid with ODD but not with children diagnosed as ADHD without comorbid ODD (Chapter 5, Section 5.3.2).

It is recognised that methylphenidate and other stimulant medications are effective in alleviating ADHD symptoms in most cases. However, the factors that differentiate between responders and non-responders or between those who experience side effects and those who don't are unknown. Recent literature suggests that these differing responses to medication may be due to individual genetic make-up. Several studies have examined medication response in ADHD individuals in relation to DAT1, resulting in three studies reporting association between the 10-repeat DAT1 allele and poor response to methylphenidate (Winsberg and Comings, 1999; Roman et al, 2002) and one with good response to the stimulant (Kirley et al, 2003).

As ADHD is recognised as having a strong genetic contribution it might be expected that there would be differences between children with a family history of ADHD and those without. Those families with ADHD in more than one individual might be expected to have a more heritable and therefore more homogeneous form of the disorder. There has been very little research into this area however, Kirley et al (2004) has reported that the 7-repeat allele of the DRD4 gene is associated with a positive family history of ADHD

In addition to variation in genetic association coupled with family history, there have also been a number of reports in relation to the sex of the parent transmitting the risk allele to the child. Evidence of increased transmission of the risk allele from fathers to ADHD children has been reported in relation to the BDNF and SNAP-25 genes. This subject of biased transmission of one parent over another is currently being extensively examined by our group. Our findings, which support the

published literature, show increased transmission of the high risk allele from fathers compared to mothers for a number of genes with established association. This work is currently being prepared for publication.

Based on the above findings, the variants typed for the fine mapping association analysis of Chapter 4 were also examined in sub groups according to the sex of the transmitting parent, a family history of ADHD, response to methylphenidate medication, and a comorbid diagnosis of ODD and CD.

5.2 Materials and Methods

For full details of laboratory methods used to genotype the variants see Chapter 2, Section 2.2.8. The TDT and ETDT (Chapter 2, Sections 2.3.2 and 2.3.3) were used to determine which sub groups showed significant association or increased ORs compared to the overall ADHD sample for a given variant. In the cases where the association appeared to be due to only one sub group of the analysis (ie only family history positive and not family history negative) the results were examined using logistic regression. This was to ascertain if the variation between the two sub groups was statistically significant. Logistic regression (2.3.6) was performed using SPSS (Version 11.0). The sub categories examined were sex of transmitting parent, the presence of a family history of ADHD, response to methylphenidate medication and the presence of comorbid ODD and CD. For further details in relation to the ascertainment of family history, medication response and comorbid conditions see Chapter 2, Section 2.1.2.

5.3 Results

5.3.1 – TDT Analysis of DRD4, DRD5 and SNAP-25 Gene Variants for Sub Groups of the Total ADHD Sample

The results of the TDT analyses for each subgroup tested are presented by gene in Tables 5.1 (DRD4), 5.2 (DRD5) and 5.3 (SNAP-25). Examination of the 120bp and -376 DRD4 gene variants showed no evidence of association with any of the subgroups tested. The C allele of the -616 SNP, which showed association with the total sample ($\chi^2=7.45$, $p=0.008$, $OR=1.63$), demonstrated an increase in the strength of association when individuals comorbid for CD were excluded from the sample ($\chi^2=9.15$, $p=0.002$, $OR=1.84$). The OR also increased from 1.6 to 1.8 for the refined phenotype. The -521 variant, which was not found to be associated in our total sample attained a level of significant association when only paternal transmissions were considered ($\chi^2=4.63$, $p=0.031$, $OR=1.69$). The results for the VNTR, which displayed no significant association with the broad DSM IV ADHD phenotype, demonstrated a significant association with ADHD individuals with a family history of ADHD ($\chi^2=9.80$, $p=0.002$, $OR=2.75$) and also with those comorbid for ODD ($\chi^2=5.77$, $p=0.016$, $OR=2.25$) and those comorbid with CD ($\chi^2=4.19$, $p=0.041$, $OR=1.65$).

In the case of the DRD5 variants, the D4S2928 variant shows association between ADHD and maternal transmission alone but not paternal transmission. Analysis of the transmission of both parents (Table 5.2) did not demonstrate significant association with ADHD and this variant. Furthermore, there is also evidence that the

variant is associated with an ADHD phenotype that is negative for comorbid ODD ($\chi^2=4.65$, $p=0.031$, $OR=1.61$) but positive for comorbid CD ($\chi^2=3.85$, $p=0.05$, $OR=2.25$). The D4S1582 variant shows suggestive evidence ($\chi^2=3.17$, $p=0.075$, $OR=1.54$) that allele 2 may be associated with ADHD comorbid with ODD rather than ADHD alone. None of the sub groups displayed stronger association with the DRD5.PCR1 variant, than the broad DSM IV phenotype ($\chi^2=11.07$, $p=0.0009$, $OR=1.6$). The T allele of the 978 SNP (which did not show association with ADHD in the total sample ($\chi^2=2.92$, $p=0.103$, $OR=1.32$) displays association with ADHD when only paternal transmission is considered ($\chi^2=4.13$, $p=0.042$, $OR=1.7$) and also in the absence of a family history of the disorder ($\chi^2=4.46$, $p=0.035$, $OR=1.61$). There is also evidence that the C allele of the 1481 SNP is associated with a 'good' response to methylphenidate ($\chi^2=6.90$, $p=0.009$, $OR=2.05$) and that the A allele of rs2066 is associated with ADHD without comorbid ODD ($\chi^2=8.76$, $p=0.003$, $OR=3.13$).

Analysis of the SNAP-25 gene, which has already been discussed in relation to the highly inconsistent findings reported in the literature and in this thesis 4.4.5, showed no association between any of the sub groups tested and the -2015, -900, 80609, 87610 or microsatellite variants. Significant association was observed between ADHD and the T alleles of the 87614 and 88268 SNPs respectively, when only paternal transmission was considered ($\chi^2=3.93$, $p=0.047$, $OR=1.86$) ($\chi^2=5.06$, $p=0.024$, $OR=1.78$). In addition, ADHD individuals with a family history of the disorder also showed significant association with allele T of the 88268 variant ($\chi^2=4.06$, $p=0.044$, $OR=1.54$).

Table 5.1 - TDT Analysis of DRD4 Variants for Sub Groups of the Total ADHD Sample

DRD4 Variants		DSM IV ADHD	Transmitting Parent		Family History		Medication Response		Comorbid ODD		Comorbid CD	
			Father	Mother	Positive	Negative	Poor/Mod	Good	Positive	Negative	Positive	Negative
Numbers (N)		178	170	178	90	82	30	68	86	88	30	144
120bp (Long)	Trans	48	24	14	28	17	11	17	22	26	14	34
	Not Trans	46	18	19	25	21	10	14	25	21	11	35
	χ^2	0.04	0.86	0.76	0.17	0.42	0.05	0.29	0.19	0.53	0.36	0.01
	p-value	0.918	0.354	0.383	0.680	0.517	0.823	0.590	0.663	0.467	0.549	0.920
	OR	1.04	1.33	0.73	1.12	0.81	1.10	1.21	0.88	1.24	1.27	0.97
-616 (C)	Trans	80	40	33	39	38	11	29	39	40	11	68
	Not Trans	49	23	19	20	29	6	21	21	26	10	37
	χ^2	7.45	4.59	3.77	6.12	1.21	1.47	1.28	5.40	2.97	0.05	9.15
	p-value	0.008	0.032	0.052	0.013	0.271	0.225	0.258	0.020	0.085	0.823	0.002
	OR	1.63	1.74	1.74	1.95	1.31	1.83	1.38	1.86	1.54	1.10	1.84
-521 (A)	Trans	94	44	30	49	40	14	36	46	45	16	75
	Not Trans	75	26	33	35	36	14	33	38	36	12	62
	χ^2	2.14	4.63	0.14	2.33	0.21	0.00	0.13	0.76	1.00	0.57	1.23
	p-value	0.166	0.031	0.708	0.127	0.647	1.000	0.718	0.383	0.317	0.450	0.267
	OR	1.25	1.69	0.91	1.40	1.11	1.00	1.09	1.21	1.25	1.33	1.21
-376 (C)	Trans	16	8	7	9	7	1	4	7	9	2	14
	Not Trans	12	7	4	4	8	3	6	6	5	2	9
	χ^2	0.14	0.07	0.82	1.92	0.07	1.00	0.40	0.08	1.14	0.00	1.09
	p-value	0.572	0.791	0.365	0.166	0.791	0.317	0.527	0.777	0.708	1.000	0.296
	OR	1.33	1.14	1.75	2.25	0.88	0.33	0.67	1.17	1.80	1.00	1.56
VNTR (7rpt)	Trans	50	20	23	33	15	13	20	27	22	6	43
	Not Trans	37	19	12	12	25	7	15	12	24	10	26
	χ^2	1.94	0.03	3.46	9.80	2.50	1.80	0.71	5.77	0.09	1.00	4.19
	p-value	0.198	0.862	0.063	0.002	0.114	0.180	0.399	0.016	0.764	0.317	0.041
	OR	1.35	1.05	1.92	2.75	0.60	1.86	1.33	2.25	0.92	0.60	1.65

Mod = moderate

Table 5.2 - TDT Analysis of DRD5 Variants for Sub Groups of the Total ADHD Sample

DRD5 Variants		DSM IV ADHD	Transmitting Parent		Family History		Medication Response		Comorbid ODD		Comorbid CD	
			Father	Mother	Positive	Negative	Poor/Mod	Good	Positive	Negative	Positive	Negative
Numbers (N)		178	170	178	90	82	30	68	86	88	30	144
D4S2928 (allele3)	Trans	89	35	44	43	46	17	33	39	53	18	74
	Not Trans	70	29	27	35	31	10	28	36	33	8	61
	χ^2	2.27	0.56	4.07	0.82	2.92	1.81	0.41	0.12	4.65	3.85	1.25
	p-value	0.153	0.454	0.044	0.365	0.087	0.179	0.522	0.729	0.031	0.050	0.264
	OR	1.27	1.21	1.63	1.23	1.48	1.70	1.18	1.08	1.61	2.25	1.21
D4S1582 (allele2)	Trans	79	31	42	47	32	15	28	43	35	13	65
	Not Trans	65	26	31	34	30	9	29	28	38	13	53
	χ^2	1.36	0.44	1.66	2.09	0.06	1.50	0.02	3.17	0.12	0.00	1.22
	p-value	0.279	0.507	0.198	0.148	0.806	0.221	0.888	0.075	0.729	1.000	0.269
	OR	1.22	1.19	1.35	1.38	1.07	1.67	0.97	1.54	0.92	1.00	1.23
DRD5. PCR1 (allele6)	Trans	114	56	54	52	59	16	45	56	55	18	93
	Not Trans	69	30	36	35	31	13	26	35	32	12	55
	χ^2	11.07	7.86	3.60	3.32	8.71	0.31	5.08	4.85	6.08	1.20	9.76
	p-value	0.0009	0.005	0.058	0.068	0.003	0.578	0.024	0.028	0.014	0.273	0.002
	OR	1.6	1.87	1.50	1.49	1.90	1.23	1.73	1.60	1.72	1.50	1.69
978 (T)	Trans	86	39	34	33	50	18	33	36	48	18	66
	Not Trans	65	23	26	33	31	10	22	33	31	9	55
	χ^2	2.92	4.13	1.07	0.00	4.46	2.29	2.20	0.13	3.66	3.00	1.00
	p-value	0.103	0.042	0.301	1.000	0.035	0.130	0.138	0.718	0.056	0.083	0.317
	OR	1.32	1.70	1.31	1.00	1.61	1.80	1.50	1.09	1.55	2.00	1.20
1481(C)	Trans	99	43	42	54	41	16	39	49	49	17	81
	Not Trans	70	28	29	38	29	10	19	35	34	10	59
	χ^2	4.96	3.17	2.38	2.78	2.06	1.38	6.90	2.33	2.71	1.81	3.46
	p-value	0.031	0.075	0.123	0.095	0.151	0.240	0.009	0.127	0.100	0.179	0.063
	OR	1.41	1.54	1.45	1.42	1.41	1.60	2.05	1.40	1.44	1.70	1.37
rs2066 (A)	Trans	40	21	15	16	21	10	16	15	25	5	35
	Not Trans	31	14	13	16	14	7	10	22	8	4	26
	χ^2	1.14	1.40	0.14	0.00	1.40	0.53	1.38	1.32	8.76	0.11	1.33
	p-value	0.342	0.237	0.708	1.000	0.237	0.467	0.240	0.249	0.003	0.740	0.249
	OR	1.29	1.50	1.15	1.00	1.50	1.43	1.60	0.68	3.13	1.25	1.35

Mod = moderate

Table 5.3 - TDT Analysis of SNAP-25 Variants for Sub Groups of the Total ADHD Sample

SNAP-25 Markers		DSM IV ADHD	Transmitting Parent		Family History		Medication Response		Comorbid ODD		Comorbid CD	
			Father	Mother	Positive	Negative	Poor/Mod	Good	Positive	Negative	Positive	Negative
Numbers (N)		178	170	178	90	82	30	68	86	88	30	144
-2015 (T)	Trans	87	35	36	39	46	15	34	50	35	10	75
	Not Trans	68	26	30	35	30	18	26	35	32	8	59
	χ^2	2.6	1.33	0.55	0.22	3.37	0.27	1.07	2.65	0.13	0.22	1.91
	p-value	0.107	0.249	0.458	0.639	0.066	0.603	0.301	0.507	0.718	0.639	0.167
	OR	1.279	1.346	1.200	1.114	1.533	0.833	1.308	1.429	1.094	1.250	1.271
-900 (G)	Trans	38	18	18	20	17	8	13	18	20	5	33
	Not Trans	33	14	17	11	19	6	12	12	18	4	26
	χ^2	0.4	0.50	0.03	2.61	0.11	0.29	0.04	1.20	0.11	0.11	0.83
	p-value	0.527	0.480	0.862	0.106	0.740	0.590	0.841	0.273	0.740	0.740	0.362
	OR	1.152	1.286	1.059	1.818	0.895	1.333	1.083	1.500	1.111	1.250	1.269
(TAAA)n (allele4)	Trans	46	24	17	15	27	7	13	23	21	6	38
	Not Trans	38	16	17	20	19	9	16	24	15	2	37
	χ^2	1.20	1.60	0.00	0.71	1.39	0.25	0.31	0.02	1.00	2.00	0.01
	p-value	0.273	0.206	1.000	0.399	0.238	0.617	0.578	0.888	0.317	0.157	0.920
	OR	1.211	1.500	1.000	0.750	1.421	0.778	0.813	0.958	1.400	3.000	1.027
(TAAA)n (allele5)	Trans	87	34	46	51	36	20	32	42	46	14	74
	Not Trans	76	36	33	35	39	14	28	39	36	14	61
	χ^2	0.4	0.06	2.14	2.98	0.12	1.06	0.27	0.11	1.22	0.00	1.25
	p-value	0.527	0.806	0.144	0.084	0.729	0.303	0.603	0.740	0.269	1.000	0.264
	OR	1.145	0.944	1.394	1.457	0.923	1.429	1.143	1.077	1.278	1.000	1.213
80609 (A)	Trans	41	16	18	20	23	8	19	16	26	9	33
	Not Trans	39	9	24	21	17	9	11	14	24	10	28
	χ^2	0.1	1.96	0.86	0.02	0.90	0.06	2.13	0.13	0.08	0.05	0.41
	p-value	0.752	0.162	0.354	0.888	0.343	0.806	0.144	0.288	0.777	0.823	0.522
	OR	1.051	1.778	0.750	0.952	1.353	0.889	1.727	1.143	1.083	0.900	1.179
87610 (G)	Trans	90	33	33	50	38	15	32	40	46	16	70
	Not Trans	68	22	28	35	31	15	27	31	36	15	52
	χ^2	3.1	2.20	0.41	2.65	0.71	0.00	0.42	1.14	1.22	0.03	2.66
	p-value	0.078	0.138	0.522	0.104	0.399	1.000	0.517	0.286	0.269	0.862	0.103
	OR	1.324	1.500	1.179	1.429	1.226	1.000	1.185	1.290	1.278	1.067	1.346

Table 5.3 continued - TDT Analysis of Subgroups of SNAP-25 Variants

SNAP-25 Markers		DSM IV ADHD	Transmitting Parent		Family History		Medication Response		Comorbid ODD		Comorbid CD	
			Father	Mother	Positive	Negative	Poor/Mod	Good	Positive	Negative	Positive	Negative
Numbers (N)		178	170	178	90	82	30	68	86	88	30	144
87614 (T)	Trans	70	28	31	38	32	12	24	38	31	12	57
	Not Trans	52	15	29	28	23	9	16	30	21	10	41
	χ^2	2.7	3.93	0.07	1.52	1.47	0.43	1.60	0.94	1.92	0.18	2.61
	p-value	0.100	0.047	0.791	0.218	0.225	0.512	0.206	0.332	0.166	0.671	0.106
	OR	1.346	1.867	1.069	1.357	1.391	1.333	1.500	1.267	1.476	1.200	1.390
88268 (T)	Trans	97	41	31	54	42	16	35	41	52	18	75
	Not Trans	75	23	29	35	37	20	24	35	39	12	62
	χ^2	2.8	5.06	0.07	4.06	0.32	0.44	2.05	0.47	1.86	1.20	1.23
	p-value	0.094	0.024	0.791	0.044	0.572	0.507	0.152	0.493	0.173	0.273	0.267
	OR	1.293	1.783	1.069	1.543	1.135	0.800	1.458	1.171	1.333	1.500	1.210

Mod = moderate

5.3.2 – Logistic Regression Analysis of Sub Groups TDT Results for DRD4, DRD5 and SNAP-25 Gene Variants

On visual inspection, where one sub group appeared to be associated with a genetic variant, compared to another, these data were further analysed using logistic regression (LR) (2.3.6) to determine if the two sub groups differed statistically in terms of the comparative strength of their association. A statistical difference between the two groups would imply that they were two distinct sub samples, one of which was associated with the variant and one that was not. The results of these analyses can be seen in Tables 5.4 (DRD4), 5.5 (DRD5) and 5.6 (SNAP-25). In the text the results of the logistic regression analysis, shown in brackets, are preceded by LR, all other results presented are from the TDT analysis.

The statistically different sub groups displaying association with the VNTR 7-repeat allele of the DRD4 gene (Table 5.4) were, those with a positive family history of the disorder ($\chi^2=9.80$, $p=0.002$, $OR=2.75$), compared to those with a negative family history of the disorder ($\chi^2=2.50$, $p=0.114$, $OR=0.60$) (LR $\chi^2=10.52$, $p=0.001$) and those ADHD individuals with comorbid ODD ($\chi^2=5.77$, $p=0.016$, $OR=2.25$) versus those without comorbid ODD ($\chi^2=0.09$, $p=0.764$, $OR=0.92$) (LR $\chi^2=3.89$, $p=0.049$). In addition, comparisons between the subgroups of ADHD with comorbid CD ($\chi^2=1.00$, $p=0.317$, $OR=0.60$) and ADHD without comorbid CD ($\chi^2=4.19$, $p=0.041$, $OR=1.65$), in relation to the 7-repeat allele of the DRD4 VNTR, as well as paternal ($\chi^2=4.63$, $p=0.031$, $OR=1.69$) versus maternal ($\chi^2=0.14$, $p=0.708$, $OR=0.91$) allele transmission, in relation to the A allele of the DRD4 -521 SNP, were almost significant with LR results of (LR $\chi^2=3.13$, $p=0.077$) and (L $\chi^2=3.09$, $p=0.079$) respectively.

For the DRD5 variants tested (Table 5.5) the only subgroups displaying statistically significant differences to each other (LR $\chi^2=8.36$, $p=0.004$) were the ADHD subgroup without ODD, which was associated with the rs2066 SNP ($\chi^2=8.76$, $p=0.003$, OR=3.13) and to the ADHD subgroup with ODD, which was not ($\chi^2=1.32$, $p=0.249$, OR=0.68). This implies that the ADHD children negative for comorbid ODD are an independent phenotype with a significant association with the A allele of the rs2066 variant.

The SNAP-25 LR analysis of the association with paternal allele transmission versus maternal allele transmission (SNPs 87614 and 88268) and a positive versus negative family history of ADHD (SNP 88268) were not found to be statistically significant (see Table 5.6).

Table 5.4 - Logistic Regression of Subgroups of DRD4 Variants

Variant	Sub Group Results			Logistic Regression	
616		Comorbid CD		χ^2	p-value
		Positive	Negative		
	Trans	11	68	0.95	0.330
	Not Trans	10	37		
	χ^2	0.05	9.15		
	p-value	0.823	0.002		
521		Transmitting Parent		χ^2	p-value
		Father	Mother		
	Trans	44	30	3.09	0.079
	Not Trans	26	33		
	χ^2	4.63	0.14		
	p-value	0.031	0.708		
VNTR		Family History		χ^2	p-value
		Positive	Negative		
	Trans	33	15	10.52	0.001
	Not Trans	12	25		
	χ^2	9.80	2.50		
	p-value	0.002	0.114		
		Comorbid ODD		χ^2	p-value
		Positive	Negative		
	Trans	27	22	3.89	0.049
	Not Trans	12	24		
	χ^2	5.77	0.09		
	p-value	0.016	0.764		
		Comorbid CD		χ^2	p-value
		Positive	Negative		
	Trans	6	43	3.13	0.077
	Not Trans	10	26		
	χ^2	1.00	4.19		
	p-value	0.317	0.041		

Table 5.5 - Logistic Regression of Subgroups of DRD5 Variants

Variant	Sub Group Results			Logistic Regression	
D4S2928		Transmitting Parent		χ^2	p-value
		Father	Mother		
	Trans	35	44	0.73	0.392
	Not Trans	29	27		
	χ^2	0.56	4.07		
	p-value	0.454	0.044		
		Comorbid ODD		χ^2	p-value
		Positive	Negative		
	Trans	39	53	0.22	0.639
	Not Trans	36	33		
	χ^2	0.12	4.65		
	p-value	0.729	0.031		
		Comorbid CD		χ^2	p-value
		Positive	Negative		
	Trans	18	74	1.18	0.277
	Not Trans	8	61		
	χ^2	3.85	1.25		
	p-value	0.050	0.264		
978		Transmitting Parent		χ^2	p-value
		Father	Mother		
	Trans	39	34	0.49	0.483
	Not Trans	23	26		
	χ^2	4.13	1.07		
	p-value	0.042	0.301		
		Family History		χ^2	p-value
		Positive	Negative		
	Trans	33	50	2.03	0.154
	Not Trans	33	31		
	χ^2	0.00	4.46		
	p-value	1.000	0.035		
1481		Medication Response		χ^2	p-value
		Poor/Moderate	Good		
	Trans	16	39	0.26	0.610
	Not Trans	10	19		
	χ^2	1.38	6.90		
	p-value	0.240	0.009		
rs2066		Comorbid ODD		χ^2	p-value
		Positive	Negative		
	Trans	15	25	8.36	0.004
	Not Trans	22	8		
	χ^2	1.32	8.76		
	p-value	0.249	0.003		

Table 5.6 - Logistic Regression of Subgroups of SNAP-25 Variants

Variant	Sub Group Results			Logistic Regression	
87614		Transmitting Parent		χ^2	p-value
		Father	Mother		
	Trans	28	31	1.84	0.175
	Not Trans	15	29		
	χ^2	3.93	0.07		
	p-value	0.047	0.791		
88268		Transmitting Parent		χ^2	p-value
		Father	Mother		
	Trans	41	31	1.94	0.163
	Not Trans	23	29		
	χ^2	5.06	0.07		
	p-value	0.024	0.791		
		Family History		χ^2	p-value
		Positive	Negative		
	Trans	54	42	0.96	0.327
	Not Trans	35	37		
	χ^2	4.06	0.32		
	p-value	0.044	0.572		

5.4 Conclusions

The results show that within the broad DSM IV ADHD phenotype of the sample there exist a number of sub phenotypes that display stronger association with the genetic variants than the total sample. Specifically these are the rs2066 SNP of the DRD5 gene, which is associated with ADHD in the absence of comorbid ODD ($\chi^2=8.76$, $p=0.003$, $OR=3.13$) and the VNTR of the DRD4 gene which is associated with a positive family history of ADHD ($\chi^2=9.80$, $p=0.002$, $OR=2.75$) as well as ADHD with comorbid ODD ($\chi^2=5.77$, $p=0.016$, $OR=2.25$). In addition LR analysis of the VNTR was almost significant (LR $\chi^2=3.13$, $p=0.077$) when comparing the sub group consisting of ADHD individuals comorbid with CD ($\chi^2=1.00$, $p=0.317$, $OR=0.06$) to those without comorbid CD ($\chi^2=4.19$, $p=0.041$, $OR=1.65$). However, this would appear to be contradictory given that CD is regarded as a more severe version of ODD and it may represent a false positive result. The ADHD with comorbid ODD is a similar phenotype to that which Holmes et al, (2002) reported association with in the combined UK and Irish analysis, which included a subset of the present sample, suggesting that sample size is a factor in preventing the result from reaching significance. If this is the case, it may also apply to the -521 SNP of the DRD4 gene, which also showed a borderline non-significant difference in its LR analysis between the sex of the transmitting parents (LR $\chi^2=3.09$, $p=0.079$).

The association between the ADHD subgroup with a family history of the disorder and the 7-repeat allele of the DRD4 VNTR implies that this variant may be more familial than the others analysed. Its association with ADHD comorbid with ODD and CD (in the Holmes et al study (2002)) is harder to interpret but may suggest that

the VNTR is associated with the symptoms of ADHD most closely related to CD and ODD (ie hyperactive-impulsive symptoms) or that the association is solely due to the diagnoses of ODD and CD, although this is unlikely. Alternatively, the ODD and CD symptoms may simply be a marker of ADHD severity and thus the strength of a genetic component. The association of the DRD5 SNP (rs2066) with the transmission of paternal alleles only is in keeping with similar findings in relation to multiple gene variants in our laboratory. This finding of increased transmission of the risk allele from fathers as opposed to mothers has also been reported in the literature in relation to the SNAP-25 gene (Mill et al, 2004a; Kustanovich et al, 2003) and the 5HT1B receptor gene, (Hawi et al, 2002; Quist et al, 2003). This paternal bias in transmission distortion has yet to be explained although growing frequency of its report in the literature supports the hypothesis that it is due to a biological mechanism as opposed to chance occurrence. Theories that could be put forward for this phenomenon include genetic imprinting, tissue specific imprinting and selection bias. Selection bias may occur due to affected mothers not bringing their children for assessment and diagnosis or for participation in research. Affected mothers may be less organised and focused in relation to presenting their children due to the nature of their own symptoms, while ADHD children with affected fathers and unaffected mothers maybe more likely to be presented to clinics and research projects.

The DRD4 VNTR results are also of interest in regard to the reports of association and non-association with this variant in the literature (Kent, 2004). Our initial study of this variant did not show association with the VNTR (Hawi et al, 2000). However, the 7-repeat allele has since been shown to be associated with various

refined phenotypes within our sample, including a positive family history of the disorder and comorbid ODD in this thesis; and comorbid CD in the Holmes et al study (2002). This suggests that the Irish DSM IV diagnosed sample, and the samples from other groups that did not report an association between the VNTR and ADHD, may have a slightly different phenotype to the DSM IV samples of the studies that did report association (or there may be genetic heterogeneity). If this is the case it may have implications for replication studies, which as discussed later is the usual approach to identifying type I errors (Chapter 7, Section 7.2.3).

Although these results are preliminary and require independent replication and confirmation, their importance should not be underestimated. This area of sub phenotypic analysis has not been extensively explored to date, mainly due to small sample sizes, which rapidly become even smaller when this type of sub group analysis is performed. However these results, if true, suggest that by continuing to use the broad DSM IV phenotype without subdivision or refinement, we are potentially losing valuable information. In addition, we may be classifying variants as ‘not associated’ with ADHD when they are in fact associated with a more refined or sub phenotype of the disorder.

The small size of these subgroups is inevitable given the modest size of the overall sample in this study. However, the fact that even with this limited power several analyses are significant and others nearly so, supports the theory that a more refined phenotype may be successful in the determination of true susceptibility variants for ADHD. The difficulties with this type of approach include obtaining a sufficiently

large sample to allow sub-division and ensuring a consistent measurement of phenotype. These issues are discussed in detail in section 7.2.1.



Chapter 6

Meta Analysis of DRD5 Microsatellite Marker DRD5.PCR1

6.1 Introduction

6.1.1 What is a Meta Analysis?

The term meta analysis is defined as the combining of multiple individual studies into one study. Although this form of analysis is usually performed on published data, the term can also be applied to the combining of raw data from more than one study.

There are two important issues to consider when combining data from multiple studies. Firstly, all of the sample data to be combined should be tested for homogeneity. Any samples found to be heterogeneous should be discarded from further analysis. Secondly, the analysis should be examined for signs of bias, most commonly selection bias and publication bias.

6.1.2 Why do a Meta Analysis?

In the field of psychiatric genetics, studies are often affected by a lack of power due to relatively small sample sizes. This is one possible explanation for the frequently observed pattern of lack of replication of significant findings. The meta analysis approach combines homogeneous data from multiple samples in an attempt to definitively confirm or refute an original significant report.

6.1.3 Background to this Study

The combined meta analysis was first proposed at a meeting of the ADHD collaborative network (Faraone, 2003) in 2000. To obtain as complete a sample as possible, and to avoid potential bias, we invited participation from all members of

this ADHD collaborative network. This network comprised of most groups conducting linkage or association studies of the ADHD phenotype. In addition, we searched PubMed using the keywords “ADHD” and “DRD5.” This revealed an earlier meta-analysis (Maher et al, 2002) that contained data from four published studies, which were already included in our proposed analysis, and from one additional unpublished sample, which we excluded because it did not meet our inclusion criteria (Chapter 6, Section 6.2.1). At the time of the analyses we were aware of five additional ADHD samples that had not been genotyped for this variant.

In the event of an overall significant association between the variant and ADHD, we proposed that, owing to the unique opportunity presented by a data set of this magnitude, we would further examine the association in relation to a number of demographic and clinical subdivisions. Three times more males than females receive a diagnosis of ADHD (Anderson et al, 1987) and it has been reported by teachers that affected boys are more inattentive and more hyperactive/impulsive than affected girls (Hartung et al, 2002). We therefore proposed analysis by the sex of the affected child. In addition, we planned to examine the sex of the transmitting parent, because the literature has suggested the possibility of imprinting at the DRD2 (Kirley et al, 2002) and SNAP-25 loci (Brophy et al, 2002). Imprinted genes are known to be a factor in various other areas of psychiatric genetics (Davies et al, 2001). Finally, recent attention has focused on the possibility that genetic analysis might aid the identification of more homogeneous diagnostic symptoms and subtypes. Waldman et al. (1998) reported that, in between-family association analyses, levels of predominantly hyperactive/impulsive symptoms were related to

the number of DAT1 high-risk alleles but that levels of the inattentive symptoms were not, whereas within-family analysis showed association between DAT1 and the combined subtype. We therefore also proposed to analyze the joint sample in relation to diagnostic subtype.

6.2 Materials and Methods

6.2.1 The Samples

In total we received clinical and genotypic data from 14 samples, 1980 affected probands and 3072 parents. Inclusion criteria for the study were the presence of a DSM-IV diagnosis of childhood-onset ADHD and the genotyping of one or both parents. Exclusion criteria were the presence of pervasive developmental disorders, fragile X syndrome, major neurological disorders, fetal alcohol syndrome, Tourette syndrome, psychosis, and a full-scale IQ score <70. The frequency of allele 10, ranged from 36.3% to 49.2%, with up to 20 distinct alleles being reported. Information on the sex of the children and the DSM-IV clinical subtype was provided along the raw genotypic data from each individual. Detailed information about each of the samples is available in Table 6.1, including size, country of origin and the instrument used to make diagnoses.

Table 6.1 – Information about Individual Participating Groups

Grp No.	Primary Investigator	No. Cases (n)	No. Parents (n)	Publication Status	Freq of Allele 10	Allele Range	Sample Origin	Instrument Of Diagnosis
1	Kratochvil CJ	41	63	Unpub	49.2	6-17	USA	KSADS ^a
2	Levy F	66	82	Unpub	35.1	7-17	Australia	ATBRs ^b
3	Kent L	69	121	Unpub	50.8	4-18	UK	CAPA ^c
4	Faraone SV	82	126	Unpub	46.5	5-18	USA	K-SADS-E ^d
5	Rohde LA	85	138	Unpub	39.7	7-19	Brazil	K-SADS-E ^d
6	Tahir E	100	193	Pub ⁱ	39.8	4-17	Turkey	KSADS ^a
7	Asherson P	103	179	Unpub	44.9	6-18	UK	CAPA ^c
8	Thapar A	106	233	Pub ^j	43.2	6-18	UK	CAPA ^c
9	Todd RD	161	271	Unpub	44.5	6-19	USA	MAGIC ^e
10	Gill M	168	325	Pub ^k & Unpub	43.6	6-17	Ireland	CAPA ^c
11	Ebstein RP	176	333	Unpub	36.3	1-16	Israel	Clinical Interview
12	Barr CL	178	268	Pub ^l & Unpub	46.4	5-17	Canada	PICS-IV ^f
13	Sinke RJ	207	295	Unpub	44.6	6-19	Holland	DISC ^h
14	Smalley SL	438	445	Unpub	38.5	5-20	USA	K-SADS-PiL ^g

a Kidi-schedule for affective disorders and schizophrenia (Kaufman et al, 1997); b Australian Twin Behavior Rating Scale (Hay et al. 2001); c Child and Adolescent Psychiatric Assessment (Angold et al, 1995); d Schedule for Affective Disorders and Schizophrenia for School-Age Children-Epidemiologic Version (Orvaschel et al. 1987); e Missouri Assessment of Genetics Interview for Children (Reich, 2000); f Parent Interview for Child Symptoms (A. Schachar, Ichowicz, unpublished data) g Schedule for Affective Disorders and Schizophrenia for School-Age-Children Present and Lifetime version (Kaufman et al. 1997); h Diagnostic Interview Schedule for Children (Shaffer et al. 2000); i (Tahir et al. 2000); j (Payton et al. 2001); k (Daly et al, 1999); l (Barr et al , 2000)

Unpub: unpublished data at time of analysis; Pub: previously published data at time of analysis; Pub & Unpub: previously published data with the addition of new unpublished data at time of analysis

6.2.2 Allele Assignment Verification

Due to the lack of standardised methodology for allele calling, at least three DNA samples were collected from each participating group, as well as the raw genotypic data. These samples were independently genotyped in our laboratory, to ensure consistent allele calling between centres.

6.2.3 Statistical Analyses

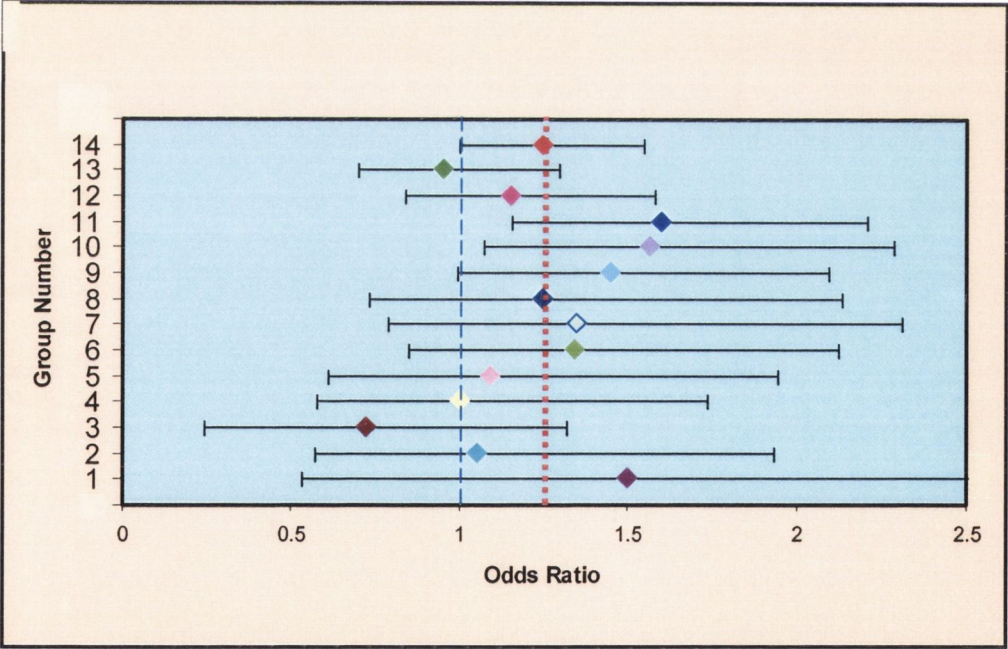
The analyses used in this study are described in full in Chapter 2. They include use of the TDT, to test the individual samples for association with ADHD and to perform the meta analysis on the combined homogeneous sample; heterogeneity testing, performed to ensure a homogeneous joint analysis; bias testing in the form of the construction of a funnel plot; and sensitivity analysis to ensure no individual sample contributed excessively to the overall result.

6.3 Results

6.3.1 Individual Group Analysis using the ETDT

All 14 participating samples were analysed individually using the ETDT (Chapter 2, Section 2.3.3). The results are displayed in Table 6.2. TDT analyses on the individual samples tested showed a significant association between two of the ADHD samples and the 148bp allele. The majority of the remainder displayed an excess of transmission of the 148bp allele but did not attain individual statistical significance. Two of the samples showed a level of transmission of allele 10 that was less than the expected transmission. Figure 6.1 shows the respective OR's and CI's for each sample.

Figure 6.1 – Odds Ratios and Confidence Intervals for Individual Groups



This figure shows the Odds Ratios and respective 95% Confidence Intervals for each sample. They are displayed in order of size with the largest sample at the top. The solid line represents an odds ratio of 1 and the dotted line an odds ratio of 1.24.

Table 6.2 – Individual Group Results

Grp No.	Primary Investigator	No. Cases (n)	χ^2	p-value	Odds Ratio	Confidence Interval	
						Lower Limit	Upper Limit
1	Kratochvil CJ	41	0.59	0.44	1.50	0.53	4.21
2	Levy F	66	0.02	0.88	1.05	0.57	1.94
3	Kent L	69	1.13	0.29	0.72	0.40	1.32
4	Faraone SV	82	0.00	1.00	1.00	0.57	1.74
5	Rohde LA	85	0.09	0.77	1.09	0.61	1.95
6	Tahir E	100	1.60	0.21	1.34	0.85	2.12
7	Asherson P	103	1.18	0.28	1.35	0.79	2.31
8	Thapar A	106	0.66	0.42	1.25	0.73	2.14
9	Todd RD	161	3.79	0.05	1.45	1.00	2.10
10	Gill M	168	11.1	0.0009	1.57	1.07	2.29
11	Ebstein RP	176	8.16	0.004	1.60	1.16	2.21
12	Barr CL	178	0.78	0.38	1.15	0.84	1.58
13	Sinke RJ	207	0.10	0.75	0.95	0.70	1.30
14	Smalley SL	438	3.80	0.05	1.24	1.00	1.55
Total		1980	16.39	0.00005	1.24	1.12	1.38

Allele range: allele 1 = 166bp, allele 20 = 128bp, allele 10 = 148bp.

6.3.2 Heterogeneity Testing

The logistic regression model assuming homogeneity between all of the samples resulted in a -2Log likelihood value of 1956.215, while the model assuming heterogeneity had a -2 log likelihood value of 1944.234. Using the formula, $(-2\text{Loglikelihood}_{\text{individual}}) - (-2\text{Loglikelihood}_{\text{combined}}) = \chi^2$, the data were tested for evidence of a significance difference between the two models which would be indicative of heterogeneity between the samples, and found that the $\chi^2 = 11.98$ with 13 degrees of freedom. This resulted in a p-value of 0.53 implying no significant difference between the two statistical models and therefore no heterogeneity between samples.

6.3.3 Combined Meta Analysis using the ETDT

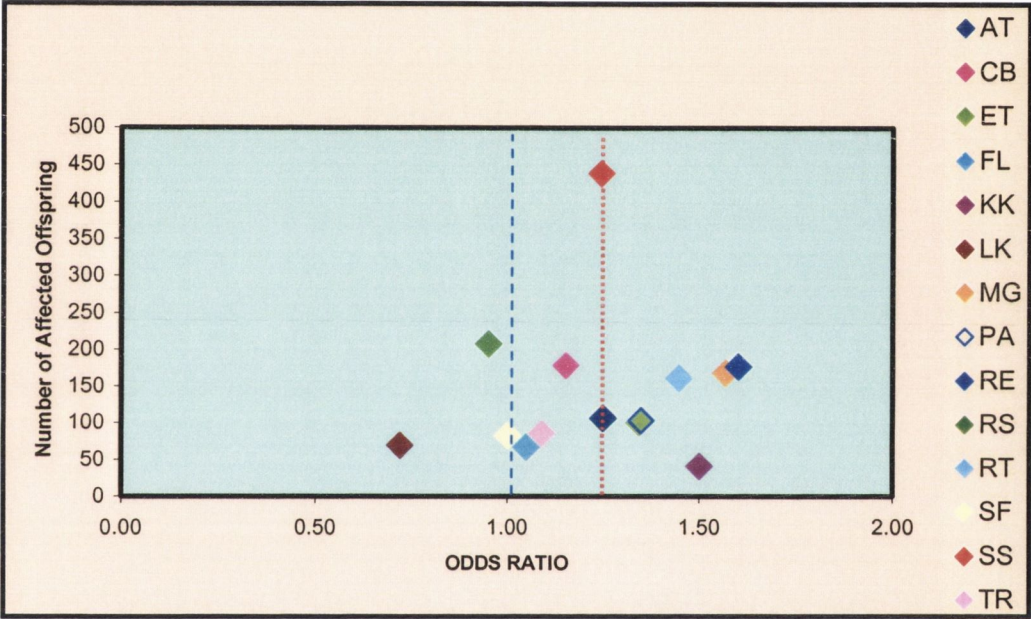
The ETDT (see 2.3.3) was applied to the combined homogeneous 1,980 probands and 3,072 parents. The analysis indicated a strongly significant association of small effect ($\chi^2_1 = 16.45$, $p = 0.00005$, $\text{OR} = 1.24$, $95\%\text{CI}$, 1.12-1.38) between ADHD and allele 10 of the microsatellite variant.

6.3.4 Bias Testing

The funnel plot (see Figure 6.2) was used to examine the data set for signs of bias. In theory, the larger samples should display ORs closer to that of the combined OR (and the true OR), than the smaller samples, resulting in a visual representation of an inverted funnel. Bias either a publication bias or a bias in the submission of data to the meta analysis would disrupt the natural symmetry of the funnel. For example, if there was a tendency not to publish small negative studies, then one side of the

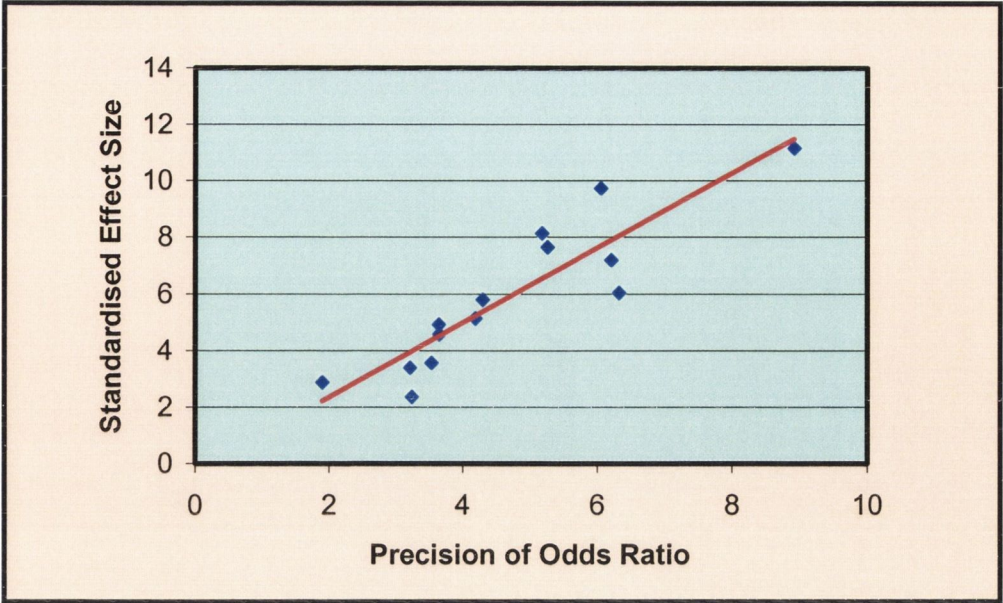
wide part of the funnel would lack data points. The plot in Figure 6.2 displays the characteristics of an inverted funnel and suggests the absence of bias. Egger's method (Egger et al, 1997) produced a scatter plot of the data with an R^2 value of 0.8036 (see Figure 6.3) implying a good fit of the data to the line. The intercept was calculated to be 0.179, with a $SE = 1.100245$, $t=0.16$, $p=0.874$ and $95\%CI= -2.218645$ to 2.575809 . As this intercept was not significant greater than zero, it implied no evidence of bias in the sample in accordance with the funnel plot.

Figure 6.2 – Funnel Plot



Funnel Plot showing the Number of Affected Offspring vs Odds Ratio. This plot depicts results consistent with the absence of publication bias due to its inverted funnel shape. The solid line represents an odds ratio of 1 and the dotted line an odds ratio of 1.24.

Figure 6.3 – Scatter Plot of Standardized Effect Size Vs Precision of the OR



This plot shows the regression of the standardized effect size vs the precision of the odds ratio. The intercept of the line will occur close to zero implying no bias in the samples.

6.3.5 Sensitivity Testing

The sensitivity analysis (see Table 6.3) showed that significant association between allele 10 of the D5 microsatellite and ADHD was maintained regardless of the data set removed, with the p-value never exceeding 0.001 and the odds ratio never fluctuating more the 0.04, in either direction, from the combined result.

Table 6.3 Sensitivity Analysis of Allele 10 of DRD5.PCR1

Subtracted Group	Odds Ratio	95% Confidence Interval	p-value
1	1.24	1.11 – 1.38	0.00007
2	1.25	1.12 – 1.39	0.00004
3	1.26	1.14 – 1.40	0.00002
4	1.25	1.12 – 1.39	0.00004
5	1.24	1.12 – 1.38	0.00007
6	1.24	1.11 – 1.38	0.00011
7	1.24	1.11 – 1.38	0.00009
8	1.24	1.12 – 1.38	0.00007
9	1.22	1.10 – 1.37	0.00027
10	1.22	1.09 – 1.36	0.00041
11	1.20	1.08 – 1.34	0.00103
12	1.25	1.12 – 1.40	0.00007
13	1.28	1.15 – 1.43	0.00001
14	1.24	1.10 – 1.40	0.00039
Total (no grp subtracted)	1.24	1.12 – 1.38	0.00005

The table shows the Odds Ratios, 95% CI and p-values for the joint dataset with the subtraction of each group in turn.

6.3.6 Subtype Analysis

The three subdivisions of the combined sample were made according to the sex of the affected child, the sex of the transmitting parent and the clinical subtype of the child. The entire combined sample was used for the first two of these analyses however clinical subtype was not available for all probands. Logistic regression analysis (Chapter 2, Section 2.3.6.2) of the sex of the child resulted in no significant difference between sexes, while the sex of transmitting parent displayed a non-significant trend towards excess maternal transmission (see Table 6.4). The 3 clinical subtypes of predominantly inattentive (n=445), combined (n=1106), and predominantly hyperactive/impulsivity (n=122) were examined separately, using ETDT, indicating ORs of 1.5, 1.3 and 0.9, respectively, with corresponding p-values of 0.0001, 0.0001 and 0.6. Comparison between the individual OR's (Table 6.5) indicated a significant difference (p = 0.035) between the ORs for the predominantly inattentive and predominantly hyperactive/impulsive subtypes.

Table 6.4 – Comparison of Sex of Affected Child and of Sex of Transmitting Parent

Analysis	χ^2	p-value	Odds Ratio	Confidence Interval	
				Lower Limit	Upper Limit
Sex of Affected Child	1.65	0.2	0.8	0.65	1.09
Sex of Trans Parent	2.63	0.1	1.2	0.96	1.48

Table 6.5 – Comparison Between Clinical Subtypes

Groups Compared	χ^2	p-value	Confidence Interval
Predominantly Inattentive & Combined	1.287	0.257	0.902 – 1.471
Predominantly Hyperactive/Impulsive & Combined	2.654	0.103	0.659 – 1.039
Predominantly Inattentive & Predominantly Hyperactive/Impulsive	4.468	0.035	0.367 – 0.963

6.4 Conclusions

Loci conferring risk with ORs <1.5 require large samples for detection and replication (Dahlman et al. 2002). In the field of psychiatric genetics this is difficult to attain, resulting in multiple small studies of variants without conclusive results. This has led to the introduction of the combined or meta analysis approach to this area of genetics. The principle concerns in combining multiple data sets are the possibility of heterogeneity between samples and publication bias. In this study no evidence of heterogeneity between the samples or publication bias was found and therefore the data from all 14 groups was combined.

Using the large combined sample, it has been determined that the OR for allele 10 of the DRD5.PCR1 variant is 1.24, but as the allele is common in the population, it has an attributable fraction of 0.07. For complex disorders involving the interaction of multiple genes and environmental factors, it is difficult to attribute an individual case to any single aetiological factor. Nevertheless, the attributable fraction is still a useful index of potential public health importance, as it is defined to be the fractional reduction in the frequency of disease if the risk factor was eliminated from the population. Sensitivity analysis showed that the omission of any of the groups, including that with the smallest p value (Group 11), that with the largest p value (Group 10) or that containing the original findings (Group 10), did little to alter the overall results, implying that no one groups data is responsible for the combined result.

The single published significant result between allele 10 of the microsatellite and ADHD (Daly et al. 1999), coupled with a number of non-significant trends in the

same direction (Barr et al. 2000; Tahir et al. 2000; Payton et al. 2001) followed a pattern familiar to association studies with small samples. It could have been concluded, incorrectly, that the original result was a false positive finding. This joint analysis, however, suggests that this is a true finding of association between ADHD and the polymorphism and that individual sample sizes lacked sufficient power for confirmation.

Subtype analyses indicate that the association of allele 10 is selective for predominantly inattentive and combined subtypes. It should be noted that while the sample size for the predominantly hyperactive/impulsive group is relatively small, power for this sample to detect association with the disorder was >75% with $\alpha=0.05$ and 85% for $\alpha=0.1$. Whether the DRD5 association is restricted to inattentive symptoms awaits further study using QTL (Curran et al. 2001) or alternative subtyping approaches.

The microsatellite variant is located 18.5kb from the transcription start site of the DRD5 gene and although no functional role has been reported to date this possibility cannot be excluded without expression testing. The DRD5 gene has also shown association with ADHD and the SNP variant rs2066 located in the 3' UTR (Chapter 5, Section 5.3.1), which may be a more likely functional candidate due to its closer proximity to the gene. However this would also require expression analysis to determine.

In conclusion, this study has shown that allele 10 of this DRD5.PCR1 microsatellite is associated with ADHD in a large combined homogeneous international sample. It

has also been indicated that the association may be confined to particular subtypes or symptom traits although it is acknowledged that this finding requires additional confirmation. In addition, the results of this joint analysis should encourage efforts to identify the functional variant or variants of this gene and promote further collaboration in the field of psychiatric genetics.



Chapter 7

General Discussion and Conclusions

7.1 - The Aims and Findings of this Thesis

The aims of this thesis were to examine the role of three dopamine related genes in the susceptibility to ADHD in an Irish sample using a variety of different molecular genetic and statistical methodologies. The genes initially chosen were the DRD5 gene, as the initial association finding with this gene had been reported by our laboratory and it was a relatively unexplored gene at the time, and the DRD4 gene, which had been extensively explored in the field and was regarded as a true susceptibility gene for ADHD at the time, but had not been fully explored in the Irish population. During the course of the PhD, the SNAP-25 gene appeared as a novel but promising new candidate gene for ADHD. As it is related to the release of dopamine into the synapse it was also included in the study.

Studies began with a mutation screen of the DRD5 gene to examine the genetic variation within the promoter and coding sequence. This information was already available for the DRD4 gene from several reports and the SNAP-25 gene as a result of a mutation screen by Mill et al (2004a). Once in possession of this information for each gene, a traditional association study, coupled with LD and haplotype analysis was performed for all three of the genes along with additional statistical analysis on the following subsets of the sample: sex of transmitting parent, family history of ADHD, methylphenidate response and the presence or absence of comorbid ODD and CD. Importantly, a multi-centre collaborative study was put together to perform a meta analysis of the associated microsatellite marker of the DRD5 gene, to definitively determine it's hypothesised role in the aetiology of ADHD.

The mutation screening of the coding and promoter regions of the DRD5 gene yielded six variants. Of these only two (978 and 1481) were present at a frequency greater than 10%, our threshold for inclusion in the fine mapping association study. In addition to providing variants for the subsequent association study, the mutation screen also highlighted multiple discrepancies between the genetic databases and the findings of this study. The databases, Ensembl and NCBI-dbSNP (www.ensembl.org, www.ncbi.nlm.nih.gov/projects/SNP) claimed the presence of 21 SNPs in the screened region, 11 of which had a frequency of 10% or greater. Attempted analyses of these 11 SNPs using the SNaPshot method of primer extension failed to detect the presence of these SNPs at all. It was noted that the majority of the SNPs unsubstantiated by this study corresponded with known variation between the real DRD5 gene and its pseudogenes. This suggests that not all of the reported sequence variations in the databases are true DRD5 SNPs.

Association analysis of the DRD5 gene variants revealed association between ADHD and allele 10 of the DRD5.PCR1 microsatellite ($\chi^2=11.07$, $p=0.001$) and the C allele of the 1481 SNP ($\chi^2=4.96$, $p=0.03$). The presence of a number of significant haplotypes was also noted. The majority of these contained allele 10 of the DRD5.PCR1 variant with the most significant consisting of allele 10 of DRD5.PCR1 and the T allele of both the 978 and 1481 SNPs ($\chi^2=13.481$, $p=0.0002$). As discussed before, other than the DRD5.PCR1 microsatellite, there has been little research by other groups on the DRD5 variants described in this thesis. Therefore the reported association with the 1481 SNP and the significant haplotype results need to be confirmed by replication.

Analysis of the DRD4 VNTR did not show significant over transmission of allele 7 to ADHD children as observed in other studies. However, a non-significant trend in this direction was clear. Over transmission of allele 7 is consistent with the meta analyses performed on this variant (Faraone et al., 2001; Maher et al., 2002) and the literature in general. Significant association was observed between allele C of the –616 variant of the promoter region of the gene and ADHD ($\chi^2=7.45$, $p=0.008$). Non-significant over transmission of the same allele was observed by Barr et al (2001) in a Canadian sample. Haplotype analysis, using TRANSMIT, revealed the presence of a significant haplotype comprising the long allele of the 120bp duplication and the C allele of the –616 SNP ($\chi^2=4.26$, $p=0.039$). These same alleles were also present in multi marker haplotypes found to be associated with ADHD in the Canadian (Barr et al, 2001) and UK (Mill et al, 2003) studies.

No individual variants at SNAP-25 were found to be associated with ADHD in our sample. However there was a significant haplotype consisting of allele G of the –900 SNP, allele 5 of the microsatellite, and alleles G, G and T of SNPs 80609, 87610 and 87614 respectively ($\chi^2=5.53$, $p=0.02$). Other recent SNAP-25 studies are inconsistent in their findings. For DRD4 and DRD5 even though some of the studies are negative a clear pattern of association or ‘trend’ is observed (see Chapter 6 Table 6.2). In the case of SNAP-25 there is no pattern emerging at this time and the Irish data does little to aid in the conclusion as to which alleles of which variants, if any, may be involved in ADHD. Further work is required to be certain, but it seems likely that SNAP-25 is not a susceptibility gene for ADHD.

Analysis of sub categories of the Irish sample, including by sex of transmitting parent, family history of ADHD, response to methylphenidate and the presence or absence of comorbid CD and ODD, have shown that a refined phenotype may reveal association not apparent in the total sample. For example, association with the 7-repeat allele of the DRD4 VNTR was demonstrated by two sub groups, the first defined by a positive family history of the disorder and the second by the presence of comorbid ODD. Whether the associations are with these specific phenotypes or whether family history and comorbid ODD index severity of ADHD remains to be determined. It is interesting in this regard to note that Kirley et al (2004) reported an increase in transmission of the 7-repeat allele ($\chi^2=3.45$, $p=0.085$) to children diagnosed with HKD under the ICD-10 diagnostic system, which is regarded as a more stringent diagnosis.

The meta analysis of the DRD5 microsatellite marker (Chapter 6), DRD5.PCR1, revealed significant association with DSM IV diagnosed ADHD children in the total sample of 1980 affected probands and 3072 parents. All 14 samples were found to be homogeneous with no evidence of selection bias (Chapter 6, Section 6.3.4). Although the finding was highly significant the OR for the variant was quite modest (OR=1.24), implying that the association finding is true but that the DRD5 gene is a gene of minor effect. This study also showed that the association may be confined to the predominantly inattentive and combined subtypes although additional confirmation of this finding is required.

7.2 - The Strengths and Limitations of this Study

There are strengths and limitations involved in all scientific studies. These may depend on multiple factors including cost, time and the availability and accessibility of necessary resources. The strengths of this thesis are: 1) the use of a homogeneous sample, 2) the stringent diagnostic methods applied to this sample, 3) the size of the sample compared to others in the field, 4) the family based design of the study and 5) the participation of so many ADHD groups in the meta analysis. The limitations of the study include: 1) the size of the sample given the small effect sizes being revealed (eg DRD5 OR=1.24), 2) the issue of multiple testing, 3) the exclusion of variants with a frequencies less than 10% and 4) the broad spectrum ADHD diagnosis. These issues are discussed in more detail below (sections 7.2.1-7.2.4).

7.2.1 – The Clinical Sample

The most important resource for a genetic association study is the sample. Ideally samples should be homogeneous, and drawn at random from the total population of individuals with the particular phenotype (to exclude bias), of a large size (to ensure detection of low frequency variants and variants conferring risk with modest ORs), and definitively diagnosed for the specific phenotype to be examined. However in reality the ideal sample is usually unattainable.

The sample used in this thesis was drawn from the Irish population. This should reduce genetic heterogeneity, as Ireland is regarded as having a genetically homogeneous population. Although post famine (1845 - 1849) there was a large migration from Ireland, resulting in genetic drift and gene flow out of the country,

we have experienced relatively little influx of people from other nationalities until the last decade. This means that as a population the Irish may possess less genetic variation than other more multi-racial countries, such as the UK and America. The advantage of this is that a more homogeneous sample is likely to contain fewer functional variants, with possible higher ORs, contributing to the development of disorder. In theory this should aid in the search for ADHD causative variants.

Some of the problems that studies of complex phenotypes have to contend with are the difficulties of sample collection, definition of phenotype and measurement of trait. As a result clinical sample collection is costly, time consuming and specialised. It is recognised that the functional variants that underlie ADHD are likely to be variants of small effect and that it will be an accumulation or interaction of many of these variants that results in the development of ADHD. The sample used in this thesis is relatively large in the field of ADHD genetics however it's potential to detect association diminishes rapidly if a subdivision of the DSM-IV ADHD phenotype is attempted. However, in comparison to samples where phenotype definition is simple (eg, a biochemical test) the size of the sample used in this thesis is quite small.

For any complex, clinically defined disorder, the method of diagnosis is extremely important. For the sample in this thesis, all ADHD individuals have been diagnosed with ADHD according to the DSM IV, via interview with the CAPA (Angold et al, 1995). This results in a very stringent and reliable diagnosis of ADHD, which is advantageous to genetic studies as the resulting sample should be more clinically homogeneous than those with a less rigorous method of diagnosis. However,

although this diagnosis is stringent it is still quite broad. The sample contains children diagnosed with all three categories of ADHD, as well as other comorbid conditions. We have already seen from section 5.3 that in the case of the DRD4 VNTR and the DRD5 SNP (rs2066) there is a significant difference between ADHD individuals with and without comorbid ODD. Furthermore the analysis of the three sub categories of ADHD in the meta analysis suggests that the DRD5 microsatellite is only associated with the predominantly inattentive and combined subtypes of ADHD (Chapter 6, Section 6.3.6). Therefore the results of this thesis suggest that a narrower, more refined phenotype may be more effective in the search for causative ADHD genetic variants than the broad DSM IV ADHD diagnosis, regardless of how stringent the diagnostic methodology.

7.2 2 – The Design and Analysis of the Sample

Association studies based on case/control or family designs are thought to be more powerful than linkage methods in detecting genes contributing to complex diseases (Risch, 2000). Furthermore, as discussed earlier (Chapter 1, Section 1.2.3), the family based design is less susceptible to population stratification and therefore false positive results. However, one major problem with this type of study is that it lacks power for low frequency variants (ie <5-10%). This issue is not exclusive to family based association studies. None of the common association study designs address this problem although linkage designs may bypass the problem if the rare variants are in the same gene. For association studies, at the present time, the only way around this situation is to increase the sample size. However, as discussed above, this is not an easy option for psychiatric disorders and must include collaboration between groups.

The 'common disease common variant' theory suggests that, as ADHD is a common disorder in the population it is likely to be the result of common risk variants. In today's society the symptoms involved in ADHD, including impulsiveness, distractibility, curiousness and hyperactivity, are not regarded as advantageous traits. However, throughout the evolution of mankind, when basic survival called for the necessity of food, safety and procreation, these traits may have been more desirable. It is therefore feasible that these traits may have been positively selected for over time resulting in the associated variants being common in today's population. Alternatively, the disorder could be due to multiple isolated mutation events that have not been selected for over time. This would fit with a 'common disease, rare variant' model such as that seen with Retinitis Pigmentosa (Hims et al, 2003). Therefore by continuing to perform association studies as we do, assuming the 'common disease, common variant' model, and using LD as an aid to screening the locus, we may be excluding a vital percentage of rare variants, which may be contributing to the disorder. How to include them in our studies is a difficult question, the answer to which returns us to the issues of study sample size and diagnosis. At the moment the most feasible method to address these low frequency variants is to increase the sample sizes and perhaps narrow the phenotype. The necessary sample size would probably be most easily accomplished via collaborative studies, however to get multiple groups to agree on a common 'narrow' phenotypes would be difficult and would most probably require additional collection and reassessment of patients, thus increasing the time and cost involved.

7.2.3 – Multiple Testing

The issue of multiple testing is not new to the field of psychiatric genetics. Formal statistical theory states that the p-value of a positive result should be corrected for the number of tests performed on the data (if not even the number of tests that *might* be performed on the data). In the field of psychiatric genetics this is usually not done. The theory behind correcting for multiple testing is that with every additional test performed the chances of a false positive result increase. Therefore the p-value should be adjusted according to the number of tests performed. If after the correction the p-value is still significant (i.e. <0.05) then the corresponding ORs and other statistical measures are regarded as reliable findings.

If corrections for multiple analyses are to be performed on a data set, it is necessary to decide how many independent tests need to be corrected for. Examples of multiple tests that definitively require correction include sub group analyses, multiple association tests and association tests on microsatellites. Although occasionally people collapse microsatellite data based on observing the frequencies of the alleles and compare one allele to the rest as a single test, this is incorrect as essentially all the other comparisons are still performed mentally (Nyholt, 2001). The situation becomes more complicated if the tests performed are correlated, as is the case of haplotype analysis with markers in LD with each other, and when sub-phenotypes overlap. In this instance each test does not provide a completely independent chance of a Type I (false positive) error and therefore the standard statistical correction will be too conservative (Ott, 1999; Nyholt, 2001).

To correct or not to correct? In this thesis, raw p-values are presented along with a record of the number of tests performed, and replication by other groups is invited. In my opinion this is the best method to address the multiple testing issue in the field of ADHD genetics. Furthermore, additional testing, as in sub group analyses, should be based as far as possible, on biological hypotheses and not performed at random. It is important that all studies not corrected for multiple testing are clearly reported as such to ensure other researchers are not misguided.

The method commonly adopted to expose false positives in this field of research is replication. Replication of a reported result in an independent sample increases the likelihood of it being a true finding. In addition, the replication study will consist of an *a priori* hypothesis and therefore only one test, overcoming the need for corrections. The downside of this method however, is that to replicate a reported finding it is estimated that the second sample to be tested should be almost twice the size of the first due to the probability of sampling variance resulting from small sample sizes (Tversky and Kahneman, 1971). What materialises in the literature as a result of this situation is a combination of significant and non-significant reports of association between the variant in question and the disorder, with no definitive answer. I believe that the best approach to deal with this situation is the one that is slowly emerging in the literature, the meta or joint analysis (Lowe et al, 2004b). This appears to be the best method by which a sufficient sample size can be obtained to definitively confirm or refute a reported association. However it does have its own drawbacks including time, cost, potential phenotypic and genetic heterogeneity, and publication bias all of which have been discussed in Chapter 6.

7.2.4 – The Meta Analysis (Collaborative Studies)

The meta analysis performed as part of this thesis was highly successful (Chapter 6) (Lowe et al, 2004b). Not only did it definitively conclude the proposed association between the DRD5.PCR1 microsatellite and ADHD, it also demonstrated the value of collaborative analyses. Although setting up the collaboration was time consuming, it was the first of its kind in the field of ADHD genetics. Never before had all ADHD groups been invited to take part in a project. Previous meta analyses had consisted of work that had already been completed and usually published, unlike this one which invited all known groups with suitable samples to participate, regardless of whether the genotyping was already done. Feedback from the field since the publication of this work has been very positive and it is hoped that the overall success of this joint analysis will help to encourage and promote further collaboration in the field of ADHD and psychiatric genetics.

7.3 - General Conclusions and Directions for the Future

7.3.1 General Conclusions

The results of this thesis concur very well with the current literature in the field of ADHD genetics, concluding that the DRD5 and DRD4 genes are susceptibility genes for ADHD. The meta analysis executed on the DRD5.PCR1 microsatellite has succeeded in confirming the association of this variant with the general ADHD diagnosis and has suggested that it may be confined to specific phenotypic subtypes. In addition, it has shown that although the process of collaboration may be complex and time consuming it can be very successful in defining the true relationship between a genetic variant and a disorder. The situation with the SNAP-25 gene is more problematic, as has already been discussed (Chapter 4, Section 4.4.5), and it has been concluded that it is unlikely to be involved in the aetiology of ADHD. The subgroup analysis of the broad DSM IV diagnosed sample strongly supports the growing opinion that a more refined phenotype may be more successful in identifying genetic risk variants. Although no obvious functional variants have been identified, the studies have advanced the field of ADHD research and highlighted the necessity for increased collaborative studies and a well-defined, probably more refined phenotype.

7.3.2 Future Directions for this Study

The results of this thesis are compelling and strongly encourage further investigation into the role of the DRD4 and DRD5 genes in relation to ADHD. At this time I believe that the next step to be taken, in conjunction with independent

replication of some of the results, should be an investigation into the areas of gene-gene and gene-environment interaction in relation to these genes. The advantage of these types of study is that they can be implemented immediately without the need for further clinical collection, which as already mentioned is time consuming and costly.

Gene-gene interaction studies involve the statistical analysis of two or more genes positioned on different chromosomes (hence not in linkage disequilibrium) that have been shown to be associated with a given trait or disease (ie ADHD). The studies are designed to evaluate the combined effect of the genes on the phenotype in question, to determine if this differs from the simple additive effect of the genes. For example if the OR associated with a variant of gene A is 2 and the OR associated with a variant of gene B is 3, what is the OR associated with possessing both variants? It may simply be the additive effect of both variants (ie $OR=5$) or a combined interactive effect that may result in the OR being larger or smaller than 5. This type of study can be used to determine if there is an interaction between the DRD4 and DRD5 genes and also between these and other ADHD susceptibility genes that do not feature in this thesis but have been examined in our laboratory (e.g. DAT1). In addition, it would be interesting to explore potential gene-gene interactions across neurotransmitter systems and examine the DRD4 and DRD5 genes in relation to some of the serotonin candidate genes (e.g. the 5-HT transporter and the 5-HT 1B receptor).

In addition to gene-gene interactions the area of gene-environment interactions is an area of growing interest as discussed in section 1.1.6. Limited information regarding

some environmental factors was collected during the initial clinical work up of the ADHD children and their families in this study. However, adding to this information to further explore potential gene-environment interactions is a relatively inexpensive process. Potential environmental factors of interest, that are easily obtainable, perhaps over the telephone, include smoking during pregnancy, alcohol consumption during pregnancy, birth weight, maternal depression and obstetric complications.

These additions to this study are achievable without further clinical recruitment and assessment. Additional opportunities for advancement of the field of ADHD genetics are more long-term. Some of these are discussed in the following section.

7.3.3 The Future of ADHD Genetic Research

The completion of the Human Genome Mapping Project (www.hgmp.mrc.ac.uk) in conjunction with the ongoing HapMap Project (www.hapmap.org) will greatly aid in the design of future association and other genetic studies. In addition, the advances in relevant biological techniques (e.g. SNP microarrays) as well as the decreasing costs of commercial sequencing and genotyping will increase laboratory productivity. However, this thesis has highlighted that the approach taken in relation to study design and sample diagnosis will be of equal importance.

The development of collaborative studies in the field of ADHD genetics, have been greatly encouraged by the ADHD Collaborative Network (Faraone 2003). This network meets annually to discuss recent updates in the field and to encourage international relationships and collaboration between groups. It was through this

network that the meta analysis in this thesis was achieved. It has also been influential in the development of the IMAGE Consortium whose aim is to perform a large scale, multi-centre ADHD genome scan, as well as further exploring existing and potential ADHD candidate genes. The development of these studies indicates that the field of ADHD genetics is beginning to recognize that the future advancement of association and linkage studies is dependent on collaboration and group studies.

In addition to the need for large multi-centre samples for linkage and association studies, a similar approach is required for the exploration of potential environmental risks and subgroups of the broad DSM IV ADHD phenotype. Chapter 5 discusses a number of epidemiological and phenotypic factors that may be associated with ADHD and explores their role in the Irish population. Although a number of significant associations have emerged from this study, collectively the work emphasizes the limitations of this type of examination in samples of this size. The majority of current ADHD samples are diagnosed according to the DSM IV and have the same limitations as this thesis's sample when subdivided. Even in the combined sample for the meta analysis of 1980 ADHD children and their parents it was not possible to definitively conclude that the association with the DRD5 microsatellite was confined to the predominantly inattentive and combined subgroups. ADHD researchers should be looking further ahead and communicating more when planning individual sample collection to reduce potential heterogeneity issues that may arise if collaborative studies are performed at a later stage.

While association, linkage, environmental and refined phenotype studies all require the need for collaboration and larger sample sizes, the less extensively explored area of expression studies in ADHD research does not. To date, little work has been done in this area, presumably due to the lack of association with obvious potential functional variants. A number of animal models for ADHD exist (1.5.1) which have been useful in highlighting neurotransmitter systems and candidate genes potentially involved in the disorder. However in vitro studies are necessary to examine the role of individual variants on gene expression, protein translation and receptor and ligand binding.

These studies are reliant on the discovery of potentially functional genetic variants, which raises the question of ‘how to know when we have found a risk variant for ADHD?’ Straightforward synonymous base pair changes provide logical candidates for functional variants, which may affect protein folding and structure and therefore protein function. However in practice, associated variants of this nature are not often observed. Association studies usually reveal variants without any obvious function and report that the true functional variant is in LD with the associated variant. I suspect, given our current limited understanding of gene regulatory systems, that there are types of functional variants that as yet we do not know exist and that true risk variants may be overlooked if we only examine associated variants with obvious potential functionality. Factors effecting gene expression are now recognized to exist in regions of DNA previously thought to be of no relevance to transcription. These include intronic alternative splicing sites as well as regulatory regions and transcription factor binding sites located 5’ and 3’ of the gene. It would be naïve to believe that there are not additional factors contributing to gene

expression that we are unaware of. A recent publication has reported the discovery of long-range regulatory regions of genes, which may even exist inside unrelated neighboring genes (Kleinjan and Heyningen, 2005) further supporting the fact that there is still much to be discovered and understood in relation to gene expression.

To return to the question ‘when will we know when we have found the causative variants?’ The answer is unclear. It is however necessary for the field to start to explore genetic variants associated with ADHD for functionality, to prevent psychiatric genetics from being left behind the rush towards genomics and proteomics. Therefore perhaps we should consider testing more definitively associated variants even if a potential reason for altered expression is not immediately apparent.

The ultimate question to be addressed is ‘what are implications of actually finding genetic risk variants for ADHD?’ The answer is not likely to be ‘curing the disorder’ however it may result in important changes for the diagnosis and treatment of individuals with ADHD. Given the complex nature of ADHD, which is recognized to be a consequence of multiple genetic risk variants and environmental factors, it is unlikely that diagnosis will ever take the form of a simple DNA test. However, correlation studies between genetic risk variants and clinical symptoms may aid in the refining of various sub phenotypes currently existing under the umbrella of ADHD. In addition, studies examining the relationship between the presence of a given risk variant or variants and response to a particular medication may enable clinicians to administer medication in a more informed manner, perhaps even based on individual genetic makeup.

7.4 *The Final Word*

The last ten years has seen considerable advances in the genetics of ADHD. I believe that this thesis reports on and discusses the major findings in the field to date. The personal research presented within it contributes to the advancement of ADHD research and concludes that the DRD4 and DRD5 genes are both susceptibility genes in the development of ADHD. The strengths and weaknesses of this thesis and the field of psychiatric genetics as a whole have been reviewed along with potential future research directions. It is concluded that future studies should be of a collaborative nature, therefore increasing the sample size and power of the study and that more consideration should be given to refining phenotypes. The future advancement of the field into functional studies should definitively conclude the role of ADHD risk variants and subsequently contribute to the improvement of the diagnosis and pharmacotherapy of ADHD, helping to improve the lives of ADHD individuals and their families.



References

Amara S, Kuhar M (1993) Neurotransmitter transporters: recent progress. *Ann Rev Neurosci* 16:73-93

Anderson JC, Williams S, McGee R, Silva PA (1987) DSM-III disorders in preadolescent children. Prevalence in a large sample from the general population. *Arch Gen Psychiatry* 44:69-76

Angold A, Prendergast M, Cox A, Harrington R, Simonoff E, Rutter M (1995) The Child and Adolescent Psychiatric Assessment (CAPA). *Psychol Med*; 25: 739-753

Arinami T, Ishiguro H, Onaivi ES (2000) Polymorphisms in genes involved in neurotransmission in relation to smoking. *Eur J Pharmacol* 410:215-226

Arnsten AF (1999) Development of the cerebral cortex: XIV. Stress impairs prefrontal cortical function. *J Am Acad Child Adolesc Psychiatry* 38:220-2

Asghari V, Sanyal S, Buchwaldt S, Paterson A, Jovanovic V, Van Tol HH (1995) Modulation of intracellular cyclic AMP levels by different human dopamine D4 receptor variants. *J Neurochem* 65:1157-65

Asherson P, Mant R, Williams N, Cardno A, Jones L, Murphy K, Collier DA, Nanko S, Craddock N, Morris S, Muir W, Blackwood B, McGuffin P, Owen MJ (1998) A study of chromosome 4p markers and dopamine D5 receptor gene in schizophrenia and bipolar disorder. *Mol Psychiatry* 3:310-20

Aston-Jones G, Rajkowski J, Cohen J (1999) Role of locus coeruleus in attention and behavioral flexibility. *Biol Psychiatry* 46:1309-20

Bakker SC, van der Meulen EM, Buitelaar JK, Sandkuijl LA, Pauls DL, Monsuur AJ, van 't Slot R, Minderaa RB, Gunning WB, Pearson PL, Sinke RJ (2003) A whole-genome scan in 164 Dutch sib pairs with attention-deficit/hyperactivity disorder: suggestive evidence for linkage on chromosomes 7p and 15q. *Am J Hum Genet* 72:1251-60

Bakker SC, Van Der Meulen EM, Oteman N, Schelleman H, Pearson PL, Buitelaar JK, Sinke RJ (2004) DAT1, DRD4, and DRD5 polymorphisms are not associated with ADHD in Dutch families. *Am J Med Genet*

Bandstra ES, Morrow CE, Anthony JC, Accornero VH, Fried PA (2001) Longitudinal investigation of task persistence and sustained attention in children with prenatal cocaine exposure. *Neurotoxicol Teratol* 23:545-59

Batstra L, Hadders-Algra M, Neeleman J (2003) Effect of antenatal exposure to maternal smoking on behavioural problems and academic achievement in childhood: prospective evidence from a Dutch birth cohort. *Early Hum Dev* 75:21-33

Barkley RA (1990) Attention deficit hyperactivity disorder. A handbook for diagnosis and treatment. Guilford, NY, USA

Barkley RA, Grodzinsky G, DuPaul GJ (1992) Frontal lobe functions in attention deficit disorder with and without hyperactivity: a review and research report. *J Abnorm Child Psychol* 20:163-88

Barkley RA (2002) Major life activity and health outcomes associated with attention-deficit/hyperactivity disorder. *J Clin Psychiatry* 63 Suppl 12:10-5

Barr CL, Feng Y, Wigg K, Bloom S, Roberts W, Malone M, Schachar R, Tannock R, Kennedy JL (2000a) Identification of DNA variants in the SNAP-25 gene and linkage study of these polymorphisms and attention-deficit hyperactivity disorder. *Mol Psychiatry* 5:405-9

Barr CL, Wigg KG, Feng Y, Zai G, Malone M, Roberts W, Schachar R, Tannock R, Kennedy JL (2000b) Attention-deficit hyperactivity disorder and the gene for the dopamine D5 receptor. *Mol Psychiatry* 5:548-51

Barr CL, Feng Y, Wigg K, Bloom S, Roberts W, Malone M, Schachar R, Tannock R, Kennedy JL (2000) Identification of DNA variants in the SNAP-25 gene and linkage study of these polymorphisms and attention-deficit hyperactivity disorder. *Mol Psychiatry* 5:405-9

Barr CL, Wigg K, Zai G, Roberts W, Malone M, Schachar R, Tannock R, Kennedy JL (2001a) Attention-deficit hyperactivity disorder and the adrenergic receptors alpha 1C and alpha 2C. *Mol Psychiatry* 6:334-7

Barr CL, Feng Y, Wigg KG, Schachar R, Tannock R, Roberts W, Malone M, Kennedy JL (2001b) 5'-untranslated region of the dopamine D4 receptor gene and attention-deficit hyperactivity disorder. *Am J Med Genet* 105:84-90

Barr CL, Kroft J, Feng Y, Wigg K, Roberts W, Malone M, Ickowicz A, Schachar R, Tannock R, Kennedy JL (2002) The norepinephrine transporter gene and attention-deficit hyperactivity disorder. *Am J Med Genet* 114:255-9

Bellgrove MA, Hawi Z, Lowe N, Kirley A, Robertson IH, Gill M (2005) DRD4 gene variants and sustained attention in attention deficit hyperactivity disorder (ADHD): Effects of associated alleles at the VNTR and -521 SNP. *Am J Med Genet.* (in press)

Biederman J, Faraone SV, Keenan K, Benjamin J, Krifcher B, Moore C, Sprich-Buckminster S, Ugaglia K, Jellinek MS, Steingard R, et al. (1992) Further evidence for family-genetic risk factors in attention deficit hyperactivity disorder. Patterns of comorbidity in probands and relatives psychiatrically and pediatrically referred samples. *Arch Gen Psychiatry* 49:728-38

Biederman J, Faraone SV, Doyle A, Lehman BK, Kraus I, Perrin J, Tsuang MT (1993) Convergence of the Child Behavior Checklist with structured interview-based psychiatric diagnoses of ADHD children with and without comorbidity. *J Child Psychol Psychiatry* 34:1241-51

Biederman J, Milberger S, Faraone SV, Kiely K, Guite J, Mick E, Ablon S, Warburton R, Reed E (1995) Family-environment risk factors for attention-deficit hyperactivity disorder. A test of Rutter's indicators of adversity. *Arch Gen Psychiatry* 52:464-70

Biederman J, Spencer T (1999) Attention-deficit/hyperactivity disorder (ADHD) as a noradrenergic disorder. *Biol Psychiatry* 46:1234-42

Biederman J, Mick E, Faraone SV, Braaten E, Doyle A, Spencer T, Wilens TE, Frazier E, Johnson MA (2002) Influence of gender on attention deficit hyperactivity disorder in children referred to a psychiatric clinic. *Am J Psychiatry* 159:36-42

Brophy K, Hawi Z, Kirley A, Fitzgerald M, Gill M (2002) Synaptosomal-associated protein 25 (SNAP-25) and attention deficit hyperactivity disorder (ADHD): evidence of linkage and association in the Irish population. *Mol Psychiatry* 7:913-7

Brunner D, Buhot MC, Hen R, Hofer M (1999) Anxiety, motor activation, and maternal-infant interactions in 5HT1B knockout mice. *Behav Neurosci* 113:587-601

Cadore RJ, Stewart MA (1991) An adoption study of attention deficit/hyperactivity/aggression and their relationship to adult antisocial personality. *Compr Psychiatry* 32:73-82

Cantwell DP (1975) Genetics of hyperactivity. *J Child Psychol Psychiatry* 16:261-4

Castellanos FX, Lau E, Tayebi N, Lee P, Long RE, Giedd JN, Sharp W, Marsh WL, Walter JM, Hamburger SD, Ginns EI, Rapoport JL, Sidransky E (1998) Lack of an association between a dopamine-4 receptor polymorphism and attention-deficit/hyperactivity disorder: genetic and brain morphometric analyses. *Mol Psychiatry* 3:431-4

Catalano M, Nobile M, Novelli E, Nothen MM, Smeraldi E (1993) Distribution of a novel mutation in the first exon of the human dopamine D4 receptor gene in psychotic patients. *Biol Psychiatry* 34:459-64

Chen WJ, Faraone SV, Biederman J, Tsuang MT (1994) Diagnostic accuracy of the Child Behavior Checklist scales for attention-deficit hyperactivity disorder: a receiver-operating characteristic analysis. *J Consult Clin Psychol* 62:1017-25

Cheon KA, Ryu YH, Kim JW, Cho DY (2005) The homozygosity for 10-repeat allele at dopamine transporter gene and dopamine transporter density in Korean children with attention deficit hyperactivity disorder: relating to treatment response to methylphenidate. *Eur Neuropsychopharmacol*: 15:95-101.

Cichon S, Nothen MM, Catalano M, Di Bella D, Maier W, Lichtermann D, Minges J, Albus M, Borrmann M, Franzek E (1995) Identification of two novel polymorphisms and a rare deletion variant in the human dopamine D4 receptor gene. *Psychiatr Genet* 5:97-103

Comings DE, Gade-Andavolu R, Gonzalez N, Blake H, Wu S, MacMurray JP (1999) Additive effect of three noradrenergic genes (ADRA2a, ADRA2C, DBH) on attention-deficit hyperactivity disorder and learning disabilities in Tourette syndrome subjects. *Clin Genet* 55:160-72

Conners CK, Sitarenios G, Parker JD, Epstein JN (1998) The revised Conners' Parent Rating Scale (CPRS-R): factor structure, reliability, and criterion validity. *J Abnorm Child Psychol* 26:257-68

Curran S, Mill J, Sham P, Rijdsdijk F, Marusic K, Taylor E, Asherson P (2001) QTL association analysis of the DRD4 exon 3 VNTR polymorphism in a population sample of children screened with a parent rating scale for ADHD symptoms. *Am J Med Genet* 105:387-93

Dahlman I, Eaves IA, Kosoy R, Morrison VA, Heward J, Gough SC, Allahabadia A, Franklyn JA, Tuomilehto J, Tuomilehto- Wolf E, Cucca F, Guja C, Ionescu-Tirgoviste C, Stevens H, Carr P, Nutland S, McKinney P, Shield JP, Wang W, Cordell HJ, Walker N, Todd JA, Concannon P (2002) Parameters for reliable results in genetic association studies in common disease. *Nat Genet* 30:149–150

Daly G, Hawi Z, Fitzgerald M, Gill M (1999) Mapping susceptibility loci in attention deficit hyperactivity disorder: preferential transmission of parental alleles at DAT1, DBH and DRD5 to affected children. *Mol Psychiatry* 4:192-6

Davids E, Zhang K, Kula NS, Tarazi FI, Baldessarini RJ (2002) Effects of norepinephrine and serotonin transporter inhibitors on hyperactivity induced by neonatal 6-hydroxydopamine lesioning in rats. *J Pharmacol Exp Ther* 301:1097-102

Davids E, Zhang K, Tarazi FI, Baldessarini RJ (2003) Animal models of attention-deficit hyperactivity disorder. *Brain Res Brain Res Rev* 42:1-21

Davies W, Isles AR, Wilkinson LS (2001) Imprinted genes and mental dysfunction. *Ann Med* 33:428-36

Diaz-Anzaldúa A, Joobar R, Riviere JB, Dion Y, Lesperance P, Richer F, Chouinard S, Rouleau GA (2004) Tourette syndrome and dopaminergic genes: a family-based association study in the French Canadian founder population. *Mol Psychiatry* 9:272-7

Egger M, Davey Smith G, Schneider M, Minder C (1997) Bias in meta-analysis detected by a simple graphical test. *BMJ* 315: 629-634

Eisenberg J, Zohar A, Mei-Tal G, Steinberg A, Tartakovsky E, Gritsenko I, Nemanov L, Ebstein RP (2000) A haplotype relative risk study of the dopamine D4 receptor (DRD4) exon III repeat polymorphism and attention deficit hyperactivity disorder (ADHD). *Am J Med Genet* 96:258-61

Eubanks JH, Altherr M, Wagner-McPherson C, McPherson JD, Wasmuth JJ, Evans GA (1992) Localization of the D5 dopamine receptor gene to human chromosome 4p15.1-p15.3, centromeric to the Huntington's disease locus. *Genomics* 12:510-6

Faraone SV, Biederman J, Milberger S (1994) An exploratory study of ADHD among second-degree relatives of ADHD children. *Biol Psychiatry* 35:398-402

Faraone SV, Biederman J (1998a) Neurobiology of attention-deficit hyperactivity disorder. *Biol Psychiatry* 44:951-8

Faraone SV, Biederman J, Mennin D, Russell R, Tsuang MT (1998b) Familial subtypes of attention deficit hyperactivity disorder: a 4-year follow-up study of children from antisocial-ADHD families. *J Child Psychol Psychiatry* 39:1045-53

Faraone SV, Biederman J, Weiffenbach B, Keith T, Chu MP, Weaver A, Spencer TJ, Wilens TE, Frazier J, Cleves M, Sakai J (1999) Dopamine D4 gene 7-repeat allele and attention deficit hyperactivity disorder. *Am J Psychiatry* 156:768-70

Faraone SV, Biederman J, Monuteaux MC (2000) Toward guidelines for pedigree selection in genetic studies of attention deficit hyperactivity disorder. *Genet Epidemiol* 18:1-16

Faraone SV, Doyle AE, Mick E, Biederman J (2001) Meta-analysis of the association between the 7-repeat allele of the dopamine D(4) receptor gene and attention deficit hyperactivity disorder. *Am J Psychiatry* 158:1052-7

Faraone SV, Sergeant, J, Gillberg C, Biederman J (2003) The Worldwide Prevalence of ADHD: Is it an American Condition? *World Psychiatry* 2:104-113

Feng J, Sobell JL, Heston LL, Cook EH, Jr., Goldman D, Sommer SS (1998) Scanning of the dopamine D1 and D5 receptor genes by REF in neuropsychiatric patients reveals a novel missense change at a highly conserved amino acid. *Am J Med Genet* 81:172-8

Fisher SE, Francks C, McCracken JT, McGough JJ, Marlow AJ, MacPhie IL, Newbury DF, Crawford LR, Palmer CG, Woodward JA, Del'Homme M, Cantwell DP, Nelson SF, Monaco AP, Smalley SL (2002) A genomewide scan for loci involved in attention-deficit/hyperactivity disorder. *Am J Hum Genet* 70:1183-96

Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, Liu-Cordero SN, Rotimi C, Adeyemo A, Cooper R, Ward R, Lander ES, Daly MJ, Altshuler D (2002) The structure of haplotype blocks in the human genome. *Science* 296:2225-9

Gainetdinov RR, Wetsel WC, Jones SR, Levin ED, Jaber M, Caron MG (1999) Role of serotonin in the paradoxical calming effect of psychostimulants on hyperactivity. *Science* 283:397-401

Gainetdinov RR, Caron MG (2000) An animal model of attention deficit hyperactivity disorder. *Mol Med Today* 6:43-4

Gillberg C, Gillberg IC, Rasmussen P, Kadesjo B, Soderstrom H, Rastam M, Johnson M, Rothenberger A, Niklasson L (2004) Co-existing disorders in ADHD -- implications for diagnosis and intervention. *Eur Child Adolesc Psychiatry* 13 Suppl 1:180-92

Giros B, Jaber M, Jones S, Wightman RM, Caron M (1996) Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature* 379:606-12

Gjone H, Stevenson J, Sundet JM (1996) Genetic influence on parent-reported attention-related problems in a Norwegian general population twin sample. *J Am Acad Child Adolesc Psychiatry* 35:588-96; discussion 596-8

Goodier IW, Pritchard DH (1966) The half-life of P32. *Int J Appl Radiat Isot* 17:121-3.

Grandy DK, Zhang YA, Bouvier C, Zhou QY, Johnson RA, Allen L, Buck K, Bunzow JR, Salon J, Civelli O (1991) Multiple human D5 dopamine receptor genes: a functional receptor and two pseudogenes. *Proc Natl Acad Sci U S A* 88:9175-9

Grandy DK, Allen LJ, Zhang Y, Magenis RE, Civelli O (1992) Chromosomal localization of three human D5 dopamine receptor genes. *Genomics* 13:968-73

Hartung CM, Willcutt EG, Lahey BB, Pelham WE, Loney J, Stein MA, Keenan K (2002) Sex differences in young children who meet criteria for attention deficit hyperactivity disorder. *J Clin Child Adolesc Psychol* 31:453-64

Hawi Z, McCarron M, Kirley A, Daly G, Fitzgerald M, Gill M (2000) No association of the dopamine DRD4 receptor (DRD4) gene polymorphism with attention deficit hyperactivity disorder (ADHD) in the Irish population. *Am J Med Genet* 96:268-72

Hawi Z, Dring M, Kirley A, Foley D, Kent L, Craddock N, Asherson P, Curran S, Gould A, Richards S, Lawson D, Pay H, Turic D, Langley K, Owen M, O'Donovan M, Thapar A, Fitzgerald M, Gill M (2002) Serotonergic system and attention deficit hyperactivity disorder (ADHD): a potential susceptibility locus at the 5-HT(1B) receptor gene in 273 nuclear families from a multi-centre sample. *Mol Psychiatry* 7:718-25

Hawi Z, Kirley A, Lowe N, Fitzgerald M, Gill M (2003a) Recent genetic advances in ADHD and diagnostic and therapeutic prospects. *Expert Rev Neurotherapeutics* 3:453-464.

Hawi Z, Lowe N, Kirley A, Gruenhage F, Nothen M, Greenwood T, Kelsoe J, Fitzgerald M, Gill M (2003b) Linkage disequilibrium mapping at DAT1, DRD5 and DBH narrows the search for ADHD susceptibility alleles at these loci. *Mol Psychiatry* 8:299-308

Hay D, McStephen M, Levy F (2001) The diagnostic genetics of ADHD symptoms and subtypes. In: Levy F, Hay D (eds) *Attention genes and ADHD*. Hove, United Kingdom, Brunner-Routledge, pp 35–37

Hechtman L, Weiss G (1983) Long-term outcome of hyperactive children. *Am J Orthopsychiatry* 53:532-41

Hechtman L, Weiss G (1986) Controlled prospective fifteen year follow-up of hyperactives as adults: non-medical drug and alcohol use and anti-social behaviour. *Can J Psychiatry* 31:557-67

Hess EJ, Collins KA, Copeland NG, Jenkins NA, Wilson MC (1994) Deletion map of the coloboma (Cm) locus on mouse chromosome 2. *Genomics* 21:257-61

Hey J (2004) What's so hot about recombination hotspots? *PLoS Biol* 2:730-3

Hims MM, Diager SP, Inglehearn CF (2003) Retinitis pigmentosa: genes, proteins and prospects. *Dev Ophthalmol* 37:109-25

Holmes J, Payton A, Barrett JH, Hever T, Fitzpatrick H, Trumper AL, Harrington R, McGuffin P, Owen M, Ollier W, Worthington J, Thapar A (2000) A family-based and case-control association study of the dopamine D4 receptor gene and dopamine transporter gene in attention deficit hyperactivity disorder. *Mol Psychiatry* 5:523-30

Holmes J, Payton A, Barrett J, Harrington R, McGuffin P, Owen M, Ollier W, Worthington J, Gill M, Kirley A, Hawi Z, Fitzgerald M, Asherson P, Curran S, Mill J, Gould A, Taylor E, Kent L, Craddock N, Thapar A (2002) Association of DRD4 in children with ADHD and comorbid conduct problems. *Am J Med Genet* 114:150-3

Jaber M, Dumartin B, Sagne C, Haycock JW, Roubert C, Giros B, Bloch B, Caron MG (1999) Differential regulation of tyrosine hydroxylase in the basal ganglia of mice lacking the dopamine transporter. *Eur J Neurosci* 11:3499-511

Jensen PS, Martin D, Cantwell DP (1997) Comorbidity in ADHD: implications for research, practice, and DSM-V. *J Am Acad Child Adolesc Psychiatry* 36:1065-79

Jiang S, Xin R, Lin S, Qian Y, Tang G, Wang D, Wu X (2001) Linkage studies between attention-deficit hyperactivity disorder and the monoamine oxidase genes. *Am J Med Genet* 105:783-8

Jones MB, Palmour RM, Zwaigenbaum L, Szatmari P (2004) Modifier effects in autism at the MAO-A and DBH loci. *Am J Med Genet* 126B:58-65

Jovanovic V, Guan HC, Van Tol HH (1999) Comparative pharmacological and functional analysis of the human dopamine D4.2 and D4.10 receptor variants. *Pharmacogenetics* 9:561-8

Kahn RS, Khoury J, Nichols WC, Lanphear BP (2003) Role of dopamine transporter genotype and maternal prenatal smoking in childhood hyperactive-impulsive, inattentive, and oppositional behaviors. *J Pediatr* 143:104-10

Kaufman J, Birmaher B, Brent D, Rao U, Flynn C, Moreci P, Williamson D, Ryan N (1997) Schedule for affective disorders and schizophrenia for school-age children-present and lifetime version (K-SADS-PL): initial reliability and validity data. *J Am Acad Child Adolesc Psychiatry* 36:980-988

Kent L, Doerry U, Hardy E, Parmar R, Gingell K, Hawi Z, Kirley A, Lowe N, Fitzgerald M, Gill M, Craddock N (2002) Evidence that variation at the serotonin transporter gene influences susceptibility to attention deficit hyperactivity disorder (ADHD): analysis and pooled analysis. *Mol Psychiatry* 7:908-12

Kent L (2004) Recent advances in the genetics of attention deficit hyperactivity disorder. *Curr Psychiatry Rep* 6:143-8

Kirley A, Hawi Z, Daly G, McCarron M, Mullins C, Millar N, Waldman I, Fitzgerald M, Gill M (2002) Dopaminergic system genes in ADHD: toward a biological hypothesis. *Neuropsychopharmacology* 27:607-19

Kirley A, Lowe N, Hawi Z, Mullins C, Daly G, Waldman I, McCarron M, O'Donnell D, Fitzgerald M, Gill M (2003) Association of the 480 bp DAT1 allele with methylphenidate response in a sample of Irish children with ADHD. *Am J Med Genet* 121:50-4

Kirley A, Lowe N, Mullins C, McCarron M, Daly G, Waldman I, Fitzgerald M, Gill M, Hawi Z (2004) Phenotype studies of the DRD4 gene polymorphisms in ADHD: Association with oppositional defiant disorder and positive family history. *Am J Med Genet* 131B:38-42

Klein RG, Mannuzza S (1991) Long-term outcome of hyperactive children: a review. *J Am Acad Child Adolesc Psychiatry* 30:383-7

Kleinjan DA, van Heyningen V (2005) Long-range control of gene expression: emerging mechanisms and disruption in disease. *Am J Hum Genet* 76:8-32

Kotler M, Manor I, Sever Y, Eisenberg J, Cohen H, Ebstein RP, Tyano S (2000) Failure to replicate an excess of the long dopamine D4 exon III repeat polymorphism in ADHD in a family-based study. *Am J Med Genet* 96:278-81

Kuhne M, Schachar R, Tannock R (1997) Impact of comorbid oppositional or conduct problems on attention-deficit hyperactivity disorder. *J Am Acad Child Adolesc Psychiatry* 36:1715-25

Kustanovich V, Merriman B, McGough J, McCracken JT, Smalley SL, Nelson SF (2003) Biased paternal transmission of SNAP-25 risk alleles in attention-deficit hyperactivity disorder. *Mol Psychiatry* 8:309-15

Kustanovich V, Ishii J, Crawford L, Yang M, McGough JJ, McCracken JT, Smalley SL, Nelson SF (2004) Transmission disequilibrium testing of dopamine-related candidate gene polymorphisms in ADHD: confirmation of association of ADHD with DRD4 and DRD5. *Mol Psychiatry* 9:711-7

LaHoste GJ, Swanson JM, Wigal SB, Glabe C, Wigal T, King N, Kennedy JL (1996) Dopamine D4 receptor gene polymorphism is associated with attention deficit hyperactivity disorder. *Mol Psychiatry* 1:121-4

Laucht M, Schmidt MH (2004) [Maternal smoking during pregnancy: risk factor for ADHD in the offspring?]. *Z Kinder Jugendpsychiatr Psychother* 32:177-85

Levitan RD, Masellis M, Basile VS, Lam RW, Jain U, Kaplan AS, Kennedy SH, Siegel G, Walker ML, Vaccarino FJ, Kennedy JL (2002) Polymorphism of the serotonin-2A receptor gene (HTR2A) associated with childhood attention deficit hyperactivity disorder (ADHD) in adult women with seasonal affective disorder. *J Affect Disord* 71:229-33

Levy F, Hay DA, McStephen M, Wood C, Waldman I (1997) Attention-deficit hyperactivity disorder: a category or a continuum? Genetic analysis of a large-scale twin study. *J Am Acad Child Adolesc Psychiatry* 36:737-44

Losier BJ, McGrath PJ, Klein RM (1996) Error patterns on the continuous performance test in non-medicated and medicated samples of children with and without ADHD: a meta-analytic review. *J Child Psychol Psychiatry* 37:971-87

Lowe N, Kirley A, Mullins C, Fitzgerald M, Gill M, Hawi Z (2004a) Multiple marker analysis at the promoter region of the DRD4 gene and ADHD: Evidence of linkage and association with the SNP -616. *Am J Med Genet* 131B:33-37

Lowe N, Kirley A, Hawi Z, Sham P, Wickham H, Kratochvil CJ, Smith SD, et al. (2004b) Joint analysis of the DRD5 marker concludes association with attention-deficit/hyperactivity disorder confined to the predominantly inattentive and combined subtypes. *Am J Hum Genet* 74:348-56

Maglott DR, Feldblyum TV, Durkin AS, Nierman WC (1996) Radiation hybrid mapping of SNAP, PCSK2, and THBD (human chromosome 20p). *Mamm Genome* 7:400-1

Maher BS, Marazita ML, Moss HB, Vanyukov MM (1999) Segregation analysis of attention deficit hyperactivity disorder. *Am J Med Genet* 88:71-8

Maher BS, Marazita ML, Ferrell RE, Vanyukov MM (2002) Dopamine system genes and attention deficit hyperactivity disorder: a meta-analysis. *Psychiatr Genet* 12:207-15

Mannuzza S, Klein RG, Bessler A, Malloy P, LaPadula M (1993) Adult outcome of hyperactive boys. Educational achievement, occupational rank, and psychiatric status. *Arch Gen Psychiatry* 50:565-76

Manor I, Eisenberg J, Tyano S, Sever Y, Cohen H, Ebstein RP, Kotler M (2001) Family-based association study of the serotonin transporter promoter region polymorphism (5-HTTLPR) in attention deficit hyperactivity disorder. *Am J Med Genet* 105:91-5

Manor I, Tyano S, Eisenberg J, Bachner-Melman R, Kotler M, Ebstein RP (2002) The short DRD4 repeats confer risk to attention deficit hyperactivity disorder in a family-based design and impair performance on a continuous performance test (TOVA). *Mol Psychiatry* 7:790-4

Manor I, Corbex M, Eisenberg J, Gritsenko I, Bachner-Melman R, Tyano S, Ebstein RP (2004) Association of the dopamine D5 receptor with attention deficit hyperactivity disorder (ADHD) and scores on a continuous performance test (TOVA). *Am J Med Genet* 127B:73-7

McCracken JT, Smalley SL, McGough JJ, Crawford L, Del'Homme M, Cantor RM, Liu A, Nelson SF (2000) Evidence for linkage of a tandem duplication polymorphism upstream of the dopamine D4 receptor gene (DRD4) with attention deficit hyperactivity disorder (ADHD). *Mol Psychiatry* 5:531-6

McEvoy B, Hawi Z, Fitzgerald M, Gill M (2002) No evidence of linkage or association between the norepinephrine transporter (NET) gene polymorphisms and ADHD in the Irish population. *Am J Med Genet* 114:665-6

Milberger S, Biederman J, Faraone SV, Guite J, Tsuang MT (1997) Pregnancy, delivery and infancy complications and attention deficit hyperactivity disorder: issues of gene-environment interaction. *Biol Psychiatry* 41:65-75

Mill J, Curran S, Kent L, Richards S, Gould A, Virdee V, Hockett L, Sharp J, Batten C, Fernando S, Simanoff E, Thompson M, Zhao J, Sham P, Taylor E, Asherson P (2001) Attention deficit hyperactivity disorder (ADHD) and the dopamine D4 receptor gene: evidence of association but no linkage in a UK sample. *Mol Psychiatry* 6:440-4

Mill J, Curran S, Kent L, Gould A, Hockett L, Richards S, Taylor E, Asherson P (2002a) Association study of a SNAP-25 microsatellite and attention deficit hyperactivity disorder. *Am J Med Genet* 114:269-71

Mill JS, Caspi A, McClay J, Sugden K, Purcell S, Asherson P, Craig I, McGuffin P, Braithwaite A, Poulton R, Moffitt TE (2002b) The dopamine D4 receptor and the hyperactivity phenotype: a developmental-epidemiological study. *Mol Psychiatry* 7:383-91

Mill J, Fisher N, Curran S, Richards S, Taylor E, Asherson P (2003) Polymorphisms in the dopamine D4 receptor gene and attention-deficit hyperactivity disorder. *Neuroreport* 14:1463-6

Mill J, Richards S, Knight J, Curran S, Taylor E, Asherson P (2004a) Haplotype analysis of SNAP-25 suggests a role in the aetiology of ADHD. *Mol Psychiatry*

Mill J, Curran S, Richards S, Taylor E, Asherson P (2004b) Polymorphisms in the dopamine D5 receptor (DRD5) gene and ADHD. *Am J Med Genet* 125B:38-42

Mitsuyasu H, Ozawa H, Takeda Y, Fukumaki Y (1999) Novel polymorphisms in the upstream region of the human dopamine D4 receptor (DRD4) gene. *J Hum Genet* 44:416-8

Morrison JR, Stewart MA (1973) The psychiatric status of the legal families of adopted hyperactive children. *Arch Gen Psychiatry* 28:888-91

Muglia P, Jain U, Macciardi F, Kennedy JL (2000) Adult attention deficit hyperactivity disorder and the dopamine D4 receptor gene. *Am J Med Genet* 96:273-7

Nebigil CG, Etienne N, Schaerlinger B, Hickel P, Launay JM, Maroteaux L (2001) Developmentally regulated serotonin 5-HT_{2B} receptors. *Int J Dev Neurosci* 19:365-72

Nguyen T, Bard J, Jin H, Taruscio D, Ward DC, Kennedy JL, Weinshank R, Seeman P, O'Dowd BF (1991) Human dopamine D5 receptor pseudogenes. *Gene* 109:211-8

Nothen MM, Cichon S, Hemmer S, Hebebrand J, Remschmidt H, Lehmkuhl G, Poustka F, Schmidt M, Catalano M, Fimmers R (1994) Human dopamine D4 receptor gene: frequent occurrence of a null allele and observation of homozygosity. *Hum Mol Genet* 3:2207-12

Nyholt DR (2001) Genetic case-control association studies--correcting for multiple testing. *Hum Genet* 109:564-7

Ogdie MN, Macphie IL, Minassian SL, Yang M, Fisher SE, Francks C, Cantor RM, McCracken JT, McGough JJ, Nelson SF, Monaco AP, Smalley SL (2003) A genomewide scan for attention-deficit/hyperactivity disorder in an extended sample: suggestive linkage on 17p11. *Am J Hum Genet* 72:1268-79

Okuyama Y, Ishiguro H, Toru M, Arinami T (1999) A genetic polymorphism in the promoter region of DRD4 associated with expression and schizophrenia. *Biochem Biophys Res Commun* 258:292-5

O'Neill MF, Heron-Maxwell CL, Shaw G (1999) 5-HT₂ receptor antagonism reduces hyperactivity induced by amphetamine, cocaine, and MK-801 but not D1 agonist C-APB. *Pharmacol Biochem Behav* 63:237-43

Orvaschel H, Puig-Antich J (1987) Schedule for Affective Disorder and Schizophrenia for School-Age Children-Epidemiological, 4th version. Nova University Center for Psychological Study, Fort Lauderdale

Ott J (1999) Analysis of human genetic linkage, 3rd Edn. Johns Hopkins University Press, Baltimore

Payton A, Holmes J, Barrett JH, Hever T, Fitzpatrick H, Trumper AL, Harrington R, McGuffin P, O'Donovan M, Owen M, Ollier W, Worthington J, Thapar A (2001) Examining for association between candidate gene polymorphisms in the dopamine pathway and attention-deficit hyperactivity disorder: a family-based study. *Am J Med Genet* 105:464-70

Petronis A, O'Hara K, Barr CL, Kennedy JL, Van Tol HH (1994) (G)n-monomonucleotide polymorphism in the human D4 dopamine receptor (DRD4) gene. *Hum Genet* 93:719

Pierce CD, Reid R (2004) Attention deficit hyperactivity disorder: assessment and treatment of children from culturally different groups. *Semin Speech Lang* 25:233-40

Popper CW (2000) Pharmacologic alternatives to psychostimulants for the treatment of attention-deficit/hyperactivity disorder. *Child Adolesc Psychiatr Clin N Am* 9:605-46

Quist JF, Barr CL, Schachar R, Roberts W, Malone M, Tannock R, Basile VS, Beitchman J, Kennedy JL (2000) Evidence for the serotonin HTR2A receptor gene as a susceptibility factor in attention deficit hyperactivity disorder (ADHD). *Mol Psychiatry* 5:537-41

Quist JF, Barr CL, Schachar R, Roberts W, Malone M, Tannock R, Basile VS, Beitchman J, Kennedy JL (2003) The serotonin 5-HT1B receptor gene and attention deficit hyperactivity disorder. *Mol Psychiatry* 8:98-102

Retz W, Retz-Junginger P, Henges G, Schneider M, Thome J, Pajonk FG, Salahi-Disfan A, Rees O, Wender PH, Rosler M (2004) Psychometric and psychopathological characterization of young male prison inmates with and without attention deficit/hyperactivity disorder. *Eur Arch Psychiatry Clin Neurosci* 254:201-8

Rietveld MJ, Hudziak JJ, Bartels M, van Beijsterveldt CE, Boomsma DI (2003) Heritability of attention problems in children: I. cross-sectional results from a study of twins, age 3-12 years. *Am J Med Genet* 117B:102-13

Rietveld MJ, Hudziak JJ, Bartels M, van Beijsterveldt CE, Boomsma DI (2004) Heritability of attention problems in children: longitudinal results from a study of twins, age 3 to 12. *J Child Psychol Psychiatry* 45:577-88

Risch NJ (2000) Searching for genetic determinants in the new millennium. *Nature* 405:847-56

Roman T, Schmitz M, Polanczyk G, Eizirik M, Rohde LA, Hutz MH (2001) Attention-deficit hyperactivity disorder: a study of association with both the dopamine transporter gene and the dopamine D4 receptor gene. *Am J Med Genet* 105:471-8

Roman T, Szobot C, Martins S, Biederman J, Rohde LA, Hutz MH (2002) Dopamine transporter gene and response to methylphenidate in attention-deficit/hyperactivity disorder. *Pharmacogenetics* 12:497-9

Rowe DC, Stever C, Giedinghagen LN, Gard JM, Cleveland HH, Terris ST, Mohr JH, Sherman S, Abramowitz A, Waldman ID (1998) Dopamine DRD4 receptor polymorphism and attention deficit hyperactivity disorder. *Mol Psychiatry* 3:419-26

Russell V, de Villiers A, Sagvolden T, Lamm M, Taljaard J (1995) Altered dopaminergic function in the prefrontal cortex, nucleus accumbens and caudate-putamen of an animal model of attention-deficit hyperactivity disorder--the spontaneously hypertensive rat. *Brain Res* 676:343-51

Russell VA (2002) Hypodopaminergic and hypernoradrenergic activity in prefrontal cortex slices of an animal model for attention-deficit hyperactivity disorder--the spontaneously hypertensive rat. *Behav Brain Res* 130:191-6

Samuel VJ, George P, Thornell A, Curtis S, Taylor A, Brome D, Mick E, Faraone SV, Biederman J (1999) A pilot controlled family study of DSM-III-R and DSM-IV ADHD in African-American children. *J Am Acad Child Adolesc Psychiatry* 38:34-9

Schachar R, Logan G (1990) Are hyperactive children deficient in attentional capacity? *J Abnorm Child Psychol* 18:493-513

Seaman MI, Fisher JB, Chang F, Kidd KK (1999) Tandem duplication polymorphism upstream of the dopamine D4 receptor gene (DRD4). *Am J Med Genet* 88:705-9

Seeger G, Schloss P, Schmidt MH (2001a) Functional polymorphism within the promotor of the serotonin transporter gene is associated with severe hyperkinetic disorders. *Mol Psychiatry* 6:235-8

Seeger G, Schloss P, Schmidt MH (2001b) Marker gene polymorphisms in hyperkinetic disorder--predictors of clinical response to treatment with methylphenidate? *Neurosci Lett* 313:45-8

Seeman P, Ulpian C, Chouinard G, Van Tol HH, Dwosh H, Lieberman JA, Siminovitch K, Liu IS, Wayne J, Voruganti P (1994) Dopamine D4 receptor variant, D4GLYCINE194, in Africans, but not in Caucasians: no association with schizophrenia. *Am J Med Genet* 54:384-90

Seeman P, Madras BK (1998) Anti-hyperactivity medication: methylphenidate and amphetamine. *Mol Psychiatry* 3:386-96

Shaffer D, Fisher P, Lucas CP, Dulcan MK, Schwab-Stone ME (2000) NIMH Diagnostic Interview Schedule for Children, version IV (NIMH DISC-IV): description differences from previous versions, and reliability of some common diagnoses. *J Am Acad Child Adolesc Psychiatry* 39:28–38

Shih JC, Chen K, Ridd MJ (1999) Role of MAO A and B in neurotransmitter metabolism and behavior. *Pol J Pharmacol* 51:25-9

Smalley SL, Bailey JN, Palmer CG, Cantwell DP, McGough JJ, Del'Homme MA, Asarnow JR, Woodward JA, Ramsey C, Nelson SF (1998) Evidence that the dopamine D4 receptor is a susceptibility gene in attention deficit hyperactivity disorder. *Mol Psychiatry* 3:427-30

Sobell JL, Lind TJ, Sigurdson DC, Zald DH, Snitz BE, Grove WM, Heston LL, Sommer SS (1995) The D5 dopamine receptor gene in schizophrenia: identification of a nonsense change and multiple missense changes but lack of association with disease. *Hum Mol Genet* 4:507-14

Solanto MV (1998) Neuropsychopharmacological mechanisms of stimulant drug action in attention deficit hyperactivity disorder, a review and integration. *Behav Brain Res* 94:127-152

Solanto MV, Abikoff H, Sonuga-Barke E, Schachar R, Logan GD, Wigal T, Hechtman L, Hinshaw S, Turkel E (2001) The ecological validity of delay aversion and response inhibition as measures of impulsivity in AD/HD: a supplement to the NIMH multimodal treatment study of AD/HD. *J Abnorm Child Psychol* 29:215-28

Sollner T, Bennett MK, Whiteheart SW, Scheller RH, Rothman JE (1993) A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. *Cell* 75:409-18

Sonuga-Barke EJ, Balding J (1993) British parents' beliefs about the causes of three forms of childhood psychological disturbance. *J Abnorm Child Psychol* 21:367-76

Sonuga-Barke EJ, Taylor E, Sembi S, Smith J (1992) Hyperactivity and delay aversion--I. The effect of delay on choice. *J Child Psychol Psychiatry* 33:387-98

Spiegelman JI, Mindrinos MN, Oefner PJ (2000) High-accuracy DNA sequence variation screening by DHPLC. *Biotechniques* 29:1084-90

Spielman RS, Ewans WJ (1996) The TDT and other family based tests for linkage disequilibrium and association. *Am J Hum Genet* 59: 983-9

Sprich S, Biederman J, Crawford MH, Mundy E, Faraone SV (2000) Adoptive and biological families of children and adolescents with ADHD. *J Am Acad Child Adolesc Psychiatry* 39:1432-7

Sunohara GA, Roberts W, Malone M, Schachar RJ, Tannock R, Basile VS, Wigal T, Wigal SB, Schuck S, Moriarty J, Swanson JM, Kennedy JL, Barr CL (2000) Linkage of the dopamine D4 receptor gene and attention-deficit/hyperactivity disorder. *J Am Acad Child Adolesc Psychiatry* 39:1537-42

Swanson JM, Sergeant JA, Taylor E, Sonuga-Barke EJ, Jensen PS, Cantwell DP (1998a) Attention-deficit hyperactivity disorder and hyperkinetic disorder. *Lancet* 351:429-33

Swanson JM, Sunohara GA, Kennedy JL, Regino R, Fineberg E, Wigal T, Lerner M, Williams L, LaHoste GJ, Wigal S (1998b) Association of the dopamine receptor D4 (DRD4) gene with a refined phenotype of attention deficit hyperactivity disorder (ADHD): a family-based approach. *Mol Psychiatry* 3:38-41

Tahir E, Yazgan Y, Cirakoglu B, Ozbay F, Waldman I, Asherson PJ (2000) Association and linkage of DRD4 and DRD5 with attention deficit hyperactivity disorder (ADHD) in a sample of Turkish children. *Mol Psychiatry* 5:396-404

Tang G, Ren D, Xin R, Qian Y, Wang D, Jiang S (2001) Lack of association between the tryptophan hydroxylase gene A218C polymorphism and attention-deficit hyperactivity disorder in Chinese Han population. *Am J Med Genet* 105:485-8

Tannock R (1998) Attention deficit hyperactivity disorder: advances in cognitive, neurobiological, and genetic research. *J Child Psychol Psychiatry* 39:65-99

Thapar A, Holmes J, Poulton K, Harrington R (1999) Genetic basis of attention deficit and hyperactivity. *Br J Psychiatry* 174:105-11

Thapar A, Harrington R, McGuffin P (2001) Examining the comorbidity of ADHD-related behaviours and conduct problems using a twin study design. *Br J Psychiatry* 179:224-9

Thorley G (1984) Review of follow-up and follow-back studies of childhood hyperactivity. *Psychol Bull* 96:116-32

Todd RD, Neuman RJ, Lobos EA, Jong YJ, Reich W, Heath AC (2001) Lack of association of dopamine D4 receptor gene polymorphisms with ADHD subtypes in a population sample of twins. *Am J Med Genet* 105:432-8

Todd RD, Lobos EA (2002) Mutation screening of the dopamine D2 receptor gene in attention-deficit hyperactivity disorder subtypes: preliminary report of a research strategy. *Am J Med Genet* 114:34-41

Tversky A, Kahneman D (1971) Belief in the law of small numbers.

Vallone D, Picetti R, Borrelli E (2000) Structure and function of dopamine receptors. *Neurosci Biobehav Rev* 24:125-32

Vila M, Przedborski S (2004) Genetic clues to the pathogenesis of Parkinson's disease. *Nat Med* 10 Suppl:S58-62

Volkow ND, Wang GJ, Fowler JS, Gatley SJ, Logan J, Ding YS, Hitzemann R, Pappas N (1998) Dopamine transporter occupancies in the human brain induced by therapeutic doses of oral methylphenidate. *Am J Psychiatry* 155:1325-31

Waldman ID, Rowe DC, Abramowitz A, Kozel ST, Mohr JH, Sherman SL, Cleveland HH, Sanders ML, Gard JM, Stever C (1998) Association and linkage of the dopamine transporter gene and attention deficit hyperactivity disorder in children: owing to diagnostic subtype and severity. *Am J Hum Genet* 63:1767-1776

Ward MF, Wender PH, Reimherr FW (1993) The Wender Utah Rating Scale: an aid in the retrospective diagnosis of childhood attention deficit hyperactivity disorder. *Am J Psychiatry* 150:885-90

Wilens TE, Biederman J, Spencer TJ (2002) Attention deficit/hyperactivity disorder across the lifespan. *Annu Rev Med* 53:113-31

Williams T, Tjian R (1991) Analysis of the DNA-binding and activation properties of the human transcription factor AP-2. *Genes Dev* 5:670-82

Wilson MC (2000) Coloboma mouse mutant as an animal model of hyperkinesia and attention deficit hyperactivity disorder. *Neurosci Biobehav Rev* 24:51-7

Winsberg BG, Comings DE (1999) Association of the dopamine transporter gene (DAT1) with poor methylphenidate response. *J Am Acad Child Adolesc Psychiatry* 38:1474-7

Wong AH, Buckle CE, Van Tol HH (2000) Polymorphisms in dopamine receptors: what do they tell us? *Eur J Pharmacol* 410:183-203

Xu C, Schachar R, Tannock R, Roberts W, Malone M, Kennedy JL, Barr CL (2001) Linkage study of the alpha2A adrenergic receptor in attention-deficit hyperactivity disorder families. *Am J Med Genet* 105:159-62

Xue Y, Gao X, Lindsell CE, Norton CR, Chang B, Hicks C, Gendron-Maguire M, Rand EB, Weinmaster G, Gridley T (1999) Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Hum Mol Genet* 8:723-30

Zimmerman H (1993) *Synaptic transmission cellular and molecular basis*. Oxford University Press, Oxford UK

Zoroglu SS, Erdal ME, Alasehirli B, Erdal N, Sivasli E, Tutkun H, Savas HA, Herken H (2002) Significance of serotonin transporter gene 5-HTTLPR and variable number of tandem repeat polymorphism in attention deficit hyperactivity disorder. *Neuropsychobiology* 45:176-81

Zoroglu SS, Erdal ME, Erdal N, Ozen S, Alasehirli B, Sivasli E (2003) No evidence for an association between the T102C and 1438 G/A polymorphisms of the serotonin 2A receptor gene in attention deficit/hyperactivity disorder in a Turkish population. *Neuropsychobiology* 47:17-20



Appendix A

Buffers and Solutions

DNA Extraction Buffers (Chapter 2, Section 2.2.1)

Lysis Buffer (10X)

- Sucrose 547.7g
- 2M Tris (pH 7.5) 25ml
- 1M MgCl₂ 25ml
- Triton X100 50ml
- H₂O up to 1L

Suspension Buffer (10X)

- 2M Tris (pH 7.5) 5ml
- 0.5M EDTA (pH 8.0) 20ml
- 5M NaCl 2ml
- H₂O up to 100ml

TE Buffer

- 2M Tris (pH 7.5) 1ml
- 0.5M EDTA (pH 8.0) 40μl
- H₂O up to 200ml

PCR Reagents (section 2.2.4)

MgCl₂ Buffer 10mM/15mM/20mM

- 1M KCl 2.5ml
- 1M Tris (pH 8.9) 0.5ml
- 1M MgCl₂ 50µl/75µl/100µl
- 1% Gelatine 0.5ml
- Triton X100 50µl
- H₂O up to 5ml

Triton and Gelatine Free MgCl₂ Buffer 10mM/15mM/20mM

- 1M KCl 2.5ml
- 1M Tris (pH 8.9) 0.5ml
- 1M MgCl₂ 50µl/75µl/100µl
- H₂O up to 5ml

Gel Electrophoresis and Visualisation (Chapter 2, Section 2.2.7)

TAE Buffer (50X)

- Trizma Base 242g
- 0.5M EDTA (pH 8.0) 100ml
- Glacial Acetic Acid 57.1ml
- H₂O up to 1L

40% Polyacrylamide Solution

- Acrylamide 39g
- Bisacrylamide 1g
- H₂O up to 100ml

Non Denaturing Polyacrylamide Gel (6%)

- 40% Polyacrylamide 9ml
- TBE (10X) 6ml
- H₂O 45ml
- 10% APS 0.5ml
- TEMED 80µl

Denaturing Polyacrylamide Gels for Use with the ABI PRISM 377

DNA Sequencer (12% and 6% (w/v))

- Long Ranger (ABI) 6ml/3ml
- TBE (10X) 3ml
- H₂O 13ml/16ml
- Urea 11g
- 10% APS 150µl
- TEMED 21µl

Denaturing Polyacrylamide Gels for Radiolabelled PCR (6%)

- 8M Urea 52ml
- Acrylamide Mix (SequaGel) 19.2ml
- TBE (10X) 8ml
- 10% APS 400µl
- TEMED 50µl

Sequagel Concentrate (National Diagnostics Inc, Atlanta, GA, USA)

Loading Dye

- Bromophenol Blue 12.5mg
- Xylene Cyanol 12.5mg
- Glycerol 15ml
- H₂O up to 50ml

Formamide Loading Dye

- Formamide 10ml
- Xylene Cyanol 10mg
- Bromophenol Blue 10mg
- 0.5M EDTA (pH 8.0) 200µl

SNaPshot Buffer

- 1M Tris (pH 9.0) 8ml
- 2mM MgCl₂ 8ml
- H₂O 34ml

DHPLC (Chapter 2, Section 2.2.9)

Buffer A

- 2M TEAA (Transgenomic) 50ml
- Acetonitrile (HPLC grade) 250μl
- H₂O up to 1L

Buffer B

- 2M TEAA (Transgenomic) 50ml
- Acetonitrile (HPLC grade) 250ml
- H₂O up to 1L

Buffer C

- Acetonitrile (HPLC grade) 250ml
- H₂O up to 1L

Buffer D

- Acetonitrile (HPLC grade) 250ml
- H₂O up to 1L



Appendix B

Primer Sequences and PCR Conditions

This Appendix lists the primers for each of the variants genotyped in this thesis. These include the primers used to genotype the DRD4 (Table I), DRD5 (Table II) and SNAP-25 (Table III) variants used in Chapter 4, the DHPLC amplicon primers used in Chapter 3 (Table IV) and the primers used to test the additional DRD5 SNPs that were present in the databases but not discovered in the mutation screening (Table V). It also describes the annealing temperature (T_m), and any additional variations to the standard PCR protocol (section 2.2).

PCR primers are labelled 'F' for the forward primer and 'R' for the reverse primer. All primers are orientated 5' to 3' and were designed using the Primer3 software (www.es.embet.org/cgi-bin/primer3_www.cgi), except where otherwise specified. SNaPshot primers, which were designed by eye based on the sequence surrounding the SNP, are listed in a similar fashion and preceded by the letter 'E'. All primers were ordered from Invitrogen (www.invitrogen.com).

Table I – Primer Sequences and Conditions used to Genotype DRD4 Variants

Variant	Primer Sequences	T _m (°C)	Variations to Standard Procedure	Reference
120bp	F-GTTGTCTGTCTTTTCTCATTGTTTCCATTG R-GAAGGAGCAGGCACCGTGAGC	58	None	Seaman et al (1999)
-616	F-TCAACTGTGCAACGGGTG R-GAGAAACCGACAAGGATGGA E- TGGTCGCGGGGGCTGAG	58	35 cycles of 94 ⁰ C, 58 ⁰ C and 72 ⁰ C for 40 sec each	Barr et al (2001)
-521	F-TCAACTGTGCAACGGGTG R-GAGAAACCGACAAGGATGGA E- CTCGCCTCGACCTCGTGCGC	58	35 cycles of 94 ⁰ C, 58 ⁰ C and 72 ⁰ C for 40 sec each	Barr et al (2001)
-376	F-TCAACTGTGCAACGGGTG R-GAGAAACCGACAAGGATGGA E- CCCGAGGGCCGGGACGCACG	58	35 cycles of 94 ⁰ C, 58 ⁰ C and 72 ⁰ C for 40 sec each	None
VNTR	F-GCGACTACGTGGTCTACTCG R-AGGACCCTCATGGCCTTG	58	40 cycles of 15 sec at 95 ⁰ C, 15 sec at 58 ⁰ C and 20 sec at 72 ⁰ C	Hawi et al (2000)

Table II – Primer Sequences and Conditions used to Genotype DRD5 Variants

Variant	Primer Sequences	T _m (°C)	Variations to Standard Procedure	Reference
D4S2928	F- ATAGACGTGTTCTGCTGG R- CTCAGGCTATTTATGGGGTG	58	30 cycles of 94 ⁰ C, 58 ⁰ C and 72 ⁰ C for 1 min each	Hawi et al (2003b)
D4S1582	F- ATCAGGGTTCTCCACACACAAA R- TTGGTTGAAACTTGTGGATATAAA	62	35 cycles of 94 ⁰ C, 62 ⁰ C and 72 ⁰ C for 1 min each	Hawi et al (2003b)
DRD5.PCR1	F- CGTGTATGATCCCTGCAG R- GCTCATGAGAAGAATGGAGTG	60	None	Daly et al (1999)
978	F- CTTCTACATCCCCGTTGC R1- CCGGAGGGCCTTCG R2- CCGGAGGGCCTTCA	61	None	Sommer et al (1993)
1481	F- AGGAGGAGGGTCCTTTTCGATC R- ATGTATTTGCGTGTGTGTGCG	60	30 cycles of 94 ⁰ C, 58 ⁰ C and 72 ⁰ C for 1 min each	Hawi et al (2003b)
3' SNP	F- CGAACCTGAAAAGAAGAAATA R- ATGGAATAGCTGTGTTTCAGTA E- TTCCCCAACTCCTGGTCAGGA	57	None	---

Table III – Primer Sequences and Conditions used to Genotype SNAP-25 Variants

Variant	Primer Sequences	T _m (°C)	Variations to Standard Procedure	Reference
-2015	F- ATCAGCAGTTGAAAACTCAGAATACC R- GCATGTTGCTGAAATTTGTTTACTTAA E- A(6)GTGTCACACTTTGGAAAA	55	35 cycles of 94 ⁰ C, 55 ⁰ C and 72 ⁰ C for 1 min each	Mill et al (2004)
-900	F- CGGCCGTGTCCCAGC R- GCGATTTGTTTTCTCCGAAGAG E- ACCCCACGGAGGCACAGA	55	35 cycles of 94 ⁰ C, 55 ⁰ C and 72 ⁰ C for 1 min each	Mill et al (2004)
micro	F- Fam-TGGAGGGATGTGGTTTGG R- AAGTTGTACACTTCAAATATGTGG	59	35 cycles of 94 ⁰ C, 55 ⁰ C and 72 ⁰ C for 1 min each	Mill et al (2002)
80609	F- AGTGCGCCTGAATCCAAGG R- CCCAGAGGACGACAGATTTC E- A(14)TGA CTG CAGTAGGGA	55	35 cycles of 94 ⁰ C, 55 ⁰ C and 72 ⁰ C for 30 sec each	Mill et al (2004)
87610	F- TTCTCCTCCAAATGCTGTCTG R- CCACCGAGGAGAGAAAATG	60	35 cycles of 94 ⁰ C for 30 sec, 55 ⁰ C for 40 sec and 72 ⁰ C for 30 sec	Barr et al (2000)
87614	F- TTCTCCTCCAAATGCTGTCTG R- CCACCGAGGAGAGAAAATG	60	35 cycles of 94 ⁰ C for 30 sec, 55 ⁰ C for 40 sec and 72 ⁰ C for 30 sec	Barr et al (2000)
88268	F- AAATTAAAGCAGATCATCTGGAGTCTATAA R- AGACTTCTAAATCATAATCATCCTTTTTTAAA E- A(18)GATTGCTCTCTC	55	35 cycles of 94 ⁰ C, 55 ⁰ C and 72 ⁰ C for 1 min each	Mill et al (2004)

Table IV – Primer Sequences and Conditions used to Amplify DHPLC Amplicons

Amplicon	Primers	T _m (°C)
D5-1	F- CTCGAGGGTCCCTTGGCTGA R- CAGACACGGCCAGAGACACG	63
D5-2	F- CATCATCTGGACCCTGCT R- TTCAGGATGGAGGCAGTG	59
D5-3	F- GGTTACTGGCCCTTTGGA R- CTGGACCGGAATGAAGGA	57
D5-4	F- TCCTTCATTCCGGTCCAG R- CGCGTGTAGGTCACGATC	57
D5-5	F- CTTCTACATCCCCGTTGC R- CAGCAACACACGAAGACC	57
D5-6	F- ATGGGGGTCTTCGTGTGT R- TGGGCAAACACCTTCTGA	57
D5-7	F- CACTCAACCCCGTCATCT R- CTCCTCGTCGTTGTCCAC	57
D5-8	F- GGAGGTGGACAACGACGA R- GCACCATATCTGTTCTCTACTAGG	57
D5-A	F- CTCGAGGGTCCCTTGGCTGA R- CACGTCGGGCTCCCAAAAGT	63
D5-B	F- GTCATGGTCGGCCTGGCATG R- AGCTGGGCAAACACCTTCTGAA	63

Hot Star Taq Polymerase (Qiagen Ltd, Crawley, West Sussex, UK) and Triton and Gelatine Free MgCl₂ buffer was used to amplify these amplicons.

Table V – Extension Primer Sequences used to Genotype DRD5 Variants Undetected by the Mutation Analysis

SNP Name	Change in Pseudo Also	Expected Bases and Freq	Extension Primer
rs2227845	Yes	T (72%) G (28%)	E- CTGGACGCCCTGGGAGGAGGAC
rs2227846	Yes	C (88%) T (12%)	E-AGTCACAGTTCTCTGCATTACGTC
rs2227843	Yes	G (84%) A (16%)	E-TACGCCATCTCTTCCTCGCTCATCA
rs2227852	Yes	G (88%) A (12%)	E-ATCAGCTTCTACATCCCC
rs2227847	No	G (87%) A (13%)	E-TCATGATCGTGACCTACACGC
rs2227853	Yes	C (89%) T (11%)	E-GGTGCAGATGCGCAGGATTTCCTCC
rs2227842	Yes	C (81%) T (19%)	E-TGGAGAGGGCCGCAGAGCACG
rs2227850	Yes	C (83%) T (17%)	E-TCTCCTTCTTGATGGAA
rs2227844	Yes	G (89%) A (11%)	E-AAGACCCCCATGATCAC

All of the SNaPshot extension reactions were from DHPLC amplicon D5-B.
Pseudo = DRD5 pseudogenes



Appendix C

Analysis Software **(Details of input files and operating commands)**

File Transfer Program

File transfer programs (FTPs) are windows based applications for transferring files between your PC (the "Local System") and an FTP site (the "Remote System"). Using FTPs, you can connect to another system from your PC, browse files and folders on both systems, and transfer files between the systems. This process is necessary when using software packages from the HGMP website, including SPLINK, TRANSMIT, FASTLINK and ETDT. Input file must be transferred to the FTP program which must be connected to the HGMP website before analysis can be performed. After analysis output files must be transferred back to the local computer from the FTP. FTPs are available on the Internet to download for free.

SPLINK

This programme was used to format the data file correctly before it was used as an input file for the TRANSMIT software.

Input file

The input file is set up as follows in Excel and saved as a text (tab delimited) file. It is a standard linkage file.

Column 1: family id (same for all members of the family)

Column 2: individual id within the family (ie father =1, mother=2, etc)

Column 3: father's id (0=unknown, otherwise same as individual id, father=1)

Column 4: mother's id (0=unknown, otherwise same as individual id, mother=2)

Column 5: sex of the child (male=1, female=2)

Column 6: affection status of child (0=unaffected, 1=unknown, 2=affected)

Column 7: variant 1, allele 1 (alleles must have individual numbers, 0=no data)

Column 8: variant 1, allele 2

Subsequent columns: may contain additional variants if available

Example of input file with two variants:

16	1	0	0	1	0	0	0	2	2
16	2	0	0	2	0	2	2	2	2
16	3	1	2	1	2	2	2	2	2
17	1	0	0	1	0	1	2	1	1
17	2	0	0	2	0	1	2	2	2
17	3	1	2	2	2	1	2	1	2

The tabs must be replaced with spaces before the file is inputted into SPLINK.

Operating Commands

- `ped2spl <input.txt> output.spl n`

An 'n' preceded by a space is entered for each variant in the file (3 variants= n n n)

Useful Flags

- `sn` used in the place of `n` will result in that marker being excluded for the output file.

This software is available for free from the UK Human Genome Mapping Project Resource Centre at www.hgmp.mrc.ac.uk.

TRANSMIT (Chapter 2, Section 2.3.5)

Input File

The input file used for this software is the output file from SPLINK (see above).

Operating Commands

- transmit <input.spl> output.out

Useful Flags

- -m (only maternal transmission is analysed)
- -f (only paternal transmission is analysed)
- -agg# (aggregates alleles with frequency less than # %)

This software is available for free from the UK Human Genome Mapping Project Resource Centre at www.hgmp.mrc.ac.uk

FASTLINK

This programme was used to format the data file correctly before it was used as an input file for the ETD software.

Input File

The input file for this program is the same as for SPLINK except that the linkage file may only contain allele data for one variant.

Operating Commands

- makeped

At the prompts enter the name of input file (input.txt) and the name of the output file (output.ped).

This software is available for free from the UK Human Genome Mapping Project Resource Centre at www.hgmp.mrc.ac.uk

ETDT (Chapter 2, Section 2.3.3)

Input File

The input file used for this software is the output file from FASTLINK (see above).

Additional Necessary Files

To use the ETDT software a LOCUS file is needed. This file is structured as follows and saved as a text tab delimited file.

```
2 0 0 5
0 0.0 0.0 0
1 2

1 2
0.999 0.001
1
0.01000 0.50000 0.50000

3 12
0.01 0.03 0.08 0.13 0.43 0.07 0.04 0.04 0.05 0.08 0.02 0.02

0.0
0.0
1 0.05 0.4
```

Operating Commands

- ped2spl input.ped locus.txt output.cal
- calctdt output.cal output.lrt
- lrttdt output.lrt output.out

This software is available for free from the UK Human Genome Mapping Project

Resource Centre at www.hgmp.mrc.ac.uk

Additional General Useful Commands for HGMP Programs

- more filename

This command allows you to view the contents of any named file from inside the HGMP site without having to transfer it back to your local computer.

- rm filename

This command removes the named file from the directory. Each file name is unique, if you attempt to name a file the same as an existing one you will be prompted that a file with that name already exists. The software will not automatically overwrite the existing file with the new one. The old file must be removed before the new one can be created or the new file must be alternative named.

Additional information about all the HGMP programs can be found at www.hgmp.mrc.ac.uk/Registered/Help

GOLD (Chapter 2, Section 2.3.4)

This software is available for downloading at [www.well.ox.ac.uk /asthma/GOLD](http://www.well.ox.ac.uk/~asthma/GOLD).

To run the program it is necessary that all input file are located in the same file as the program itself.

Input File

The input file is set up as follows in Excel and saved as a text (tab delimited) file.

Only parental data is used in the analysis.

- Column 1: family id (same for all members of the family)
- Column 2: individual id within the family (ie father =1, mother=2)
- Column 3: row of zeros (indicating parental status)
- Column 4: affection status of parent (0=unaffected, 1=unknown, 2=affected)
- Column 5: sex of the child (male=1, female=2)
- Column 6: leave blank
- Column 7: variant 1 genotypes (alleles must have a space between them, 0 0 = no data)
- Column 8: leave blank
- Column 9: variant 2 genotypes (alleles must have a space between them, 0 0 = no data)
- Subsequent columns: may contain additional variants

Example of input file with three variants:

94	2	0	0	2	2 2	2 2	2 2
94	1	0	0	2	2 3	1 2	2 2
95	2	0	0	2	2 4	2 2	1 2
95	1	0	0	2	3 4	1 2	2 2
96	2	0	0	2	4 4	2 2	1 2
96	1	0	0	2	3 3	1 1	2 2

Additional Necessary Files

An additional file known as a map file is also necessary. This file is created as follows in note pad and saved as a text tab delimited file:

- Row 1 contains the headings of the rows (Markerid, Name, Position)
- Column 1: Markerid (defines the position of the variant in the pedigree file)
- Column 2: Name (provides the name of the variant)
- Column 3: Position (provides the physical position of the variant)

Example of input file with three variants:

Markerid	Name	Position
1	m1	1000
2	m2	2000
3	m3	3000

NB Variants must be ordered in both files according to their physical order on the chromosome.

Operating Command

- `ldmax -pinput.txt -mmap1.txt -t0.05`

Logistic Regression (Chapter 2, Section 2.3.6)

SPSS (version 11.0) was used to run the below data file

Input File

The input file is set up as follows in an SPSS data file.

Column 1: transmission data (0=not transmitted, 1=transmitted)

Column 2: binary code for variable 1 (eg 0=female, 1=male)

Column 3 +: binary code for variable 2 etc

Final Column: number of transmissions (or non transmissions) for variable (data used to weight analysis)

Example of input file with three variables:

1	1	0	0	30
0	1	0	0	24
1	0	1	0	43
0	0	1	0	32
1	0	0	1	34
0	0	0	1	28

Operating Commands

- Under *Data* select *Weight Cases* and weight by the last column.
- Under *Analyse* select *Binary Regression*
- Enter the first column as the *Dependent*
- Enter the variable(s) as the *covariates*
- Under *Options* select the *95% CI* if desired
- Press *OK* to run analysis