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An examination of Serotonergic candidate genes in Attention Deficit Hyperactivity Disorder

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for the degree of Doctor of Philosophy

Department of Psychiatry

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Trinity College

2008

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Declaration

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Summary

Attention Deficit Hyperactivity Disorder (ADHD) is a common, highly heritable childhood disorder. It is characterised by symptoms of inattention, hyperactivity, and impulsivity. Heritability of ADHD is estimated to be between 70-90%. The disorder is clinically heterogeneous and the aetiology remains largely unknown. It is widely believed that ADHD is a polygenic disorder with multiple genes of small/moderate effect.

The serotonergic neurotransmitter system has been implicated in the aetiology of ADHD through animal, pharmacological, and molecular studies. A number of serotonergic genes have been implicated as potential susceptibility loci for the disorder.

The relationship between ADHD and eight serotonergic candidate genes was examined in this investigation using a sample of 178 DSM-IV diagnosed ADHD probands and their parents. A highly significant association was observed between marker rs1843809 of the TPH2 gene and ADHD ($\chi^2 = 13.47$, $p = 0.0003$, OR = 2.45). A haplotype containing this marker and markers rs1386496 and rs1386494 was found to be more significantly associated with ADHD ($\chi^2 = 16.86$, $p = 0.0000$) further indicating the importance of this region in the predisposition to ADHD. Associations were also observed between ADHD and the MAO-A and 5-HT2A genes.

The IMAGE study has also reported an association between marker rs1843809 and ADHD but was with the opposite allele to that observed in this thesis. An evolutionary based haplotype analysis of the Irish ADHD and the IMAGE samples was performed in an attempt to explain the conflicting findings. This was accomplished using a relatively novel method of grouping haplotypes through the use of evolutionary relationships between haplotypes. The results pointed to a region of the gene that potentially harbours a functional variant that has not yet been identified, in both the Irish ADHD and the IMAGE Dublin samples. This finding suggests that this region of the TPH2 gene potentially represents a susceptibility region for ADHD in the Irish population. Evidence was also found to

suggest that variants of the TPH2 gene are correlated with quantitative impulsive scores that were measured in the entire sample.

Sub group analysis identified sub phenotypes that displayed stronger association with some variants than with the total sample. For example markers rs363220 of VMAT2 and the CA repeat of MAO-B showed no association with the combined subtype but showed a significant association with the inattentive subtype ($\chi^2 = 5.44$, $p = 0.0391$, OR = 8 and LRS = 13.3, df = 6, $p = 0.038$ respectively). In addition, analysis of ADHD probands with comorbid oppositional defiant disorder showed significant associations with markers rs1228814 and rs1157585 of the 5-HT1B gene (table 3.66: $\chi^2 = 4.88$, $p = 0.035$, OR = 1.65 and $\chi^2 = 4.56$, $p = 0.048$, OR = 1.6 respectively) though no association was observed with the total ADHD sample. The results of this thesis suggest that without subdivision or refinement of the broad ADHD DSM-IV phenotype it is possible that valuable information will be lost.

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Attribution of work

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

DNA extraction and quantification of the sample was performed by Dr Ziarah Hawi. The genotyping of the MAO-A gene and part of the MAO-B variants outlined in Domschke et al, 2005. This work was performed by Dr Katharina Domschke. The genotyping of the SERT promoter SNP 5-HTTLPR, VNTR, and 3'UTR SNP for a subset of the sample used in this thesis was performed in collaboration with Dana Kilroy. The genotyping of the VMAT2 gene, TPH1 gene and one of the TPH2 SNPs (rs4570625) was carried out commercially by K-Bioscience Genotyping Services. The genotyping of five TPH2 variants from the IMAGE study was carried out by the IMAGE consortium

Publications Relating to this Thesis

Sheehan K, Lowe N, Kirley A, Mullins C, Fitzgerald M, Gill M, Hawi Z (2005) Tryptophan hydroxylase 2 (TPH2) gene variants associated with ADHD. *Molecular Psychiatry* **10**:944-949.

Domschke K, Sheehan K, Lowe N, Kirley A, Mullins C, O'Sullivan R, Freitag C, Becker T, Conroy J, Fitzgerald M, Gill M, Hawi Z (2005) Association analysis of the monoamine oxidase A and B genes with attention deficit hyperactivity disorder (ADHD) in an Irish sample: preferential transmission of the MAO-A 941G allele to affected children. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics* **134**(1):110-114.

Sheehan K, Hawi Z, Gill M, Kent L (2007) No association between TPH2 gene polymorphisms and ADHD in a UK sample. *Neuroscience Letters* **412**(2): 105-107.

Abbreviations

3'UTR	3' Untranslated Region
[^{3}H]TBZOH	Dihydrotetrabenazine
5-HIAA	5-hydroxyindolacetic acid
5-HT	5-hydroxytryptamine, serotonin
5-HTP	5-hdroxytrytophan
5-HTT	Serotonin Transporter
5-HTTLPR	Serotonin Transporter promoter polymorphism
5-HT1B	Serotonin receptor 1B
5-HT2A	Serotonin receptor 2A
6-OHDA	6-hydroxydopamine-lesioned
aa	Amino acid
AAAD	Aromatic Amino Acid Decarboxylase
ABI	Applied Biosystem International
ADHD	Attention Deficit Hyperactivity Disorder
ADHD-CD	Attention Deficit Hyperactivity Disorder with comorbid Conduct Disorder
AEI	Allelic Expression Imbalance
ANOVA	Analysis of Variance
Arg	Arganine
ATD	Acute Tryptophan Depletion
APA	American Psychiatric Association
ADHD-ODD	Attention Deficit hyperactivity Disorder with comorbid Oppositional Defiant Disorder
BDNF	Brain Derived Neurotrophic Factor
BH_4	Tetrahydrobiopterin
bp	Base Pair
CAPA	Child and Adolescent Psychiatric Assessment
CD	Conduct Disorder
CNS	Central Nervous System
COMT	Catechol *O*-methyltransferase
CPT	Continuous Performance Test
CSF	Cerebrospinal Fluid
DAT1	Dopamine Transporter

DAT-KO	Dopamine Transporter Knock Out
DBH	Dopamine Beta Hydroxylase
DDC	Dopa Decarboxylase
df	Degrees of freedom
DMLO	Dim Light Onset Melatonin
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
ddNTP	Dideoxy nucleotide triphosphate
DRD1	Dopamine D1 receptor
DRD2	Dopamine D2 receptor
DRD3	Dopamine D3 receptor
DRD4	Dopamine D4 receptor
DRD5	Dopamine D5 receptor
DSM	Diagnostic and Statistical Manual
DTNBP1	Dysbindin
DZ	Dizygotic
eHap	Evolutionary-based Haplotype Analysis Package
ETDT	Extended Transmission Disequilibrium Test
EM	Expectation Algorithm
F	Forward Primer
FAM	5-carboxyfluorescin
FEN	Fenfluramine
FH+	Family History Positive
FH-	Family History Negative
H_20	Water
HEX	Hexachlorofluorescein
HHRR	Haplotype-based Haplotype Relative Risk
His	Histadine
ICD-10	International Statistical Classification of Diseases and Related Health problems, tenth revision
IMAGE	International Multicentre ADHD Gene project
IS	Impulsive Subjects
Kb	Kilobase
kDa	KiloDaltons
LD	Linkage Disequilibrium
LNAA	Large Neutral Amino Acid
LOD	Logarithm of Odds
LRS	Likelihood Ratio Score

M	Molar concentration
MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
Mb	Megabase
MD	Major Depression
mg	milligrams
ml	millilitres
MPH	Methylphenidate
MZ	Monozygotic
NED	A proprietary fluor from Perkin Elmer/Applied Biosystem International
ng	nanogram
NIMH	National Institute of Mental Health
NIS	Non-Impulsive Subjects
NT	Not transmitted
NSRI	Noradrenalin Serotonin Reuptake Inhibitor
OCD	Obsessive Compulsive Disorder
ODD	Oppositional Defiant Disorder
OR	Odds Ratio
PCR	Polymerase chain reaction
PDD	Pervasive Developmental Disorder
PET	Positron Emission Topography
PH	Phenylalanine Hydroxylase
Phe	Phenylalanine
PLP	Pyridoxal Phosphate
PRL	Prolactin
Pro	Proline
R	Reverse primer
RFLP	Restriction Fragment Length Polymorphism
ROX	6-carboxy-X-rhodamine
SAP	Shrimp Alkaline Phosphatase
s.d.	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SERT	Serotonin Transporter
SHR	Spontaneously Hypertensive Rat
SLC18A2	Solute Carrier Family 18 (Vesicular Monoamine Transporter), Member 2
SNP	Single nucleotide polymorphism
SSRI	Selective serotonin reuptake inhibitor
T	Transmitted

TDT	Transmission Disequilibrium Test
TE	Tris-EDTA
TH	Tyrosine Hydroxylase
TPH	Tryptophan Hydroxylase
TPH1	Tryptophan Hydroxylase 1
TPH2	Tryptophan Hydroxylase 2
Trp	Tryptophan
TS	Tourette's Syndrome
UTR	Untranslated region
V	Volts
Val	Valine
VMAT2	Vesicular Monoamine Transporter 2
VNTR	Variable Number Tandem Repeat
WKY	Wistar Kyoto
WURS	Wender Utah Rating Scale
µl	Microlitre

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Chapter 1

Introduction

1.1 General Overview of Attention Deficit Hyperactivity Disorder

1.1.1 Definition of Attention Deficit Hyperactivity Disorder

Attention Deficit Hyperactivity disorder (ADHD) is a childhood onset psychiatric disorder. It is characterised by symptoms of inattention, hyperactivity, and impulsivity that are developmentally inconsistent with the child's age. There are three subtypes of ADHD; predominantly hyperactive-impulsive, predominantly inattentive and combined type. Symptoms including distractibility, lack of concentration, excessive fidgeting, and difficulty staying seated should be manifest in all areas of life including school and home settings. These symptoms and their associated problems seriously disrupt the life of the affected child and their family.

ADHD is frequently carried into adolescence and adulthood despite being considered as a childhood disorder. The disorder may persist into adolescence in as high as 80% of cases and has been reported to continue in 30-40% of adults (Faraone, 2003; Mannuzza et al, 1998). While symptoms of hyperactivity and impulsivity appear to decrease with age, symptoms of inattention remain the same throughout the lifetime.

ADHD type symptoms were first documented by Hippocrates in 493 BP when he described patients with "quickened responses to sensory experiences, but also less tenaciousness because the soul moves on quickly to the next impression". He attributed this condition to an "overbalance of fire and water". In 1902 Dr George Still recorded cases of impulsiveness with "marked inability to concentrate and sustain attention" and deemed it to be a "morbid deficit of moral control" (Still, 1902). A flu pandemic in 1918 resulted in many survivors suffering from encephalitis. Some of these patients displayed behavioural problems similar to ADHD which led to suggestions that ADHD was due to injury to the brain. In 1960 Stella Chess described "Hyperactive Child Syndrome", the first introduction of the concept that hyperactivity was not being caused by brain damage.

The disorder was first entered in the Diagnostic and Statistical Manual of Mental Disorders, second edition (DSM-II, American Psychiatric Association, 1968) and was termed "hyperkinetic reaction of childhood". In 1980, the name Attention Deficit Disorder (ADD) +/- hyperactivity was first introduced in DSM-III (American Psychiatric Association, 1980) with ADD and ADHD being separate diagnoses. In the 1994 DSM-IV (American Psychiatric Association, 1994) the disorder was officially named as ADHD and was divided into the three subtypes of predominantly inattentive, predominantly hyperactive/impulsive, and combined type.

The criteria for a diagnosis of ADHD according to DSM-IV are shown in table 1.1. In order to be diagnosed as having one of the three subtypes, a minimum six out of nine symptoms of inattention and/or a minimum six out of nine symptoms of hyperactivity-impulsivity are required. The symptoms must be manifest in two or more settings such as school or home and there must be clear evidence of impairment of social, academic, or occupational functioning. As DSM-IV now requires the presence of symptoms in two or more settings, it minimizes the chance that a child whose behaviour is simply reactive to a given situation, parent, or teacher will be diagnosed with ADHD. Children with predominantly hyperactive-impulsive ADHD may exhibit aggressive behaviours while those with predominantly inattentive type may be more withdrawn. As such, children with inattentive ADHD often go unrecognized and/or unassisted as they tend to be less disruptive than the other subtypes of the disorder (U.S. Department of Education, 2003).

In the International Statistical Classification of Diseases and Related Health problems, tenth revision, (ICD-10, World Health Organisation, 1992), ADHD is categorised under Hyperkinetic disorders. This category is characterised by early onset; a combination of overactive, poorly modulated behaviour with marked inattention and lack of persistent task involvement; and pervasiveness over situations and persistence over time of these behavioural characteristics (WHO, 1993). The ICD-10 uses the same 18 core symptoms as the DSM-IV for diagnosing ADHD but the operational criteria are slightly different. An ICD-10 diagnosis calls for the presence of symptoms from all three domains of hyperactivity, inattention, and impulsivity while the DSM-IV does not. The ICD-10 has a special provision

for a combined conduct category when there is a co-morbid conduct disorder which is not present in the DSM-IV (Swanson et al, 1998).

Table 1.1 DSM-IV diagnostic criteria for ADHD (derived from the Diagnostic and Statistical Manual of Mental Disorders, Fourth edition)

DSM-IV Criteria for Attention Deficit/Hyperactivity Disorder

A. According to the DSM-IV, a person with Attention Deficit/Hyperactivity Disorder must have either (1) or (2):

(1) Six (or more) of the following symptoms of inattention have persisted for at least 6 months to a degree that is maladaptive and inconsistent with developmental level:

Inattention

(a) Often fails to give close attention to details or makes careless mistakes in school work, work, or other activities
(b) Often has difficulty sustaining attention in tasks or play activities
(c) Often does not seem to listen when spoken to directly
(d) Often does not follow through on instructions and fails to finish schoolwork, chores, or duties in the workplace (not due to oppositional behavior or failure to understand instructions)
(e) Often has difficulty organizing tasks and activities
(f) Often avoids, dislikes, or is reluctant to engage in tasks that require sustained mental effort (such as schoolwork or homework)
(g) Often loses things necessary for tasks or activities (e.g., toys, school assignments, pencils, books, or tools)
(h) Is often easily distracted by extraneous stimuli
(i) Is often forgetful in daily activities

(2) Six (or more) of the following symptoms of hyperactivity-impulsivity have persisted for at least 6 months to a degree that is maladaptive and inconsistent with developmental level:

Hyperactivity

(a) Often fidgets with hands or feet or squirms in seat
(b) Often leaves seat in classroom or in other situations in which remaining seated is expected
(c) Often runs about or climbs excessively in situations in which it is inappropriate (in adolescents or adults, may be limited to subjective feelings or restlessness)
(d) Often has difficulty playing or engaging in leisure activities quietly
(e) Is often "on the go" or often acts as if "driven by a motor"
(f) Often talks excessively
(g) Often blurts out answers before questions have been completed
(h) Often has difficulty awaiting turn
(i) Often interrupts or intrudes on others (e.g., butts into conversations or games)

B. Some hyperactive-impulsive or inattentive symptoms that caused impairment were present before age 7 years.
C. Some impairment from the symptoms is present in two or more settings (e.g., at school [or work] and at home).
D. There must be clear evidence of clinically significant impairment in social, academic, or occupational functioning.
E. The symptoms do not occur exclusively during the course of a Pervasive Developmental Disorder, Schizophrenia, or other Psychotic Disorder and are not better accounted for by another mental disorder (e.g., Mood Disorder, Anxiety Disorder, Disassociative Disorder, or a Personality Disorder).

Based on these criteria, three specific subtypes of ADHD are identified:

Attention Deficit/Hyperactivity Disorder, Combined Type: if both Criteria A1 and A2 are met for the past 6 months.

Attention Deficit/Hyperactivity Disorder, Predominantly Inattentive Type: if Criterion A1 is met but Criterion A2 is not met for the past 6 months.

Attention Deficit/Hyperactivity Disorder, Predominantly Hyperactive-Impulsive Type: if Criterion A2 is met but Criterion A1 is not met for the past 6 months.

1.1.2 ADHD: Categorical versus quantitative

The concept of ADHD as a discrete category is being re-evaluated and there is growing evidence that it may be an extreme of a set of behavioural traits quantitatively distributed in the population which, if true, will have implications for the classification of the disorder, identification of genes involved, diagnosis and the treatment of ADHD (Levy et al, 1997). ADHD is diagnosed categorically but measures of attention and activity are continuously distributed in the general population. 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).

1.1.3 Epidemiology

The prevalence of ADHD in the general population (diagnosed according to DSM-IV) has been estimated to range as high as 6-9% (Swanson et al, 1998; Faraone et al, 2003). Rates however vary across the world with North America having a higher prevalence rate compared to Europe. However, a report from the USA indicates that prevalence rates are as high in non-U.S. populations with the highest prevalence rates found when DSM-IV is utilized in diagnosis of the disorder (Faraone et al, 2003). Due to the numerous changes in the definition of ADHD over the past 20 years and the subsequent clinical difficulties in diagnosing the disorder, there have been challenges in estimating the exact prevalence of the disorder. This is compounded by the fact that the symptoms of ADHD are at the end of a quantitative scale of behavioural traits and characteristics and what is meant by 'clear evidence of impairment of social, academic, or occupational functioning' depends on factors other than the child and their symptoms alone.

Boys are 4-9 times more likely to be diagnosed with ADHD than girls. This sex difference is found in all cultures although prevalence rates may differ (NIMH, 1999). Girls were also found to be less at risk for co-morbid major depression, conduct disorder (CD) and oppositional defiant disorder (ODD) than boys with ADHD (Biederman et al, 2002). Levy et al (2005) assessed gender differences in

ADHD symptom co-morbidity with ODD, CD, separation anxiety disorder, generalized anxiety disorder, speech therapy, and remedial reading using a sample of twins and siblings from the Australian Twin ADHD Project. The group reported higher rates of ODD and CD in males while females had higher rates of separation anxiety disorder. Externalizing disorders would therefore appear to be more common in males with ADHD and internalizing disorders more common in female ADHD cases. This would mean that females are more likely to go undiagnosed as they are not disruptive and would therefore attract less attention to their behaviour. Biederman et al (2002) reported that girls are 2.2 times more likely to be diagnosed with the inattentive subtype of ADHD and are therefore less likely to have a learning disability and less likely to manifest problems in school or at home.

1.1.4. Associated Medical and Psychological Disorders

It is widely recognised that children and adults with ADHD also experience additional psychiatric difficulties. Some of these conditions have overlapping symptoms and can therefore create difficulties during the diagnostic process. A number of co-morbid disorders are associated with ADHD in 40-60% of all cases. Common associated conditions include oppositional defiant disorder, conduct disorder, learning disabilities, Tourette's syndrome and depression.

1.1.4.1. Oppositional Defiant Disorder

Children with Oppositional Defiant Disorder (ODD) display frequent and consistent hostile and uncooperative behaviours when compared with children of the same age and developmental level. Prevalence of ODD is estimated to be 10-14% of all school-age children (Cohen et al, 1993). Approximately 32-60% of children with ADHD have co-morbid ODD (August et al, 1996, Kadesjo et al, 2003). Symptoms of ODD include: excessive arguing with adults, frequent anger, and resentment, revenge seeking behaviour, active defiance, and refusal to comply with adult rules and requests. These behaviours are usually observed in multiple settings but are most often reported at home and school.

1.1.4.2. Conduct Disorder

Conduct disorder (CD) refers to a collection of emotional and behavioural issues displayed by some young people. Prevalence of CD is estimated to be 4-16% in children (Cohen et al, 1993). The disorder results in difficulty obeying rules and conforming to social convention. Children with CD often display symptoms of aggression towards people and animals, serious violations of rules, deceitfulness, stealing, and destruction of property. If the disorder is left untreated these children may be unable to adapt to the demands of adulthood thereby permanently affecting their family and social lives.

1.1.4.3. Depression

ADHD and depression co-occur beyond what would be expected by chance. Children comorbid for both disorders are more depressed than those with ADHD alone. These children suffer a significant impairment in academic and social functioning (Blackman et al, 2005). Mick et al, 2003 found evidence for a distinct familial subtype of ADHD and major depression (MD) which occurred more often in female members of families in the study.

1.1.4.4. Tourette's Syndrome

Tourette's syndrome (TS) is a neuropsychological disorder characterised by motor and vocal tics and is associated with behavioural abnormalities. Seventy-five percent of cases are male and the age of onset is usually between the ages of 2-14 years. A diagnosis of TS according to the American Psychiatric Association (APA) requires both vocal and motor tics over a period of more than one year, voluntary suppression of symptoms and onset between 2-15 years of age. Approximately 10% of cases of TS have a family history of TS.

ADHD occurs in approximately 49-83% of TS patients and up to 50% of children with ADHD have a family history of chronic tics (Knell and Comings, 1993). A study by Spencer et al (1998) reported that children with TS and ADHD had more additional co-morbid disorders and lower psychosocial function than children with ADHD alone. They concluded that TS and ADHD appears to be a more severe condition than ADHD. Affected families are at an increased risk of co-morbid ADHD and TS which may indicate an overlap in pathophysiology or neurobiology of the two disorders (Stewart et al, 2006).

1.1.5. Treatment of ADHD

There are two main methods utilised in the treatment of ADHD. The predominant choice is stimulant medication (e.g. Methylphenidate, more commonly known as Ritalin®). Stimulants were found to be effective in up to 70% of ADHD cases (Wilens and Biederman, 1992). Other types of medication including MAOIs, SSRIs, and NSRIs have also been prescribed. Non-pharmacological interventions include educational remediation and individual and family psychotherapies are also used in conjunction with stimulant medication

1.1.5.1. Pharmacotherapy

Stimulant medications such as methylphenidate and amphetamines remain the most common form of medication prescribed to ADHD patients due to the well documented efficacy of these drugs in controlling symptoms of the disorder. A review of Methylphenidate (MPH) trials reported an overall improvement of ADHD symptoms in 65-75% of subjects compared to an improvement in symptoms of 4-30% in subjects using a placebo (Pliszka, 2007). Stimulant medications are thought to act on the central nervous system (CNS) and in peripheral tissues by enhancing neurotransmission of dopamine and noradrenaline. These medications are required to be taken 2-3 times during the day as the effects peak approximately one hour following each dose and are ineffectual after approximately four hours (Swanson et al, 1998). This can lead to problematic side effects such as insomnia if administration is not timed correctly. New long-acting or slow release preparations that can last for 8-12 hours (such as Ritalin LA® and Concerta®) can help with issues ADHD children may experience with taking medication during the day. These medications may also help reduce any sleep difficulties caused by side effects of the drugs.

A meta-analysis by Faraone and Wilens (2003) confirmed that there is a protective effect against later substance use disorder conferred on ADHD cases that take stimulants during childhood. Stimulant treatment appears to decrease the risk by 50%, which brings the levels well within the normal population risk for substance use disorders.

1.1.5.2. Nonpharmacological intervention

Counselling and behaviour therapy can be beneficial if a parent is reluctant to place their child on medication or in the case of children who do not respond to any type of drug therapy. The aim of behaviour therapy is to change the affected child's home and school environment in order to bring about a change in their behaviour. Strategies involve introducing a more structured day and limiting distractions in the immediate environment of the child. Parents and teachers are both trained in techniques to improve children behaviour. These techniques include positive reinforcement, where the child is rewarded for behaving in the desired manner, and response cost, where the child is punished by withdrawal of a reward or privilege in response to problem behaviour (AAP, 2001). These methods have been reported to successfully change the behaviour of children with ADHD (Pelham et al, 1998). Parent and child education and counselling are used to help the affected child and their family manage the effects of ADHD. Educating children as to the nature of ADHD and how the disorder affects their lives at present may help them deal with the disorder in the future. Counselling methods are available and include teaching strategies for self-monitoring their attention, organizing school work, completing homework, resolving conflict and how to deal with parents and teachers. For parents, counselling may involve teaching them about ADHD and providing them with skills to help deal with the day-to-day management of their child with ADHD (Pierce and Reid, 2004).

1.2 Genetic Studies and Attention Deficit Hyperactivity Disorder

1.2.1 Family, Twin, and Adoption Studies

Although the exact aetiology of ADHD has not been well established, a strong genetic component has been implicated in the disorder. The heritability of ADHD (a statistical description of the fraction of variance in a trait due to genetic differences) has been estimated to be between 70-81% (Stevenson et al, 1992; Gjone et al, 1996; Martin et al, 2002).

Family studies have demonstrated a strong genetic component in the aetiology of ADHD. Concordance rates among siblings of ADHD probands has been estimated to be 32% and between 15-20% and 25-30% in their mothers and fathers respectively. Relatives of probands with attention deficit hyperactivity disorder were also found to have an increased risk for antisocial disorders, major depressive disorder, substance dependence, and anxiety disorders (Biederman et al, 1992). As might be expected with complex non Mendelian disorders, there is a decline in concordance rates in second degree relatives (Faraone et al, 1994).

The basic theory behind twin studies is that monozygotic (MZ) twins are genetically identical whereas dizygotic twins share approximately 50% of their genes. A disorder that has a genetic influence should therefore have a higher concordance rate in MZ twins than in dizygotic (DZ) twins, assuming that environmental influences are similar for both MZ and DZ twin pairs. In ADHD, twin studies have confirmed that MZ twins are significantly more concordant (50%-80%) than DZ twins (~30%) (Goodman and Stevenson, 1989; Gilger et al, 1992). The use of twin studies has also found that rater effects can bias concordance rate estimates depending on whether a diagnosis is made based on teacher or parent reports. Sherman et al (1997) compared ratings based on mother and teacher reports in a large, unselected twin sample. Concordance rates based on reports from mothers showed a high degree of within MZ pair similarity ($r = 0.67$) and complete discordance within DZ pairs ($r = 0.0$). In contrast to this, concordance rates based on teachers' reports found more modest rates for both MZ

(r = 0.71) and DZ twin pairs (r = 0.49). The difference in rates may be due to mothers underreporting ADHD or teachers over-reporting symptoms. Mothers also tend to exaggerate the contrast between their offspring, increasing discordance in DZ pairs. These findings indicate that the extent of genetic influence on ADHD varies according to whose report diagnoses are based upon.

To date, there have been relatively few adoption studies in relation to ADHD. An important issue with adoption studies is that adoptive parents may have to undergo psychiatric evaluation prior to adopting a child therefore potentially reducing the prevalence of mood and anxiety disorders found in this group in comparison to biological parents. Sprich et al. (2000) found that only 6% of adoptive parents of ADHD probands had ADHD compared to 18% of biological parents and 3% of parents of control children. The authors also reported high rates of mood and anxiety disorders among the biological parents compared to the adoptive parents indicating that these parental disorders are not a result of the stress of raising a child with ADHD.

Adoption studies may be important in elucidating any shared environmental component in the aetiology of ADHD. Reports have shown that adoptive relatives of ADHD probands who are exposed to the same environmental conditions are at a reduced risk of the disorder compared to biological relatives (Morrison and Stewart, 1973; Cantwell, 1975). Cadoret et al. (1991) reported that psychiatric problems and socioeconomic stress in adoptive families correlated significantly with various clinical outcomes of ADHD in a study involving 283 male ADHD adoptees. However, overall, environmental factors play a small part in the aetiology of ADHD.

1.2.2. Environmental Factors and ADHD

The observation that the concordance rate in MZ twins is not 100% indicates that environmental factors may contribute to the development of ADHD. However, the high MZ concordance rates reported for ADHD suggest that the role of the environment is limited. Environmental factors such as premature birth, complications during delivery, maternal alcohol consumption, tobacco and drug use and prenatal neurological damage have been linked to ADHD (NIMH, 1999). In support of this, behavioural characteristics similar to ADHD symptoms have been reported in animals exposed to caffeine, nicotine, and ethanol in utero. These stimulants can cross the placental barrier and reach the human foetal brain (Eriksson et al, 2000; Sobotka et al, 1989).

One of the first studies on the environmental effects on ADHD reported that mothers of methylphenidate (MPH) sensitive children smoked 2-3 times as many cigarettes during pregnancy than mothers of healthy children. Naye et al (1994) found that children whose mothers smoked during pregnancy had a higher incidence of hyperactivity, shorter attention spans, and lower scores on spelling and reading tests. Fergusson et al (1993) carried out a 15-year longitudinal study on 1,265 children in New Zealand. They reported a small but significant increase in childhood problem behaviours with children whose mothers smoked more than 20 cigarettes per day during pregnancy. Cornelius et al (2001) studied the effect of maternal smoking on 10 year old children's performances on measures of learning and memory and problem solving. They reported that prenatal exposure to nicotine was significantly associated with deficits in learning and memory and increased impulsivity.

A retrospective study by Mick et al (2002) reported that ADHD children were 2.1 times more likely to have been exposed to cigarettes and 2.5 times more likely to have had alcohol exposure in utero than controls though no association was found with drug use. Thapar et al (2003) also observed significant association with maternal smoking during pregnancy. As smoking itself is highly heritable, they suggested that the observed association may be due to a set of genes that influence both the putative risk factor and the outcome. A case–control study on children born between 1976 and 1982 in the U.S. state of Minnesota (n = 5,701) was carried

out in 2004 by St Suaver and colleagues. Of these children 305 were diagnosed with ADHD. The group examined birth certificates of all the children for risk factors for ADHD. They reported that pregnancy and labour characteristics, low birth weight, and the presence of a twin birth were not associated with ADHD. However, they did confirm that male sex was associated with an increased risk for ADHD and low maternal and paternal education levels increased this risk in males compared to females. High maternal and paternal education levels were found to be associated with a decreased risk for ADHD.

It is important in these types of studies to adjust the findings for potential confounding variables such as parental psychopathology, parental ADHD, birth weight, psychosocial adversity, alcohol and drug usage and parental IQ. Nevertheless, smoking and alcohol consumption during pregnancy appear to be the most influential environmental risk factors for children with ADHD.

1.2.2.1. Gene-environment interaction

Gene-environment interaction examines whether an individual's genetic architecture contributes to a reaction towards an environmental stimulant or if it makes an individual more likely to become exposed to certain environmental factors. While there is little evidence that a shared environment contributes towards a familial occurrence of ADHD, the environment may still have an influence over the outcome of an individual with ADHD.

A study regarding the effect of parental smoking in conjunction with a DAT1 VNTR showed children homozygous for the 480bp allele, whose mothers smoked while pregnant, showed an association with hyperactivity. Children either homozygous for the allele or exposed to smoking in utero displayed no association with the examined traits (Kahn et al, 2003).

Thapar et al (2005) examined a possible gene and environment interaction between ADHD with early onset antisocial behaviour and low birth weight using the valine/methionine variant in the catechol *O*-methyltransferase gene (COMT). There was a significant COMT x birth weight interaction ($p = 0.006$) with probands possessing the val/val genotype being more vulnerable to the effects of

low birth weight. Brookes et al (2006) reported a gene-environment interaction between the dopamine transporter (DAT1) and maternal use of alcohol during pregnancy. A haplotype containing the DAT1 10/6 repeats was found to mediate the effects of maternal alcohol consumption during pregnancy. To date there have been no reported findings on gene-environment interaction between genes in the serotonin pathway and ADHD.

1.3. Serotonin as a potential candidate in ADHD aetiology

Dysregulation of catecholamine neurotransmitter pathways (dopaminergic, serotonergic and noradrenergic) has been suggested as a cause of ADHD. Supportive evidence for this comes from the behaviour and biochemistry of animal models, pharmacology of stimulant medications used in the treatment of ADHD, neuroimaging studies, and molecular studies. This thesis focuses on the involvement of the serotonergic pathway in ADHD.

1.3.1. Serotonin

Serotonin (5-HT) is a neurotransmitter involved in a variety of functions including circadian rhythm entrainment, appetite, temperature regulation, attention, locomotion, aggression, pain sensitivity, sleep-wake cycle, stress, and sensorimotor reactivity. A conventional theory for the involvement of serotonin in behaviour proposes that an increase in serotonin function limits the behavioural reaction to an external arousing stimulus whereas a decrease in serotonin results in a lack of control over a response to diverse external stimuli (Lucki, 1998).

1.3.1.1 Serotonin biosynthesis

Serotonin is synthesized from the dietary amino acid tryptophan. The ingested tryptophan competes with other amino acids such as tyrosine, valine, and leucine for transportation across the blood-brain barrier via the large neutral amino acid (LNAA) transporter (Blows, 2000). Inside the neuron, tryptophan is converted to 5-Hydroxytryptophan by the enzyme Tryptophan Hydroxylase 2 (TPH2) in the presence of Fe^{2+}, O_2 and tetrahydrobiopterin (BH_4). Aromatic Amino Acid Decarboxylase (AAAD), also known as Dopa Decarboxylase (DDC), catalyses the production of 5-Hydroxytryptamine (serotonin) from 5-Hydroxytryptophan. This step requires pyridoxal phosphate (PLP) as a co-factor for AAAD. Vitamin B6 is crucial for the creation of PLP, therefore it is an important vitamin involved in

serotonin biosynthesis. The rate of serotonin production therefore depends on a number of factors including free plasma tryptophan (Trp) levels, the ratio of Trp to other large neutral amino acids in the plasma, the activity of LNAA and the gene expression of TPH2 (Nishizawa et al, 1997).

The levels of serotonin in the CSF may be a sign of spinal cord serotonin metabolism and can also be influenced by the transport of 5-HIAA in and out of the CSF and the rate of serotonin catabolism. Nishizawa et al. (1997) assessed serotonin synthesis levels in the brain using positron emission tomography and acute tryptophan depletion on eight male and seven female subjects. This method as described in Diksic et al (1990) uses α-[^{11}C] methyl-L-tryptophan as a tracer which is converted to α-[^{11}C] methylsertotonin and accumulates in serotonergic neurons. It is not a substrate for monoamine oxidase and does not cross the blood-brain barrier so all levels measured are a reflection of true serotonin metabolism levels in the brain. The mean rate of serotonin synthesis was found to be approximately 52% higher in normal males than in normal females (Nishizawa et al, 1997).

1.3.1.2 Tryptophan Depletion

Lowering levels of tryptophan in the brain correlates with increases in aggressive tendencies, impulsivity, and decreases in cooperativeness in human subjects. Serotonin synthesis in the brain relies on the availability of free tryptophan therefore enhancing or depleting levels of dietary tryptophan can affect serotonin levels. This process is carried out using the acute tryptophan depletion (ATD) method whereby subjects ingest a combination of amino acids devoid of tryptophan. Any subsequent behavioural changes could be seen as a direct result of lower serotonin function in the CNS. Studies have been carried out on nonclinical samples to assess the effect of tryptophan depletion on personality and behaviour. Details of studies examining ATD and impulsivity are described in Chapter 4 (section 4.2).

1.3.2. ADHD and Serotonin

1.3.2.1. Biochemical findings

Evidence for the serotonergic hypothesis for ADHD emerged when Coleman (1971) observed a decreased concentration of serotonin in the blood of children with ADHD. In addition, there have been reports that children with ADHD have lower 5-HIAA levels circulating in the CSF than controls. 5-HIAA is the metabolite of serotonin and is excreted in the urine. Krusei et al. (1990) assessed 5-HIAA CSF levels in relation to aggression, impulsivity, and social functioning in 29 children with disruptive behaviour disorders. They reported low levels of 5-HIAA in these patients when compared to age-, sex- and race-matched patients with Obsessive Compulsive Disorder (OCD).

Spivak et al. (1999) measured the levels of serotonin, norepinephrine, dopa and lipids and the degree of hyperactivity, impulsiveness, aggressiveness, and lack of concentration in boys with ADHD of low and high severity. They reported that the blood serotonin levels were lower in boys with ADHD of high severity compared to boys with low severity ADHD. The group hypothesised that children with more severe forms of ADHD may have different levels of serotonin turnover than those with a milder form. A reduction in CSF 5-HIAA levels may be indicative of decreased serotonin synthesis, decreased serotonin release or an increase in serotonin transport into serotonergic neurons. Cook et al. (1995) measured the whole blood serotonin levels in mothers and their children who have ADHD with or without co-morbid CD or ODD. No difference was found in serotonin levels amongst all groups (from different ethnic backgrounds) with ADHD, except those with co-morbid ODD or CD. It was suggested by the authors that ADHD children with co-morbid ODD or CD may therefore represent a more clinically homogenous subgroup.

Schulz et al. (2001) examined whether age or ADHD affects the association between serotonin and aggression in 46 males 7-11-years old. Central serotonin levels were measured by prolactin (PRL) response to fenfluramine (FEN, a drug that increases the levels of serotonin) which in turn induces prolactin secretion. The

group reported no association between central serotonin function and aggression in disruptive boys. The same group (Schulz et al, 2002) published a study examining whether seasonal variations in central serotonin function are the cause of discrepancies in studies of central serotonin and aggression in ADHD. However, they observed no differences in serotonin levels in annual, bi-annual, 3-monthly, 2-monthly, monthly and bi-monthly rhythms in the sample of 54 aggressive ADHD boys and 39 non-aggressive ADHD boys. Measures of aggression were based on parental responses to ratings of behaviour meaning results may be biased.

A reduction in serotonergic function has been linked to ADHD symptoms such as impulsivity (Halperin et al, 1997; Lucki, 1998). Relatively low platelet serotonin levels have also been reported in patients with ADHD (Rapport et al, 1974). Overall, these findings suggest that a dysfunction of the serotonergic system may be involved in the aetiology of ADHD.

1.4. The search for ADHD candidate genes

1.4.1. Linkage Studies

Linkage scans are used to identify regions of the genome possibly involved in an inherited disease condition or disorder. They are usually carried out using families with two or more affected individuals by testing approximately 400-500 markers spaced across the genome by 5-10 Mb. A logarithm of odds (LOD) score of greater than 3.3 is generally considered significant with odds of 1 in 1000 that the finding was by chance (Sklar, 2005). There have been several linkage scans for ADHD to date (Fisher et al, 2002; Bakker et al, 2003 and Ogdie et al, 2003, Arcos-Burgos et al, 2004 and Hebebrand et al, 2006).

Ogdie et al, (2003) reported linkage on the short arm of chromosome 16 (16p13), suggestive linkage on chromosome 17 (17p11) and weaker evidence of linkage on chromosomes 5 (5p13), 6 (6q14), 11 (11q25) and 20 (20q13). This study used an expansion of the original sample used by Fisher et al, 2002. Bakker et al. (2003) found suggestive linkage on chromosomes 15 (15q15.1) and 17 (17p13) and nominal linkage on chromosomes 3 (3q13.32), 4 (4p16.3), 5 (5p13.1), 6 (6q26), 9 (9q33.3), 10 and 13 (13q33.3). More recently, Arcos-Burgos et al (2004) examined linkage in extended multigenerational pedigrees and observed linkage on chromosomes 4 (4q13.2), 5 (5q33.3), 11 (11q22) and 17 (17p11) and suggestive linkage on chromosome 8 (8q11.23). In addition, Hebebrand et al (2006) identified nominal evidence for linkage to chromosomes 6q, 7p, 9q, 11q, 12q, and 17p. It has to be stressed that of all the above linkage scans studies the most significant findings were reported on chromosomes 5p13, 11q22-25, and 17p11. This overlap in linkage signals between studies supports the theory that these regions are potential susceptibility loci for ADHD. It should be noted that the studies were underpowered to identify genes that are responsible for less than approximately 15% of the variance of a complex disorder such as ADHD (Waldman and Gizer, 2006). This may explain the limited overlap between reported linkage regions in the linkage scan results published to date.

1.4.2. Animal models

Animal models can help with deciphering the molecular basis of a disorder by highlighting molecular pathways, potential mutagens, the roles of individual genes, or the regions of the brain involved in a disorder. These models are more often genetically homogenous and their environment can be controlled more easily than clinical cases (Sagvolden et al, 2005). Researchers also have greater control over animal models in regards to variables such as diet and learning history. Rodent models are more useful than primate models as they are genetically more homogenous, more is known about their neurobiology, and they are much less expensive to maintain. Though the nervous system of rodent models is simpler, they exhibit basic behavioural mechanisms similar to humans (Russell et al, 2005).

A number of animal models have been used to study the underlying mechanisms of ADHD including the coloboma mouse, the Naples hyperexcitable rat, the cerebellar stunted rat, and the acallosal mouse. The most frequently used model of ADHD however is the spontaneously hypertensive rat (SHR) which is selected from outbred Wistar Kyoto (WKY) rats (Okamoto and Aoki, 1963). These rodents display impaired sustained attention, motor impulsiveness and hyperactivity that is absent in novel, non-threatening situations but increases with time when reinforcers are infrequent, similar to that of ADHD cases (Russell et al, 2005). In addition the SHR model has comparable brain pathology to ADHD cases as SHR have a smaller brain volume than controls, in particular a smaller hippocampus, occipital cortex, and prefrontal cortex (Bendel and Eilam, 1992).

Serotonin function in SHR models has been examined in a number of studies. Kaehler et al. (1999) found that stressors invoked similar serotonin release in both SHR and WKY. Pollier et al. (2000) reported that acute administration of citalopram (a selective serotonin reuptake inhibitor) reduced the hyperactivity of SHR in an elevated plus-maze test. In addition, Stocker et al. (2003) suggested that brain serotonin function was altered in the SHR model when administration of fenfluramine (serotonin transporter inhibitor) induced less prolactin secretion in SHR compared to WKY.

Another potential rodent model of ADHD is the 6-hydroxydopamine-lesioned (6-OHDA) mice. These mice are generated through the disruption of the central dopaminergic pathways by lesioning with 6-hydroxydopamine (Avale et al, 2004). They display hyperactivity and impaired learning in spatial discrimination tasks. These behaviours improve post Methylphenidate (MPH) or d-amphetamine administration consistent with their clinical effects in ADHD (Luthman et al, 1989). The hyperactivity of this mouse model is accompanied by an increase in serotonin transporter binding in the striatum. In addition, inhibitors of serotonin transporter (5-HTT) significantly reduce the hyperactive state indicating that serotonin transporters are involved in the calming affect of psychostimulants in these mice (Davids et al, 2002).

Additional rodent models include the dopamine transporter knock out (DAT-KO) mice as described by Gainetdinov et al. (2000). This model displays symptoms of hyperactivity similar to that seen in ADHD. The hyperactivity induced by an increase in dopamine levels was found to be attenuated by increasing serotonin levels (described in detail later, section 1.4.4). Rodent models are a useful means for supplying information about the identification and disruption of novel candidate genes in ADHD. In particular, they point to the involvement of the serotonergic pathway in aspects of ADHD characteristics such as hyperactivity and impaired attention.

1.4.3. Dopaminergic System Genes and ADHD

Dopaminergic imbalance has been suggested to play a role in the predisposition to ADHD. Stimulant medications, such as methylphenidate (Ritalin ®), are the most commonly prescribed and effective treatments for ADHD. The principle site of action for methylphenidate was identified as the dopamine transporter (DAT1). In addition, animal models, neuroimaging studies in adult ADHD patients and molecular studies have also implicated dopamine dysregulation in ADHD aetiology. The dopaminergic system has a direct influence on motor control, endocrine function, reward, and control (Vallone et al, 2000). It influences these areas through DAT1, five G-protein coupled receptors (DRD1, DRD2, DRD3, DRD4, and DRD5) and several related enzymes involved in the biosynthesis and degradation of dopamine, such as tyrosine hydroxylase (TH), dopamine beta hydroxylase (DBH) and dopa decarboxylase (DDC). The observed association between methylphenidate and dopamine has led the majority of previous candidate gene studies to concentrate primarily on the role of dopamine system genes in the development of ADHD. It is generally accepted now that DAT1, DRD4, and DRD5 are susceptibility loci for ADHD, though biological proof is still required to confirm these findings.

1.4.4. Serotonergic Genes and ADHD

Several lines of evidence suggest the involvement of the serotonergic system in the aetiology of ADHD. A study by Gainetdinov et al (2000) observed that mice that have the dopamine transporter knocked out (DAT-KO mice) expressed remarkable hyperactivity possibly due to a 5-fold increase in extracellular dopamine levels in the striatum. However, DAT-KO mice only become more active when placed in a novel environment. In their familiar environment, these animals act like normal littermates. Crucially there is no corresponding increase in dopamine levels when these mice are exposed to a novel environment which suggests that there are other regulators of the hyperlocomotor effects seen in DAT-KO mice. Following administration of amphetamines (Adderall® and Dexedrine®), methylphenidate

(Ritalin®), and cocaine, the hyperactivity of knockout mice was found to be significantly reduced. However, the dopamine levels of the DAT-KO mice did not show any changes following methylphenidate administration. This would suggest that psychostimulants do not affect the dopamine system of these mice and therefore the calming effect must be due to their action on other neurotransmitters targeted by these drugs. To test this hypothesis nisoxetine, a selective inhibitor of the norepinephrine transporter, was given but this had no effect on hyperactivity.

Gainetdinov et al (2000) followed up on these findings by administering Fluoxetine (Prozac ®, a serotonin re-uptake inhibitor) to the mice which resulted in a remarkable reduction in hyperactivity. The authors also reported that other drugs that activate serotonin receptors, e.g. quipazine (a serotonin receptor agonist), and precursors of serotonin such as tryptophan and 5-Hydroxytryptophan that increase brain serotonin levels also produce a calming effect in hyperactive mice. The results of these studies indicate that hyperactivity induced by a hyperdopaminergic state can be reduced by increasing serotonergic tone enough so as to inhibit behaviour (Gainetdinov and Caron, 2000).

In recent years there has been an increase in studies have since been carried out on serotonergic candidate genes (Table 1.2) in relation to ADHD with most attention was focused on the serotonin transporter gene (5-HTT). Potential serotonergic candidate genes that may be involved in the aetiology of ADHD include; (1) precursor genes which affect the rate of serotonin biosynthesis; (2) metabolite genes which control serotonin degradation; (3) receptor genes that are involved in receiving serotonin signals and (4) transporter genes involved in the re-uptake of serotonin from the synapse back into the pre-synaptic neuron or into the post-synaptic neuron.

Table 1.2 Serotonergic candidate gene association studies in ADHD.

Gene	Function of Protein	Genetic Variant	Positive Associations (Specific variant)	No Association
5-HTT	Involved in the re-uptake of serotonin from the synaptic cleft	5-HTTLPR	Seeger *et al.* (2001) Manor *et al.* (2001) Kent *et al.* (2002) Retz *et al.* (2002) Zoroglu *et al.* (2002) Beitchman *et al.* (2003) Curran *et al.* (2005) Li *et al.* (2006) Li *et al.* (2007)	Cadoret *et al.* (2003) Langley *et al.* (2003) Johann *et al.* (2003) Beitchman *et al.* (2003) Zhao *et al.* (2005) Xu *et al.* (2005) Kim *et al.* (2005) Curran *et al.* (2005) Wigg *et al.* (2006) Heiser *et a.* (2006) Banerjee *et a.* (2006) Li *et al.* (2006) Guimaraes *et al.* (2006)
		VNTR Intron 2	Zoroglu *et al.* (2002) Banerjee *et al.* (2006)	
		3'UTR	Kent *et al.* (2002)	
5-HT1B	A serotonin receptor	G861C	Hawi *et al.* (2002) Quist *et al.* (2003) Li *et al.* (2005)	Mill *et al.* (2005) Li *et al.* (2005) Heiser *et al.* (2006) Smoller *et al.*(2006) Ickowicz *et al.* (2007)
		A-161T	-	
		T-102C	-	
5-HT2A	A serotonin receptor	His452TYR	Quist *et al.* (2000) Hawi *et al.* (2002) Guimaraes *et al.* (2007)	Quist *et al.* (2000) Zoroglu *et al.* (2003) Bobb *et al.* (2005) Heiser *et al.* (2006) Guimaraes *et al.* (2007)
		T102C	Levitan *et al.* (2002)	
		A-1438G	Li *et al.* (2006)	

MAO-A	Involved in serotonin degradation	$(CA)_n$ Intron 2	Jiang *et al.* (2001) Payton *et al.* (2001)	Domscke *et al* (2005) Lawson *et al.* (2003)
		G941T	Xu *et al.* (2007) Domschke et al(2005)	
		rs979606	-	
		30bp promoter VNTR	Manor *et al.* (2002)	
MAO-B	Involved in serotonin degradation	$(CA)_n$ Intron 2	-	Jiang *et al.* (2001) Domschke *et al.* (2005)
		rs1799836	-	
TPH1	Rate-limiting enzyme of serotonin biosynthesis in the periphery	A218C	-	Tang *et al.* (2001) Li *et al.* (2003) Li *et al.* (2006)
		G-6526C	-	
TPH2	Rate-limiting enzyme of serotonin biosynthesis in the brain	rs1843809	Sheehan *et al.* (2005) Brookes *et al.* (2006)	Sheehan *et al.* (2006)
		rs1386493	Sheehan *et al.* (2005) Brookes *et al.* (2006)	
		rs4570625	Walitza *et al.* (2005)	
		rs1007023	Brookes *et al.* (2006)	

1.5. Linkage Disequilibrium mapping, Association Testing and Haplotype Analysis

From the above discussion, it is clear that the serotonergic system plays a role in the development of ADHD. This could be due to a primary defect or variation in the serotonergic system itself or, alternatively, the effects could be downstream alterations due to other changes. Assuming the former, then the serotonergic system genes become important candidates for ADHD. Once potential candidate genes have been identified, there are a number of approaches available to examine if a gene is associated with a disorder. These include association testing, linkage disequilibrium mapping, haplotype testing, and mutation screening.

Linkage disequilibrium (LD) is the non-random association between alleles at adjacent loci and is a powerful alternative to linkage analysis for mapping and detecting genes of minor effect (Risch, 2000). LD occurs when a given allele at a locus occurs in a population with another allele at a different locus more frequently than would be expected by chance. It can occur when recombination (the formation of a new combination of genes on a chromosome as a result of crossing over) is reduced in the area or from a founder effect where sufficient time has not passed to reach equilibrium since one of the markers was introduced into a population. When two alleles are in complete LD, the allele found at one locus exactly predicts the allele found at the other locus. If one of these alleles predisposes to a disease then the second allele would also be found more frequently in cases than controls. In theory, testing a large number of markers around a candidate gene may lead to the identification of a polymorphism that is associated with the disease allele. This is the basis of association testing and LD mapping. However, this does not necessarily mean that there has to be a single disease-causing allele or haplotype.

It was thought that LD could extend up to 100 Kb either side of common SNPs but it has been shown that it can extend only a few Kb either side. Reich et al. (2001) proposed that long-range LD may be due to a population undergoing a bottleneck or an extreme founder effect whereby a population decreased to a great extent during a certain period so that only a few ancestral haplotypes gave rise to the haplotypes present today. Simulations in the Reich et al (2001) revealed the

presence of a severe bottleneck that occurred approximately 27,000-53,000 years ago that could have generated the LD patterns seen in the Utah sample used in the study. A follow-on study using a Swedish cohort found nearly identical LD patterns leading the authors to conclude that the similarity may be due to a similar historical event in European history before the separation of North American and European ancestors. The causal events of this long-range LD was postulated to have been either the Last Glacial Maximum (30,000-15,000 years ago) where Europe was greatly de-populated and recolonised by a small number of founders or due to a severe bottleneck during the founding of Europe. Using a sample from the Yoruban population it was discovered that the Nigerian and European populations show the same allelic combinations at short distances. D' (see below for calculation) values were found to have the same sign and a similar magnitude that indicates a common LD generating event at a time in human history.

Genetic association studies utilize LD in an attempt to locate functional variants in a gene that may contribute towards a disorder. The LD structure of the area surrounding a polymorphism associated with a disorder is examined. The two most common measures of LD are the absolute value of D' and r^2.

Lewontin (1964) developed the first commonly used measure of LD as D. It was defined it as the difference between observed and expected frequencies if two loci are segregating at random. D calculates the frequency of recombination between two loci and therefore gives an indication of the physical distance between them. Consider two tightly linked loci, each with two alleles (A and a at locus 1; B and b at locus 2). The frequency of a two-marker haplotype would be P_{AB}. If random assortment is assumed then $P_{AB} = (P_A \text{ X } P_B)$. D can therefore be calculated using the equation

$$D = (P_{AB} - (P_A \text{ x } P_B))$$

D' is calculated by dividing D by its maximum possible value, adjusting for the allele frequencies at the two loci. If D' = 1, and $r^2 = 1$, the loci are in complete LD, making one of the loci redundant for gene mapping. D' values vary from 0 to 1 which makes values approaching 1 or 0 easy to interpret as either complete LD or

complete linkage equilibrium. Intermediate values of D' are therefore interpreted with caution. D' values can fluctuate if a small number of samples are used in a study or if rare alleles are examined (Gabriel et al, 2002).

An alternative method for measuring LD is r^2. It is calculated by dividing D^2 by the sum of the haplotype frequencies. Therefore

$$r^2 = \frac{(P_{AB} - P_A P_B)^2}{P_A P_a P_B P_b}$$

When $r^2 = 1$, in perfect LD, the two loci have not been separated by recombination and have the same allele frequency. An advantage of the measure is that individual values of r^2 are more easily interpreted and the calculation is less inflated in small samples.

While it was once thought that LD was relatively consistent across the genome it has been shown that this is not the case. There are regions of high LD and low recombination interspersed with regions of low LD and high recombination known as recombination hotspots (Hey, 2004). This results in the formation of haplotype blocks, sites of closely located variants that are inherited together in blocks. Haplotype patterns across 51 autosomal regions spanning 13 Mb of the human genome were characterized as part of The SNP Consortium Allele Frequency Projects using samples from Africa, Europe, and Asia. The genome was found to be separated into haplotype blocks defined as sizeable regions within which there are only a few common haplotypes and which have little evidence of historical recombination. The boundaries of the blocks and the haplotypes within were found to be highly correlated across all populations studied (Gabriel et al, 2002).

The International HapMap project (http://www.hapmap.org/) was created to develop a haplotype map of the human genome and has generated genome-wide and densely spaced sequence variation data in several human populations from Europe, Africa, and Asia. It defines haplotype blocks as regions in the genome between 10 and 100kb that have between two to four haplotypes defining >90% of all chromosomes. Awareness of the underlying haplotype structure of the genome

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. As little as 6-8 randomly chosen common markers were found to be sufficient to identify common haplotypes in a block with little evidence of additional common haplotypes when using up to 17 markers (Gabriel et al, 2002). Therefore haplotypes can be tested using a small number of tagging SNPs and still capture most of the genetic variation across extended regions (Gabriel et al, 2002).

Testing the transmission of haplotypes to affected individuals may help to identify regions of genes that may contain functional polymorphisms. Regions between markers may contain the functional marker of interest therefore transmission of a two marker haplotype may be more significant than transmission of either marker alone.

1.5.1. Linkage studies versus association studies

It was recently realized in the middle of the last decade that the power of a linkage study in the case of complex polygenic disorders is relatively weak. This was partly attributed to the fact that each of the functional variants involved may only play a minor role in the development of the disease under investigation. Association studies however have a greater power to detect variants of small magnitude for a comparable sample size (Risch, 2000). The two forms of association testing are the case-control test and the family based association design. The case-control method examines the frequencies of high and low-risk alleles of a candidate gene for a disorder in affected groups (cases) and unaffected groups (controls). Cases and controls are matched on a number of background characteristics e.g. age, ethnicity or sex. The theory is that the cases will have higher frequencies of the high-risk allele for the disorder than the controls. A basic χ^2 test yielding a statistical difference between the two groups implies an association between the tested allele of the variant and the disorder. However, case-control tests can be biased by population stratification where different allele frequencies and differing rates of the disorder are found between groups which can lead to false positive results. Differences between ethnic groups are thought to be the most common source of population stratification though it can be caused by any form of population structure that varies between the cases and controls (Waldman and Gizer, 2006).

The Transmission Disequilibrium Test (TDT) (Spielman et al, 1993) (section 2.3.2) and the Haplotype-based Haplotype Relative Risk (HHRR) are family-based association tests designed to overcome the problem of population stratification. These tests are based on families where the sample consists of at least one affected child and one or both biological parents. Here the alleles transmitted from the parents to the affected child (cases) are contrasted against the non-transmitted alleles (controls) from the same parents which provide matched cases and controls thus avoiding bias. HHRR contrasts the transmitted and untransmitted alleles from both homozygous and heterozygous parents whereas TDT only contrasts transmissions from heterozygous parents. This difference gives TDT advantages over HHRR

1. The TDT is a test of association and linkage but HHRR is a test of association only.
2. The TDT is robust to the biasing effects of population stratification but HHRR is not completely so.
3. The TDT allows for the inclusion of multiple affected offspring from a family in a test of linkage, whereas HHRR cannot due to the violation of the assumption of observational independence in a test of association. (Waldman and Gizer, 2006)

In addition, the TDT does not require the collection of data on multiple family members or unaffected siblings. One main disadvantage of the TDT is that it can only detect linkage between a marker and the disease-locus if association due to LD is present (Spielman et al, 1993). An additional disadvantage to the TDT is a loss of power as the calculation is only performed in heterozygous parents. Therefore only a fraction of entire sample can be used. In addition for both the TDT and HHRR it is not always possible to collect parental samples in cases such as adult onset disorders like Huntington's and Alzheimer's disease.

1.6. Aims of the Current Investigation

It is clear that there is strong genetic component in the aetiology of ADHD. Like many complex diseases/disorders, the mode of inheritance is not known. However, it is widely believed that the disorder is polygenic with several genes (each of minor or moderate effect) contributing to the development of the disorder.

Several lines of evidence suggest that interaction with, and balance between dopaminergic and serotonergic neurotransmission is important in the development of ADHD. Until recently, the focus of the majority of molecular studies was concentrated on the dopaminergic pathway genes though a large body of evidence suggests the importance of serotonergic system genes in ADHD. The main aim of this thesis is to identify potential genetic risk factors for ADHD in the serotonergic pathway using a candidate gene approach in the following ways:

1. Perform linkage disequilibrium mapping on serotonergic candidate system genes in an Irish ADHD population along with haplotype analysis to narrow down the region of association. Candidate genes involved had either *a priori* evidence to suggest their possible involvement in ADHD or had been reported to be associated with ADHD in other samples.

2. Conduct association analyses between the resulting genotypic data of the candidate genes in relation to a number of subcategories including ADHD subtype (Inattentive, Hyperactive-Impulsive or Combined type ADHD), sex of transmitting parent (parent of origin effect), family history of ADHD and the presence of a co-morbid disorder such as ODD and CD.

3. Conduct an evolutionary-based association analysis using haplotype data from the Irish population and from the International Multicentre ADHD Genetics (IMAGE) study in order to explain why the risk allele from TPH2 marker rs1843809 was reversed in these samples.

4. Identify a role for the TPH1 gene in the aetiology of ADHD using reports of sleep disturbances in ADHD probands.

5. Test several candidate-gene markers for association with impulsive behaviours given that considerable research suggests that ADHD should be seen as the extreme end of a number of continuous behavioural dimensions.

CHAPTER TWO

Materials and Methods

2.1. Sample Description

2.1.1. The Irish ADHD Sample

The sample described herein was obtained with ethical approval from the South Western Area Health Board ethics committee. Families with clinically diagnosed ADHD children were recruited from child guidance clinics and ADHD support groups around Ireland (The Irish National Council of ADHD Support Groups) or through referrals by Professor Michael Fitzgerald, Consultant Child and Adolescent Psychiatrist. In all, 178 Irish ADHD nuclear families were ascertained and diagnosed according to DSM-IV criteria. DNA from both parents and the affected child (or children) was available for 170 trios; the remaining 8 families consisted of mother-child duos. The age range of the probands was between 5 and 14 years, with males accounting for 88% of cases.

2.1.1.1. Clinical Diagnosis

To establish DSM-IV diagnoses, one or both parents were interviewed using the Child and Adolescent Psychiatric Assessment (CAPA) (Angold et al, 1995). To fulfil DSM-IV ADHD criteria for symptom pervasiveness, information about ADHD symptoms at school was also obtained from teachers using a semi-structured teacher telephone interview (Holmes et al, 2004), which involved the teacher's opinions about DSM-IV symptoms of ADHD and impairment shown in a classroom setting. ADHD diagnostic subtypes were derived from DSM-IV diagnostic classification (Combined, Inattentive, and Hyperactive-impulsive types). Symptom dimensions as well as comorbid disorders such as Oppositional Defiant Disorder (ODD) and Conduct Disorder (CD) were obtained from the CAPA. A positive family history of ADHD was defined by at least one parent scoring 36 or greater on the 25 item subscale of the Wender Utah Rating Scale (WURS). This scale is 96% sensitive and specific for a retrospective diagnosis of childhood

ADHD in a parent (Ward et al, 1993). In all, 30% of the parents have a history of ADHD.

With regard to DSM-IV ADHD, 112 children (62.6%) met research criteria for ADHD combined type, 21 (14.6%) for ADHD inattentive type and 11 (6.1%) for ADHD hyperactive-impulsive type. From the total sample 87 children (48.6%) met the criteria for ODD and 30 children (16.8%) met criteria for CD.

2.1.2. STAR Sample

Families in the STAR sample were recruited as part of a naturalistic, prospective study of stimulant response in ADHD. Participating families were recruited between January 2003 and June 2006 from community-based child and adolescent mental health clinics in the Republic of Ireland (South Western Area Health Board, North Eastern Health Board, Lucena clinics, Midlands Health Board) and from two outpatient clinics at children's hospitals (Our Lady's Hospital for Sick Children, Crumlin and Temple Street Children's Hospital). Referrals received from child and adolescent psychiatrists underwent an initial telephone screening to determine eligibility and willingness to consider participation in the study. Of 132 families eligible to participate, 130 families provided consent, of which 108 families met inclusion criteria. Inclusion criteria were: age between 4 and 15 years, meeting DSM-IV criteria, stimulant medication naïve status, availability of one or both biological parents, and willingness to provide a blood or saliva sample for genetic analysis. Exclusion criteria were: IQ less than 70, epilepsy, Fragile X Syndrome, Foetal Alcohol Syndrome, significant drug abuse during pregnancy, primary diagnosis of a Pervasive Developmental Disorder (PDD) (children not meeting full criteria for PDD were included in the study sample), Tourette's Syndrome, current psychosis or bipolar disorder and current treatment with other non-stimulant psychotropic medications. The STAR sample was used to analyse TPH1 variants in relation to sleep disturbances in children with ADHD (Chapter 4, section 4.1).

2.1.3. The UK ADHD sample

A replication study was carried out on the TPH2 gene using an ADHD sample from the UK (ascertained by Dr Lindsey Kent at St Andrews university). The sample was recruited from several child psychiatry clinics in the UK and consists of 63 parent-proband trios, 44 mother-child duos, and one father-child duo. Probands were Caucasian, born in the UK and aged between 5-16 years (mean age = 11.1 years, s.d = 3.0). Cases were diagnosed using the Child and Adolescent Psychiatric Assessment (CAPA). All probands fulfilled DSM-IV diagnostic criteria for ADHD.

2.1.4 International Multicentre ADHD Genetics study (IMAGE) Sample

The IMAGE sample was collected as follows: European Caucasian subjects were recruited from 11 specialist centres in eight countries: Ireland, United Kingdom, Belgium, Germany, Holland, Israel, Spain, and Switzerland. Ethical approval for the study was obtained from the National Institute of Health registered ethical review boards for each centre. Detailed information sheets were provided and informed consent obtained from the majority of children and from all of their parents. All ADHD probands and their siblings were aged 5-17 years at the time of entry into the study and access was required to one or both biological parents for DNA collection. Entry criteria for probands were a clinical diagnosis of DSM-IV combined subtype ADHD and having one or more full siblings available for ascertainment of clinical information and DNA collection. Exclusion criteria applying to both probands and siblings include autism, epilepsy, IQ <70, brain disorders and any genetic or medical disorder associated with externalising behaviours that might mimic ADHD (Brookes et al, 2006).

The final data set comprises 674 DSM-IV combined type probands with 808 siblings. One hundred and two of the siblings also had combined type ADHD making a total data set of 776 affected individuals. DNA was available for both parents in 614 families (90%) and from one parent in 49 families (7%). Ninety four per cent of the ADHD cases were male subjects. The age range for both probands

and siblings varied from 5 to 17 years with the mean age of 11.2 (s.d. = 2.7) for probands and 11.2 (s.d. = 3.1) for siblings. Prior to entry into the study, all probands underwent clinical evaluations by a paediatrician or child psychiatrist and both existing and new patients were included in the study. Wherever possible families withdrew stimulant medication for one week prior to research assessments to allow for more accurate ascertainment of the current level of ADHD symptoms and behaviours. Probands were excluded from the study if the last medication free period was more than two years ago (Brookes et al, 2006).

2.2 Biological Techniques

All pertinent solutions and buffers used in the experimental procedures of this thesis are described in full in Appendix I.

2.2.1 DNA Extraction

2.2.1.1 DNA Extraction from Blood

Approximately 6ml of blood was collected from consenting affected individuals and their parents in 10ml EDTA tubes. The blood samples, and back up samples if collected, were stored at –70°C until extraction. Before extraction, samples were gradually thawed on ice for approximately three hours.

The blood was added to a 50ml falcon tube with 12.5ml of lysis buffer and 6.5ml of sterile water. The sample was placed on ice for 30 minutes, with occasional inversion, and then centrifuged for 15 minutes at 4000rpm at room temperature. The supernatant was poured off leaving ~ 2ml of supernatant and the pellet in the falcon tube. The samples were then re-suspended and lysis buffer was added to make the volume up to 25ml. The sample was re-incubated on ice for 10 minutes with occasional inversion before being spun again at 4000rpm for 15 minutes. The supernatant was decanted off and the pellet re-suspended in 2ml suspension buffer. The sample was transferred to a 15ml falcon tube containing 150µl 10% (w/v) sodium dodecyl sulphate (SDS) and 60µl proteinase K (10mg/ml) and incubated overnight at 37°C.

Following this, an equal volume of buffer saturated phenol was added to the samples, which were thoroughly mixed, and centrifuged at 4000 rpm for 10 minutes. The aqueous layer was removed and extracted twice more using 2ml of a 1:1 phenol chloroform solution and chloroform (24:1, chloroform : isoamyle alcohol) respectively. DNA precipitation was achieved by adding 50µl 3M Sodium Acetate (PH 5) and two volumes of 100% ethanol (stored at -20°C). The DNA was then removed, using sterile Pasteur pipettes, to labelled eppendorf tubes. The DNA

The DNA pellet was washed three times in 70% ethanol to remove any residual sodium acetate, air dried over night then re-suspended in 250µl TE Buffer before quantification.

2.2.1.2 DNA Extraction from Cheek Cells

Cheek swabs were collected from those unable or unwilling to give blood using sterile cytology brushes (CytoSoft, Medical Packaging Corporation) and the samples extracted within two weeks of collection. Samples were kept at 4°C prior to extraction.

To extract the DNA two cheek swab brush heads were placed in an eppendorf containing 500µl of 50mM NaOH and vortexed for 60 seconds. The tubes were then incubated at 95°C for 10 minutes followed by addition of 50µl of 1M Tris (pH 8). The samples were then vortexed for 30 seconds. 200µl of Instagene Matrix (Biorad Labs, 2000 Alfred Nobel Dr., CA, USA) was added and the samples were incubated for a further 30 minutes at 56°C. The samples were mixed by vortexing for 10 seconds and heated for 8 minutes at 100°C. The brush heads were removed and the tubes were centrifuged at 12000rpm for 3 minutes. The supernatant containing the DNA was transferred to a labelled tube and stored at -20°C when not in use.

2.2.2. DNA Quantification

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 (ng/µl) was calculated using the formula that an OD of 1 corresponds to 50µg/ml of DNA at 260nm. The ratio of OD_{260}:OD_{280} was used as a control for the presence of RNA.

2.2.3 Polymerase Chain Reaction (PCR)

Details of PCR amplification, primer sequences, and optimum annealing temperatures for all markers are provided in Appendix II. In general 50ng of DNA was added to a PCR master mix containing 1.5mM of $MgCl_2$, 200 μM of each dNTP, 20 p of forward and reverse primers and 0.5-1U Taq Polymerase in a final volume of 25μl. In the case of samples undergoing genotyping by the SNaPshot method (Applied Biosystems, Foster City, CA, USA), the same reaction was prepared but all volumes were adjusted to a final volume of 12.5μl. All PCR reactions were performed on a MJ Research DNA Engine Thermal Cycler and consisted of an initial denaturing step of 3.5 min at 95°C typically followed by 30 cycles of 30 sec at 94°C, 30 sec at the optimum annealing temperature and 30 sec at 72°C. This was followed in all cases by a final extension step of 5 min at 72°C.

2.2.4 Gel Electrophoresis and DNA Visualisation

Five microlitres of PCR amplified samples were mixed with 3μl loading dye prior to being loaded on a gel along with appropriate size standards. The samples were then electrophoresed on 2% (w/v) agarose gels containing 0.02mg/ml ethidium bromide. The gels were run in TAE buffer (X1) at 70V for approximately 45-60 minutes. The gels were then visualised on an ultra violet illuminator and photographed using a Polaroid camera.

2.2.5. Genotyping

2.2.5.1. Restriction Fragment Length Polymorphism (RFLP) Analysis

A number of markers (in the 5-HT1B and 5-HTT genes) were analysed using PCR and restricting fragment length polymorphism (RFLP) procedures. This technique involves amplification of the region surrounding the marker of interest. Digestion of the PCR product using the appropriate restriction enzyme and buffer (supplied by New England Biolabs, Beverly, MA, USA) permits determination of the allelic form of the marker. Details of the enzymes used and the digestion conditions (according to the recommendations by the manufacturer) can be found in Appendix II. The appropriate enzymes for each marker were either determined from the literature or obtained by electronically digesting the PCR product sequence using the web based restriction analysis program Restriction Mapper version 3 (www.restrictionmapper.org).

2.2.5.2. SNaPshot

The SNaPshot method of primer extension (Applied Biosystems International [ABI], Foster City, CA, USA) employs the principle of extending an unlabelled oligonucleotide primer by 1 base pair in the presence of fluorescently labelled ddNTPs. Each of the 4 ddNTPs is tagged by a different fluorescent dye (6-carboxy-X-rhodamine (ROX) = Red = dTTP; 5-carboxyfluorescin (FAM™) = blue = dGTP, hexachlorofluorescein (HEX) = green = dATP and NED™ = yellow (black) =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 first step of the SNaPshot primer extension reaction involves treatment of 5µl 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 excess dNTPs and primers respectively (1U SAP,

0.5µl SAP dilution buffer and 1U Exo I per 5µl PCR product). Conditions for this treatment step are 37°C for 30 min followed by 80°C for 15 min. Ten micro litter primer extension reaction is then set up consisting of 1.5µl treated PCR product, 0.4µl extension primer [5pmol], 1µl SNaPshot buffer (see Appendix I 1.3.2), 1.5µl SNaPshot Ready Reaction Mix (Applied Biosystems, Foster City, CA, USA) and 5.6µl H_2O. 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 0.5µl SAP dilution buffer per 5µl DNA) before being analysed on an ABI 3100 Genetic Analyzer using Genemapper (version 3.0) software package.

2.2.5.3. Genotyping with Applied Biosystem 3100 Genetic Analyzer (ABI 3100)

Genotyping of the majority of markers was performed using the SNaPshot technique on an Applied Biosystems 3100 Genetic Analyzer. This is a multi-colour fluorescence-based DNA analysis system using capillary electrophoresis. For each sample, 1µl of the pooled dilutions was mixed with 0.1µl of LIZ120 Size Standard (Applied Biosystems, Foster City, CA, USA, min band size = 15bp, max size = 120bp) and 9.4µl of Hi-Di® Formamide Solution (Applied Biosystems, Foster City, CA, USA). Genotyping was performed using the ABI 3100 data collection software and Genemapper v.2.7. At this point samples that had failed to amplify were identified and were re-amplified under optimized PCR conditions and genotyped again in the same manner. After two failed genotyping attempts, samples were excluded from analysis.

2.2.5.4 TaqMan Allelic Discrimination

Allelic discrimination using the ABI 7900 HT was performed for two markers in the 5-HTT gene. This technique is based on the design of two TaqMan probes (Applied Biosystems, Foster City, CA, USA), one specific for the wild-type allele, the other for the mutant allele. Both of these probes are fluorescently tagged (with FAM [Blue] and VIC [Green]). These probes are degraded during the amplification process by the 5' → 3' Exonuclease activity of the polymerase. During this degradation process the fluorescent group becomes separated from the quencher group, thus leading to increased fluorescence. The binding efficiency of the wild-type probe to the mutant allele and vice-versa is very low due to mismatch within the TaqMan probe and target sequence. Consequently mismatched binding is much reduced. The markers genotyped using this technique were included in the "assays-on-demand" product listing. In brief, 2.25µl of DNA (approximately 25ng of DNA) was added to 2.5µl of TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, USA) and 0.25µl of the specific probe. The samples were then run on the ABI 7900HT using the absolute quantification programme, which was modified to run for 10 mins at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 min at 60°C. Samples were analysed using the Sequence Detection System (SDS) allele discrimination programme (Applied Biosystems, Foster City, CA, USA).

2.2.6. DNA Sequencing

Sequence analysis of exons four and five of the TPH2 gene was conducted in 32 samples on both the forward and reverse strands. Samples were chosen based on their genotypes for marker rs1843809 of the TPH2 gene. Fifteen probands homozygous for the A allele, fifteen heterozygous for the A/C alleles and the two probands that were homozygous for the C allele were used for sequencing. Samples were sent for commercial sequencing to MWG biotech (MWG Biotech, Ebensburg, Germany) following a post PCR cleanup step.

2.2.6.1. Post PCR Cleanup

A post PCR clean up step used carried out in order to remove the original PCR primers and unlabelled dNTPs to ensure that they did not interfere with the subsequent sequencing reaction. The cleanup process was performed using the Nucleospin Extract II kit (Machery-Nagel, www.mn-net.com, Germany). The first step involves mixing 100µl of PCR product with 200µl of buffer NT. This mixture was loaded into a Nucleospin® Extract II column in a 2ml eppendorf and centrifuged for 1 minute at 11000 x g in order to bind the DNA to the column. The flow-through was discarded and the column was placed back in the eppendorf. Buffer NT3 (600µl) was added to the column and centrifuged again for 1 minute at 11000 x g to wash the silica membrane. The flow-through was discarded and the column returned to the eppendorf. The silica membrane was dried by centrifuging for 2 minutes at 11000 x g to remove any residual buffer NT3 (as residual ethanol in this buffer would inhibit subsequent reactions). The column was then placed in a clean eppendorf. Twenty microlitres of elution buffer NE was added and the column was left to incubate at room temperature for 1 minute to increase the yield of eluted DNA. The column was then centrifuged for 1 minute at 11000 x g. The eluted DNA was air-dried and was ready for sequencing.

2.3. Statistical Tests

2.3.1. Hardy Weinberg Equilibrium

Genotype data from parents with no history for ADHD was examined to ensure that they conformed to Hardy Weinberg (H-W) equilibrium. The Hardy Weinberg principle assumes that allele frequencies should conform to the following mathematical equation:

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

Where p^2 = P(AA), the probability of being an AA homozygote
2pq = P(AB), the probability of being an AB heterozygote
q^2 = P(BB), the probability of being a BB homozygote

H-W equilibrium can be tested for using the χ^2 statistic according to the equation:

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

Where O = the observed frequency of the allele
E = the expected frequency of the genotype

A significant χ^2 value is indicative of deviation from the Hardy Weinberg principle. This may result from either genotyping errors or from lack of conforming to the Hardy Weinberg assumptions, which are as follows: 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 test of association in the presence of linkage used in family based studies. The test examines a single allele of a marker to determine whether it is transmitted from heterozygous parents to affected offspring more often than would be expected by chance, i.e. on more than 50% of occasions. It is similar to a case-control χ^2 test in the sense that alleles not transmitted act as controls providing matched cases and controls. This method eliminates the problem of population stratification, which can lead to false positive or false negative results in case-control design studies.

The TDT χ^2 statistic is calculated using the McNemar equation of

$$\chi^2 = \frac{(a-b)^2}{(a+b)}$$

Where a = the number of high risk alleles transmitted from heterozygous parents to affected offspring.

b = the number of high-risk alleles not transmitted from heterozygous parents to affected offspring.

2.3.3. Odds Ratio (OR)

The OR is also a measure of the strength of association between a marker and a disorder. It measures the probability that disorder is present compared with the probability that it is absent. It is calculated according to the following formula

$$OR = \frac{a}{b}$$

a = Number of allele or haplotype transmissions by heterozygous parents to affected probands.

b = Number of allele or haplotype non-transmissions by heterozygous parents to affected probands.

2.3.4 Linkage Disequilibrium Measurement

Linkage disequilibrium between markers was calculated from the genotypic data of the parents only using the GOLD program (Graphical Overview Of Linkage Disequilibrium) and the Haploview program (http://www.broad.mit.edu/mpg/haploview/). GOLD is a software package that gives a graphical representation of LD in genetic data and is available online at www.well.ox.ac.uk/asthma/GOLD. The LD relations between markers are expressed as D' which ranges between 0 (no LD) and 1 (perfect LD). D' values (Chapter 1, section 1.5.) are generated with relevant χ^2 statistics and p value significance. The program runs in MS-DOS mode and utilizes an input file with population genotype data for a series of markers (both microsatellite and SNP data). Details on how to use this program, and the structure of the input files are available in Appendix III.

2.3.5. Haplotype Analysis

Haplotype analysis in this thesis was conducted using the program TRANSMIT available at the LITBIO site (www.litbio.org). The theory underlying the method is described in Clayton and Jones (1999). This program tests for association between genetic marker and disease by examining the transmission of markers from parents to affected offspring. The main features which differ from other similar programs are:

1. It can deal with transmission of multi-locus haplotypes even if phase is unknown.
2. Parental genotypes may be unknown.

The tests are based on a score vector which is averaged over all possible configurations of parental haplotypes and transmissions consistent with the observed data. Data from unaffected siblings, or siblings whose disease status is unknown, may be used to narrow down the range of possible parental genotypes which need to be considered. The program produces the following asymptotic chi-squared tests:

1. For each haplotype or allele, a test on 1-df for excess transmission of that haplotype.
2. A global test for association on H-1 df, where H is the number of haplotypes for which transmission data are available.

Haplotype analysis for MAO-A and MAO-B were conducted using the program FAMHAP (version 16). In this program, an expectation algorithm (EM) is implemented to estimate transmitted and nontransmitted haplotype frequencies. FAMHAP takes the smallest p value found among the combinations as a test statistic. The transmission/nontransmission status is then permuted to obtain the distribution of the test statistic. The empirical p value is the fraction of permutation replicates resulting in a test statistic greater than or equal to the test statistics of the real data. The program was run on genotypes from parent-child trios where genotype information for all four MAO-A SNPs was obtainable.

Details of the necessary input files and operational commands are available for both programs are described in Appendix III.

2.3.6 Kruskal-Wallis Testing

Kruskal-Wallis testing, measured by the H statistic, was performed to test if different alleles in a variant were associated with different impulsivity scores. It is an extension of a two-sample t-test, where more than two sample populations (in the case of SNP there will be three populations, 2 homozygous and 1 heterozygous) can be tested simultaneously, in which non-parametric methods must be used. It was necessary to use non-parametric method as the different allele groups had different variances and sample sizes, and therefore could not be tested using the normal one-way analysis of variance (ANOVA) test. A Kruskal-Wallis test, in comparison to multiple t-tests, reduces the probability of making a type I error, i.e. a false positive where one rejects the null hypothesis when the null hypothesis is true. In the test the null hypothesis is that $mean_{grp1} = mean_{grp2} = mean_{grp3}$. The alternative hypothesis is that either ($mean_{grp1} \neq (mean_{grp2} = mean_{grp3})$) or (($mean_{grp1}$

$= \text{mean}_{grp2}) \neq \text{mean}_{grp3})$ or $((\text{mean}_{grp1} = \text{mean}_{grp3}) \neq \text{mean}_{grp2})$ or $(\text{mean}_{grp1} \neq \text{mean}_{grp2} \neq \text{mean}_{grp3})$. Kruskal-Wallis testing was performed using SPSS (version 15).

2.3.7 Evolutionary based association analysis

The eHap software was used to analyse multilocus data as haplotypes and to determine whether there is any association between haplotypes and phenotypes. eHap version 3 is available for free at http://wpicr.wpic.pitt.edu/wpiccompgen/. Network version 4.201 (available for free at www.fluxus-engineering.com) was used to create a graphical output of haplotype trees from the data produced from eHap. The structure of the input files required for eHap and other necessary details of how to use this program are available in Appendix III of this thesis.

Chapter 3

Association studies of serotonergic candidate genes with ADHD

3.1. General Introduction

Fine mapping of a gene or region involves the selection, genotyping, and analysis of variants in the region immediately surrounding an associated variant. This may help to identify the boundaries of the association or indicate variants that are more strongly associated, as an aid to the identification of the functional variant or variants. Techniques include single variant testing; haplotype analysis and the establishment of the linkage disequilibrium (LD) relationships between variants in the region.

The candidate gene approach has been successful in ADHD with associations identified and replicated with variants at several genes. This chapter presents association analyses for several genes in the serotonergic pathway that have been studied in ADHD (5-HT1B and 5-HTT); genes that have been implicated in ADHD (TPH1, MAO-A, MAO-B, 5-HT2A and VMAT2) and one that was a novel candidate gene (TPH2).

3.1.1 The Serotonin System

The production of serotonin, also known as 5-hydroxytryptamine (5-HT) results from the hydroxylation of tryptophan by the enzyme tryptophan hydroxylase (TPH) (Figure 3.1). This step, which is the rate-limiting step in the synthesis of serotonin, leads to the formation of 5-hydroxytryptophan (5-HTP). The decarboxylation of this product results in the formation of serotonin. Serotonin can then be released from a synaptic vesicle across a nerve synapse, whereupon it stimulates a reaction in the post-synaptic nerve, before being degraded or taken back up into the presynaptic cell by the serotonin transporter (5-HTT). After re-uptake, serotonin can be recycled by storage in another synaptic vesicle or it can be broken down by the enzyme monoamine oxidase (MAO) leading to the formation of 5-hydroxyindoleacteic acid (5-HIAA), which is excreted by the kidneys. Evidence for the potential involvement of serotonin in the aetiology of ADHD is discussed in detail in chapter 1 (section 1.3.2).

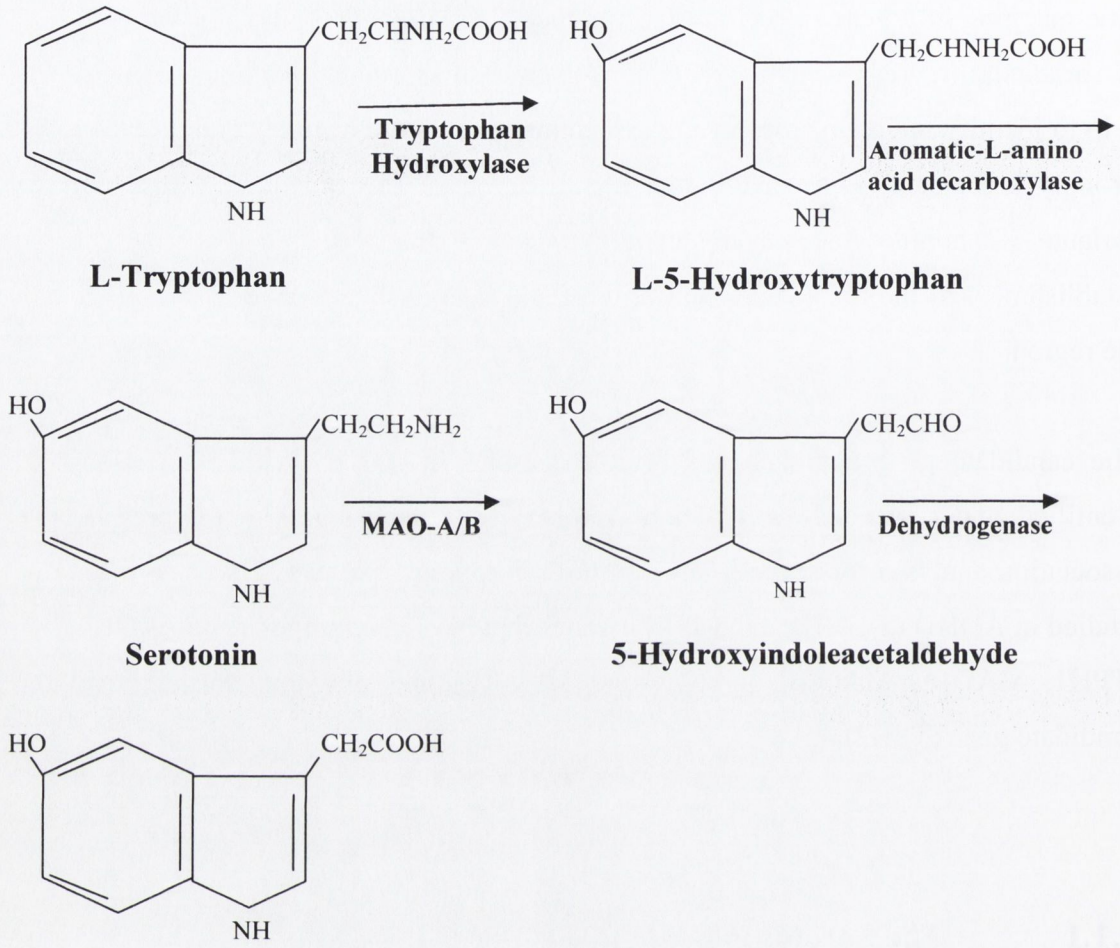


Figure 3.1 Serotonin metabolic pathway in the brain

3.2. Materials and Methods

3.2.1. Samples

Analysis of all genes was carried out on the Irish ADHD sample of 178 trios (Chapter 2, section 2.1.1.). DNA was obtained using blood samples and buccal swabs and extracted as described in section 2.2.1. Individual genotyping assays were carried out as described in section 2.2.5 using standard PCR conditions and reagents unless specified. A complete list of all primers and PCR conditions used in this thesis is provided in Appendix II. Attempted replication of the original TPH2 association reported in this study was then carried out on a UK sample (n =108, see chapter 2, section 2.1.3.).

3.2.2. Statistics

All markers were tested for Hardy-Weinberg equilibrium. LD was measured using the D' statistic in Haploview (section 2.3.4. for further details). Transmission of alleles was examined using the TDT (section 2.3.2.). Haplotypes analysis was conducted using TRANSMIT (chapter 2, section 2.3.5.).

MAO-A and MAO-B are x-linked genes therefore genotype information from fathers was used to assess the frequency of transmissions of alleles to female ADHD probands only. TDT was used for single marker analysis and extended TDT (ETDT) using TDTPHASE for multi-allelic markers for both genes. Linkage disequilibrium (LD) between the markers was assessed using the program GOLD. Only parents (in female probands) and mothers (in male probands) genotypes were used to assess LD. Hardy-Weinberg equilibrium was examined using the online site (http://kursus.kvl.dk/shares/vetgen/_Popgen/genetik/applets/ kitest.htm). Haplotype analysis was conducted using the program FAMHAP (version 16, Becker and Knapp, 2004) (section 2.3.5.).

It is important to emphasize that statistical analyses are not corrected for multiple testing for any of the examined genes.

3.3. Candidate genes

3.3.1. Tryptophan Hydroxylase

Tryptophan Hydroxylase (TPH) is the rate-limiting enzyme in serotonin biosynthesis. There are two forms of the enzyme, TPH1 and TPH2, resulting from the transcription of two separate genes. Both enzymes are members of the tetrahydropterin-dependent amino acid hydroxylase family along with phenylalanine hydroxylase and tyrosine hydroxylase (Fitzpatrick, 1999).

3.3.1.1. Tryptophan Hydroxylase 1

The gene for Tryptophan Hydroxylase 1 (TPH1) is located on chromosome 11 covering a region of 21kb and contains 10 exons. Transcription of the gene produces a protein of 444 amino acids (aa) of 51 kDa (www.genecards.org/cgi-bin/carddisp.pl?gene=TPH1). It is expressed in the pineal gland, gut, liver, spleen, lungs and thymus. The basic function of the enzyme is catalyzing the biopterin-dependent monooxygentaion of tryptophan to 5-hydroxytryptophan, which is then decarboxylated to form serotonin (5-hydroxytryptamine).

The two most common TPH1 variants analysed in association studies are A-218C (rs1800532) and A-6526G (rs4537731). Both are located in the promoter region of the gene but their function has not yet been identified There have been several association studies between ADHD and TPH1 (Chapter 1, Table 1.2). As TPH1 is not highly expressed in the brain, the *a priori* evidence for its involvement with neuropsychiatric disorders is weak.

However, TPH1 is highly expressed in the pineal gland where serotonin is involved in the synthesis of melatonin. Melatonin is a key factor in the sleep-wake cycle with high levels found at night and low levels during the day. Sleep problems have been reported in medication naïve children with ADHD. The prevalence rate of this problem is debated but in the Irish ADHD sample approximately 26% of children

have reported sleeping problems including early morning waking and initial or middle insomnia. Analysis of this gene in relation to sleep disorders in ADHD is further discussed in chapter 4 (chapter 4, section 4.1.).

3.3.1.2. Tryptophan Hydroxylase 2

The gene for Tryptophan Hydroxylase 2 (TPH2) is located on chromosome 12q21.1. It is a large gene of 93,595 Kb in length and consists of 11 exons. Transcription of the gene produces a protein of 490 aa with a molecular weight of 56kDa (www.genecards.org/cgi-bin/carddisp.pl?gene=TPH2). This protein shows a high resemblance (71%) to that produced by the TPH1 gene.

The presence of a second form of TPH was only relatively recently identified by Walther and Bader (2003). Gene targeting was used to functionally ablate the Tph gene in mice. The resulting animals (tph-/-) were found to be deficient in serotonin in the periphery and in the pineal gland but had close to normal levels of serotonin in the brain. These mice also displayed no significant behavioural differences in the elevated plus maze and hole board tests that are indicative of serotonin-related behaviour. Subsequent analysis revealed the presence of a second, neuronal, form of TPH now known as TPH2. The group found 150 times more Tph2 mRNA in the brain compared to Tph1. A consequent study by Zill et al (2004) on post-mortem specimens confirmed that TPH2 mRNA was exclusively present in the human brain but was not found in any of the peripheral tissues examined (i.e. heart, lung, kidney, duodenum, liver, and adrenal gland).

A study involving a mouse strain with a C1473G transversion in Tph2, leading to a Pro447 to Arg447 substitution, found that mice homozygous for the 1473G allele had a 50% reduction in serotonin biosynthesis in the frontal cortex and a 70% reduction in the striatum (Zhang et al, 2004). Kulikov et al (2005) additionally studied the C1473G polymorphism in 10 inbred mouse strains and observed that Tph2 activity was higher in the midbrain of mice homozygous for 1473C than those homozygous for the G allele. The homozygous animals for the G allele also exhibited an increased number of attacks toward other male mice. In humans a functional mutation in exon 11 of TPH2 which replaces the highly conserved Arg441 with His441, resulting from a G to A transition at position 1463, was found

to result in an 80% reduction in serotonin production when TPH2 is expressed in human PC12 cells (cells that synthesise and store serotonin and dopamine). In a case control study of unipolar depression Zhang et al (2005) observed this mutation in only nine of the 87 cases and three of the 287 controls. However, replication studies have failed to find this mutation in a combined total of 388 patients with bipolar disorder, 1743 patients with unipolar depression or in the 1080 controls from several population samples (Zhou et al, 2005; van den Bogaert et al, 2005; Glatt et al, 2005). Scheuch et al (2007) have recently reported that a polymorphism in the putative promoter region of the gene results in a 22% decrease in TPH2 transcriptional activity in primary serotonergic neurons.

Prior to the present investigation, no association studies between TPH2 and ADHD had been reported. However, two subsequent studies have been published. In the first Walitza et al (2005) observed preferential transmission of markers rs4570625 ($p = 0.049$) and rs11178997 ($p = 0.034$) and a haplotype consisting of these two markers ($p = 0.064$) to ADHD probands. In the second study, Brookes et al (2006) found significant association between TPH2 variants rs1843809 and rs1386493 and ADHD in the International Multicentre ADHD Gene project (IMAGE) sample.

3.3.2. Serotonin Transporter

The serotonin transporter gene (5-HTT) is mapped to chromosome 17 at position 17q11.2. It contains 14 exons and is 37.8Kb in length. The gene produces a protein of 630 aa of 70.3kDa (www.genecards.org/cgi-bin/carddisp.pl?gene=5-HTT). This protein is the target of psychomotor stimulants such as cocaine or amphetamines. 5-HTT controls the levels of serotonin in the synaptic cleft through rapid reuptake of 5-HT into the nerve terminal.

The serotonin transporter gene has been the most extensively studied serotonergic gene in ADHD to date (Chapter 1, Table 1.2). There are three variants common to most studies. They include:

1. A 44bp insertion / deletion in the promoter region. Heils et al (1995) reported that the basal activity of the long allele (insertion/insertion, LL) is three fold higher than that of the short allele (deletion /deletion, SS). The long allele therefore removes serotonin more efficiently from the synaptic cleft compared to the short. This made the serotonin transporter a good candidate gene for involvement in the hyposerotonergic hypothesis of ADHD. In keeping with this theory, it would be expected that the L allele should be the associated allele in ADHD. Interestingly, several studies have reported an over-representation of the L allele or the L/L genotype in children with ADHD (Seeger et al , 2001; Manor et al, 2001; Kent et al, 2002 [pooled analysis]; Zoroglu et al, 2002; Retz et al, 2002; Curran et al, 2005; Li et al, 2006). Beitchman et al (2003) reported that aggressive children with ADHD (n = 29) were more likely to have one or two copies of the L allele compared to aggressive non-ADHD children (n = 9) (89.7% vs. 55.6%, p = 0.041). In contrast to these findings, Li et al (2007) have reported over-transmission of the S allele to ADHD probands ($\chi^2 = 5.75$, p = 0.016). It needs to be pointed out that eleven other studies failed to find an association between the 5-HTTLPR and ADHD (Chapter 1, Table 1.2).

2. A variable number tandem repeat (VNTR) element of 17 bp in intron 2 (Ogilvie et al, 1996) containing nine (250 bp), 10 (267 bp) or 12 (301 bp) repeat alleles. It is not certain how the VNTR influences serotonin function, although the 12 repeat has been reported to drive higher expression of the transporter in embryonic mouse rostral hindbrain (Mackenzie and Quinn, 1999). To date two studies have reported significant association between the 12 repeat and ADHD (Zoroglu et al, 2002; Banerjee et al, 2006). Eight studies did not confirm this association (Table 1.2).

3. A G to T transversion in a putative polyadenylation site in the 3' untranslated region (3' UTR) of the gene. The function of this variant is not yet known. In a pooled analysis, Kent et al (2002) reported preferential transmission of the T allele to ADHD probands ($\chi^2 = 4.06$, $p = 0.04$). Four other investigations did not confirm this association (Chapter 1, Table 1.2).

3.3.3. Vesicular Monoamine Transporter 2

The gene for vesicular monoamine transporter 2 (VMAT2) is located on chromosome 10 at position 10q26.11. VMAT2 is involved in the ATP-dependent transport of serotonin by drawing serotonin into synaptic vesicles prior to secretion from the neuron via exocytosis (www.genecards.org/cgi-bin/carddisp.pl?gene=VMAT2).

Hypoactivity of serotonin transmission in ADHD children could be associated with decreased expression of VMAT2. The binding capacity of [^{3}H]TBZOH (dihydrotetrabenazine, a VMAT2 inhibitor) was found to be 17% lower in ADHD children compared to controls even though the affinity of [^{3}H]TBZOH for VMAT2 binding sites was found to be the same in both groups (Toren et al, 2005). However, an association study of 51 ADHD candidate genes by Brookes et al (2006) did not report any association between VMAT2 and ADHD in the IMAGE phase I study.

3.3.4. Serotonin receptor 1B

The serotonin receptor 1B (5-HT1B) is located on chromosome 6 at position 6q13. The intronless gene produces a protein of 390 amino acids of 43.6kDa. It is one a number of receptors for serotonin (www.genecards.org/cgi-bin/carddisp.pl?gene =5-HT1B).

5-HT_{1B} knockout mice have been found to exhibit increased impulsivity (Brunner and Hen, 1997). In addition, the 5-HT1B receptor agonist RU2496 had no effect on these knockout mice while it led to increased anxiety and locomotion in normal mice, indicating that this effect is mediated by 5-HT_{1B} receptors (Saudou et al, 1994). Hawi et al (2002) reported association, in a multi-centre study, between the G allele of G861C and ADHD children ($\chi^2 = 6.4$, $p = 0.014$). There was also a significant transmission of the G allele from fathers to affected probands ($\chi^2 = 6.04$, $p = 0.018$). These findings were partly supported by Quist et al (2003) who observed a trend towards association of the G allele with ADHD ($\chi^2 = 2.91$, $p = 0.09$). In addition, Quist et al also observed a similar bias towards transmission of the risk allele from fathers ($\chi^2 = 4.80$, $p = 0.03$). Li et al (2005) reported a marginal association of the G allele with ADHD ($\chi^2 = 3.76$, $p = 0.052$). In addition they also observed a trend towards association with a haplotype composed of the G allele of G861C and the A allele of A-161T ($\chi^2 = 2.95$, $p = 0.087$). Smoller et al (2006) reported no overall association between 21 SNPs in and around 5-HT1B and ADHD. However, they observed association with a haplotype block encompassing the gene with the inattentive subtype of ADHD (global $p < .01$). Six other studies reported no association between certain 5-HT1B variants and ADHD (Table 1.2) although not all variants were common to each study.

3.3.5. Serotonin receptor 2A

The gene for the serotonin receptor 2A (5-HT2A) is physically mapped to chromosome 13 at position 13q14-q21. The gene produces a multi-pass membrane protein of 471 aa of 52.6 kDa. It is one of many receptors for serotonin (www.genecards.org/cgi-bin/carddisp.pl?gene=5-HT2A). 5-HT2A is thought to be paternally imprinted in some individuals (polymorphic imprinting). Kato et al (1996) observed that the 5-HT2A gene is paternally imprinted in human fibroblasts therefore transcription is from the maternal allele only. A study by Bunzel et al (1998) reported that 4 out of 18 individuals had monoallelic expression of the 5-HT2A gene in a series of 41 tissue samples from the human adult brain (taken from patients undergoing surgery for treatment-refractory-epilepsy). However, the remaining 14 patients had biallelic expression which suggested that imprinting of the 5-HT2A gene is polymorphic, at least in the human population. Fukuda et al (2006) also reported monoallelic and unequal allelic expression of the 5HT2A gene in human brain and peripheral lymphocytes. It is therefore plausible that the gene may be imprinted in some ADHD probands although no studies have examined this to date.

Several studies have reported association between the His452Tyr variant of 5-HT2A and ADHD children (Quist et al, 2000; Hawi et al, 2002; Guimaraes et al, 2007). In addition there have been reported associations between the T102C SNP (Levitan et al, 2002) and ADHD and the A-1438G SNP (Li et al, 2006) and functional remission of ADHD. These SNPs are in complete LD with each other. Four other studies did not find association between 5-HT2A and ADHD (Table 1.2).

3.3.6. Monoamine Oxidase A

The Monoamine oxidase A gene (MAO-A) gene is located on chromosome X at position Xp11.3. The gene produces a protein of 527 aa and 59.7kDa. MAO-A catalyzes the oxidative deamination of biogenic and xenobiotic. MAO-A preferentially oxidizes biogenic amines such as serotonin, epinephrine, and norepinephrine (www.genecards.org/cgi-bin/carddisp.pl?gene=MAOA).

MAO-A inhibitors such as tranylcypromine have been shown to be efficacious in the treatment of ADHD (Zametkin et al, 1985). In addition, Brunner et al (1993) described a rare loss-of-function point mutation in the MAO-A gene that resulted in a highly impulsive and aggressive behavioural phenotype. MAO-A knockout mice have also been observed to exhibit significantly increased aggressive behaviour accompanied by elevated levels of serotonin, noradrenalin, and dopamine (Cases et al, 1995). Children with ADHD have been found to have significantly lower levels of platelet MAO-A activity associated with increased impulsivity and inattention (Shekim et al, 1986).

Several studies have reported association between MAO-A variants and ADHD (Chapter 1, Table 1.2).

3.3.7. Monoamine Oxidase B

The Monoamine Oxidase B (MAO-B) gene maps to chromosome Xp11.23, in close proximity to the MAO-A gene. Like MAO-A, MAO-B catalyzes the oxidative deamination of neurotransmitters. Both MAO-A and MAO-B genes exhibit an identical exon-intron structure as well as high sequence similarity (~70%) and are functionally related to each other. Pharmacological studies have shown preliminary evidence that MAO-B inhibitors such as selegiline or deprenyl have a beneficial effect in the treatment of ADHD (Feigin et al, 1996; Akhondzadeh et al, 2003). Administration of deprenyl has also been shown to significantly reduce impulsiveness in spontaneously hypertensive rats (SHR) (Boix et al, 2003).

No association between MAO-B variants and ADHD has been reported (Table 1.2).

3.4. Phenotype analysis

The use of the DSM-IV as operational diagnostic criteria for ADHD has lead to an improved reliability in the diagnosis of ADHD. This should result in more homogenous clinical samples which would be advantageous for the development of genetic studies of ADHD. However, there remain considerable inconsistencies in reported findings between groups. This may be attributed to small sample sizes resulting in lack of power to detect minor effects and to the presence of false positive findings. However, it is also possible that the DSM-IV diagnosis may be too broad as a phenotype or may represent a clinically heterogeneous group of conditions. Examining samples according to subgroups, for example ADHD subtype or perhaps taking a quantitative approach to a specific set of ADHD symptoms may result in more replicable association findings.

3.4.1. Parent of Origin effect

Evidence of increased paternal transmission of a risk allele to ADHD children has been reported in relation to several genes of the dopaminergic and serotonergic pathways (Hawi et al, 2005). Two additional studies have reported preferential paternal transmission of risk alleles from the 5-HT1B gene to ADHD probands (Quist et al, 2003; Smoller et al, 2006). Kent et al (2005), examining the brain-derived neurotrophic factor (BDNF) gene and ADHD, reported evidence for the preferential paternal transmission of the G allele of marker rs6265 to ADHD probands (paternal transmissions: OR = 3.2, p = 0.02; maternal transmissions: OR = 1, p = 1). Increased paternal relative to maternal transmissions can be indicative of genomic imprinting, a situation in which genes from the mother and father have differential patterns of expression in their offspring. There is evidence that the 5-HT2A is paternally imprinted in some individuals (section 3.3.5) but no evidence for imprinting has been reported for any other genes in the serotonergic pathway.

3.4.2. Family history of ADHD

As ADHD is recognised to have a strong genetic contribution, it might be expected that there would be differences between ADHD children with a family history (i.e. one or both parents have/had the disorder) compared to those with no history of the disorder. A study by August and Stewart (1983) defined two subtypes of childhood hyperactivity i.e. family history positive (FH+) and family history negative (FH-). They observed that children in both subgroups were equally deviant on measures of the core components of childhood hyperactivity. However, the children with a positive family history were, in addition, deviant on dimensions of conduct disturbance and had a high prevalence of siblings with CD. In contrast to this, children with a negative family history of the disorder had increased learning and academic problems and their siblings were found to have more attentional and learning disabilities but no evidence of CD. A study by Goos et al (2007) observed that ADHD probands with a maternal history of ADHD had higher ratings of behavioural disorders such as ADHD, conduct disorder and oppositional symptoms compared to probands with a paternal history of the disorder. Smalley et al (2000) reported that 55% of families with affected sibling pairs have at least one parent with a lifetime diagnosis of ADHD. The frequency increased to 63% in families with at least one affected girl while it decreased to 45% in families with at least one affected boy ($p = 0.02$) indicating that females require a greater genetic load than males in order to develop ADHD.

3.4.3 ADHD subtype

There are three phenotypes of ADHD according to DSM-IV: combined (both inattentive and hyperactive-impulsive symptoms) predominantly inattentive and, least common, predominantly hyperactive/impulsive. The combined type is the most commonly diagnosed form, accounting for 50-75% of all ADHD individuals; the predominantly inattentive type averages 20-30% of cases and the predominantly hyperactive/impulsive type accounts for less than 15% of cases (Wilens et al, 2002). Individuals are diagnosed if they have 6 or more symptoms out of the 9 listed in DSM-IV under the inattentive or hyperactive/impulsive categories persisting for 6 months or more (Table 1.1). It is possible that

individuals with different subtypes could be aetiologically heterogeneous and may have different genetic background. Recent reports on the 5-HT1B gene have found that while there may not be an overall association of ADHD with the G861C SNP, sub-dividing the sample into the categories above results in a positive association between the G allele and the inattentive ADHD subtype (Li et al, 2005; Smoller et al, 2006). Manor et al (2001) found evidence for a significant decrease in the S/S genotype of the serotonin transporter variant, 5-HTTLPR (section 3.3.2) in a group of children with combined type ADHD compared to controls (10.29% vs. 30.88%). Similarly, Brookes et al (2006) also reported association between the TPH2 gene and ADHD combined type individuals. In addition a meta-analysis by Lowe et al (2004) reported significant association between the 148-bp allele of a DRD5 microsatellite and the predominantly inattentive and combined subtypes.

3.4.4 Comorbid Disorders

ADHD children with co-morbid oppositional defiant disorder (ODD) have been found to be more severely affected than those with ADHD or ODD alone. In addition they also were observed to have an earlier age of onset, to be more aggressive and antisocial, and to suffer greater academic and social impairment (Carlson et al, 1997). Kadesjo et al (2003) reported that children with ADHD and ODD had consistently higher rates of ADHD symptoms compared to those with ADHD only. Clinical studies have also shown that ADHD comorbid with conduct disorder (CD) is a more severe condition with a poorer outcome than ADHD alone (Barkley, 1990; Jensen et al, 1997; Kuhne et al, 1997). Family studies have also suggested that ADHD-CD may represent a familial subtype (Faraone et al, 1998). In addition, Thapar et al (2001) using a twin study design, suggested that children with ADHD-CD may represent a more genetically loaded and more clinically severe group that children with ADHD alone. In support of this theory, Cook et al (1995) reported that serotonin levels in children with ADHD-ODD or ADHD-CD were different to children with ADHD alone.

Based on the above findings, the variants typed for the genes described in section 3.3 above were also examined in sub groups according to the sex of transmitting parent, family history of ADHD, ADHD subtype and a co-morbid diagnosis of ODD and CD.

3.5. Results

The observed genotype frequencies did not differ from those expected according to Hardy-Weinberg equilibrium for all examined markers. Tabulated below are the results of single variant association analyses and phenotype analyses for all variants in the eight genes analysed in this investigation.

3.5.1. TPH1

Transmission disequilibrium analysis (TDT) on the four examined variants showed no significant association with ADHD (Table 3.1). However, a trend towards overtransmission of the C allele of A-218C (rs1800532) to ADHD cases was observed (χ^2 = 3.13, p = 0.093, OR = 1.37). This allele also showed a significant association with the ADHD inattentive subgroup (Table 3.46: χ^2 = 6.25, p = 0.021, OR = 4.33). Allele A of rs211102 also showed a trend towards association with the inattentive subgroup (Table 3.46: χ^2 = 3.77, p = 0.09, OR = 3.33). In addition the G allele of rs10488683 showed a trend for association with probands with comorbid Conduct Disorder (CD) (Table 3.70: χ^2 = 4, p = 0.077, OR = 3). Analyses of paternal transmissions (Table 3.10) and maternal transmissions (Table 3.16), a positive family history for ADHD (Table 3.30), combined type ADHD (Table 3.38), hyperactive type ADHD (Table 3.54), and probands with comorbid Oppositional Defiant Disorder (ODD) (Table 3.62) did not reveal any significant associations between TPH1 and ADHD.

LD analysis of the markers is presented in figure 3.3 with D' values ranging from 0.32-1. Markers rs10488683 and rs211102 were found to be in complete LD (D' = 1). The weakest LD was observed between rs4537731 and rs10488683 (D' = 0.32).

Haplotype analysis on all markers (Table 3.80) showed association with the under transmission of three haplotypes; one containing markers rs4537731 and rs1800532 (χ^2 = 4.79, p = 0.0286), another containing both these markers and marker rs10488683 (χ^2 = 3.87, p = 0.0492) and a haplotype containing all four markers (χ^2

= 3.76, p = 0.0525). The global tests however did not reach the threshold of significance at $p < 0.05$.

3.5.2. TPH2

TDT analysis of the 14 examined markers is presented in table 3.2. A highly significant association between allele A of the marker rs1843809 and ADHD was observed ($\chi^2 = 13.47$, p = 0.0003, OR = 2.45). A significant but weaker association was also observed with marker rs1386497 ($\chi^2 = 4.2$, p = 0.048, OR = 1.45). In addition, a trend towards association was observed for allele G of marker rs1386494 ($\chi^2 = 3.2$, p = 0.093, OR = 1.5).

Stratifying the data by sex of transmitting parent (Tables 3.11 and 3.17) showed that the odds ratio between rs1843809 and ADHD is greater for paternal than for maternal transmissions ($\chi^2 = 10.94$, p = 0.00132, OR = 3.71 and $\chi^2 = 4.57$, p = 0.047, OR = 2.1 respectively). However, the pattern of paternal transmission of alleles was not significantly different to that of maternal transmissions ($\chi^2 = 0.019$, p = 1). Furthermore, significant associations between alleles C of rs1386493 and A of marker rs1487279 were observed when paternal transmissions were considered separately (rs1386493 paternal: $\chi^2 = 4.59$, p = 0.04, OR = 1.88 and rs1487279 paternal: $\chi^2 = 5.12$, p = 0.03, OR = 1.94). Again, there was no statistical difference between paternal and maternal ORs (rs1386493: $\chi^2 = 0.62$, p = 0.51; rs1487279: $\chi^2 = 1.1$, p = 0.36). A trend toward increased transmission of allele A of marker rs1386497 ($\chi^2 = 4.09$, p = 0.058, OR = 1.75) and a significant overtransmission of allele T of marker rs1487279 ($\chi^2 = 7.69$, p = 0.0078, OR = 2.25) was observed for maternal transmissions only. Maternal transmission of the T allele of marker rs1487279 was not significantly different to paternal transmissions ($\chi^2 = 1.98$, p = 0.2). However, paternal transmission of the C allele of this marker (rs1487279) was found to be significantly overtransmitted when compared to maternal transmissions ($\chi^2 = 6.48$, p = 0.015).

Analyses of the sample based on family history for ADHD shows a significant association of allele A of rs1843809 with a negative family history of the disorder (Table 3.23: $\chi^2 = 10.37$, p = 0.002, OR = 2.64). Overtransmission of allele A was found to be significantly different from family history negative compared to family

history positive for the disorder (χ^2 = 11.52, p = 0.0009). Allele C of marker rs1386493 exhibited a trend towards association when a negative family history of the disorder was considered (χ^2 = 3.46, p = 0.081, OR= 1.55). The C alleles of markers rs10748186 and rs10879345 were found to be over transmitted from parents with a positive history of ADHD (Table 3.31: χ^2 = 7, p = 0.0125, OR = 3 and χ^2 = 4.83, p = 0.041, OR = 2.18 respectively). Both alleles were significantly undertransmitted from parents with a positive family history of the disorder compared to transmission form parents with no known history of ADHD (χ^2 = 4.41, p = 0.048 and χ^2 = 5.38, p = 0.027 respectively).

Dividing the sample into DSM-IV subtypes shows a strong association between the A allele of rs1843809 and the combined subtype (Table 3.39: χ^2 = 9.68, p = 0.0026, OR = 2.57). There was also a significant association between the G allele of rs2129575 (Table 3.47: χ^2 = 5.4, p = 0.035, OR = 4) and a trend towards association of the G allele of rs4570625 (χ^2 = 4.57, p = 0.057, OR = 3.67) and allele T of marker rs1487275 (χ^2 = 3.85, p = 0.075, OR = 2.25) with the inattentive subtype. No association was found with the hyperactive subgroup (Table 3.55).

TDT analysis of cases with co-morbid ODD (n = 85) shows a significant association with marker rs1843809 (Table 3.63: χ^2 = 6.43, p = 0.016, OR = 2.5) and a trend towards significance with allele A of marker rs1386497 (χ^2 = 3.95, p = 0.063, OR = 1.71). In contrast, no distortion in the transmission of any of the examined markers was observed when analyses were conducted on cases with co-morbid CD (Table 3.71).

LD relationships between markers, measured as r^2 (figure 3.2) and D' (figure 3.3) are presented. Notably two blocks of LD can be observed using D' values. The first comprises markers 1-11 with D' measures between 0.38 and 0.91. The second block consists of markers rs1487275 and rs1487279 with D' of 0.77. A break of LD Between markers 11-13 (D' = 0.33 for markers 11 and 12 and D' = 0.14 between markers 12 and 13) can also be seen indicating a possible recombination hot spot within this 18kb region.

Two, three, four, five, six and seven marker haplotypes have been analysed in sliding windows using the program Transmit (Table 3.81 for select haplotype analysis results). Increased transmission of a number of haplotypes was observed

with the most significant over transmission found with a haplotype comprising alleles A, T and G of markers rs1843809, rs1386496 and rs1386494 respectively (χ^2 = 16.86, p = 0.0000). This suggests that there may be an as yet uncharacterised functional variant in the region of these three markers that is increasing susceptibility to ADHD in this sample.

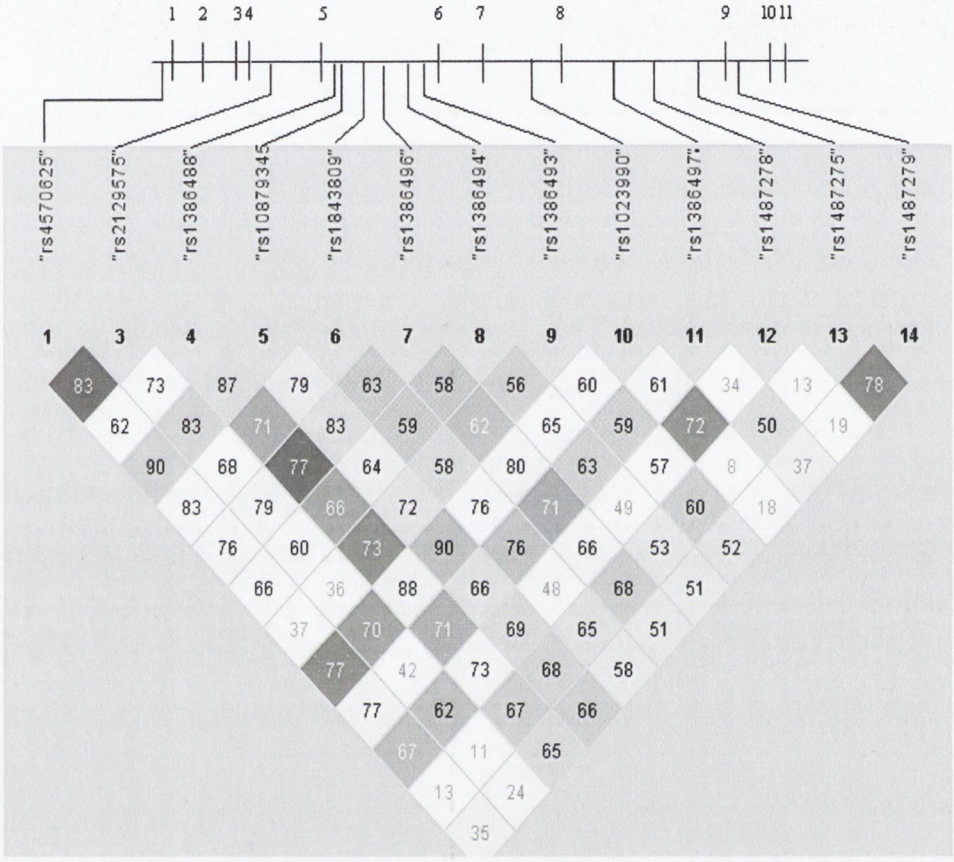


Figure 3.2 **Gene map of TPH2 (showing exons 1-11) including positions of markers analysed.** Also includes r^2 measures of linkage disequilibrium.

3.5.2.1 Replication Analysis in a UK ADHD Sample

TDT analysis of the UK sample was conducted on four examined markers (the four markers that exhibited the most significant association at individual marker or haplotype level in the Irish population) (Table 3.3). There was no significant difference in the transmission of alleles to ADHD cases. Linkage disequilibrium (LD), measured as D', shows that the examined markers are in significant LD ranging from 0.57-0.98 (Figure 3.4). These D' findings are comparable to those found in the Irish sample (Figure 3.5).In addition; there was no significant over

transmission of haplotypes to ADHD probands. A power study was performed on this replication analysis using the Genetic Power Calculator (http://pngu.mgh.havard.edu/~purcell/gpc) (Purcell et al, 2003). Given a total sample size of 108 families the power of this replication was calculated to be 52.4% to detect an association at a p value <0.05.

3.5.3. 5-HTT

Five DNA variants covering the gene (approximately 39 Kb) from the promoter to the 3'UTR were investigated. TDT analyses of the examined variants are presented in table 3.4. No overall association was observed between any of the analysed markers and ADHD. This includes the long allele of 5-HTTLPR which was reported to associate with ADHD in several previous studies. However, there was a trend towards association of the T allele of the 3'UTR SNP to ADHD children with co-morbid ODD (Table 3.64: $\chi^2 = 3.85$, p = 0.064, OR = 1.6). A trend towards association between the 12 repeat allele of the intron 2 VNTR and ADHD probands with co-morbid CD (Table 3.72: $\chi^2 = 3.46$, p = 0.089, OR = 1.92) was also observed. Analysing the data by the sex of transmitting parent (Tables 3.12 and 3.18), family history of the disorder (Tables 3.24 and 3.32) and ADHD subtype (Tables 3.40, 3.48 and 3.56) found no significant difference in the transmission of alleles to ADHD cases.

LD analysis shows that the D' relations among examined markers ranges between 0.21-0.94 (Figure 3.6). A single block is evident, covering a region of 21 Kb that consists of markers rs1872924, rs4325622, and the 3'UTR SNP. D' values range between 0.69-0.94. The promoter variant (5-HTTLPR) and the VNTR (intron 2) were observed to be in very weak LD with each other and with the other examined markers.

Two, three, four and five marker haplotype analyses showed no evidence of increased transmission of any haplotype to ADHD probands (Table 3.82).

3.5.4. VMAT2

TDT analysis of the six examined VMAT2 markers is presented in table 3.5. A trend towards over transmission of the C allele of the marker rs363226 to ADHD cases was observed ($\chi^2 = 3.78$, p = 0.063, OR = 1.42). Stratifying the data by sex of transmitting parent (Tables 3.13 and 3.19) showed a trend towards preferential maternal transmission of allele C of marker rs363226 ($\chi^2 = 3.63$, p = 0.076, OR = 1.7). This allele was also over transmitted to children with inattentive type ADHD (Table 3.49: $\chi^2 = 5.44$, p = 0.0391, OR = 8). No distortion in the transmission of any of the examined markers was observed when analyses were conducted by parent of origin, family history of ADHD (Tables 3.25 and 3.33), combined or hyperactive-impulsive ADHD subtypes (Tables 3.41 and 3.57 respectively), and the presence of comorbid ODD or CD (Tables 3.65 and 3.73 respectively).

LD analysis shows the presence of two haplotype blocks in this gene (Figure 3.7). The first is composed of markers rs2283135 and rs363387 which are in complete LD (D '= 1). The second contains markers rs363226 and rs363279 (D' = 0.94). These two blocks are separated by 22 kb, the D' values between them range from 0.3-0.49.

Examination of two marker haplotypes revealed increased transmission of a haplotype containing allele C of marker rs363220 and allele G of marker rs4552045 (Table 3.83: $\chi^2 = 4.68$, p value = 0.0305). Three-marker analysis found increased transmission of a haplotype constructed from the G allele of rs363387, the C allele of rs363220, and the G allele of rs4552045 ($\chi^2 = 3.91$, p value = 0.048). A haplotype composed of alleles C, G and C of markers rs363220, rs4552045, and rs363226 was also observed to be associated with ADHD ($\chi^2 = 3.9$, p value = 0.0483). Also, there was a trend towards over transmission of a haplotype composed of all six markers ($\chi^2 = 3.59$, p value = 0.058). Although individual haplotypes were observed to be over-transmitted, global tests did not reach the threshold of significance at $p < 0.05$.

3.5.5. 5-HT1B

Transmission disequilibrium test conducted on 5 markers covering the gene and the surrounding 5'and 3' regions showed no significant association between ADHD and any of the examined markers (Table 3.6). A previous study by our group (Hawi et al, 2002) on the first half of the current sample reported association with the SNP G861C (rs6296) ($\chi^2 = 9$, $p = 0.0037$). This however was not confirmed as the entire sample was not observed to be significantly associated with ADHD (Table 3.6: $\chi^2 = 2.16$, $p = 0.165$, OR = 1.27). It was also reported by Hawi et al (2002) that there was preferential paternal transmission of the 861G allele to ADHD children ($\chi^2 = 4.5$, $p = 0.052$). Once again this finding was not confirmed in the extended sample (Table 3.14: $\chi^2 = 2.4$, $p = 0.117$, OR = 1.5). No association was found when transmission of alleles of maternal origin was considered alone (Table 3.20). Stratifying the data based on family history of ADHD showed a trend towards association of the G allele to probands from parents with no history of the disorder (Table 3.26: $\chi^2 = 3.32$, $p = 0.08$, OR = 1.49). No association was found with transmissions from parents with a positive history of ADHD (Table 3.34). Dividing the sample by ADHD subtypes did not show any significant associations with 5-HT1B (Tables 3.42, 3.50, and 3.58). Analysis of ADHD probands with comorbid ODD showed significant over transmission of allele C of marker rs1228814 and allele T of marker T-261G (rs1157585) to affected children (Table 3.66: $\chi^2 = 4.88$, $p = 0.035$, OR = 1.65 and $\chi^2 = 4.56$, $p = 0.048$, OR = 1.6 respectively). TDT analysis on probands with comorbid CD did not show any association between this subgroup and the 5-HT1B gene (Table 3.74).

LD relations (measured as D') between analysed markers were high, ranging from D' = 0.79-0.89 (Figure 3.8) except for between G861C (rs6296) and rs6297 (D' = 0.49).

There were no significant differences in the transmission of haplotypes to affected offspring (Table 3.84). However there was a trend towards association between a haplotype made up of the A-T-A alleles of markers rs1228814, rs1157585 and rs130058 and ADHD ($\chi^2 = 3.09$, p value = 0.0788). The global test did not reach the threshold of significance at $p < 0.05$.

3.5.6 5-HT2A

TDT analysis on six markers covering approximately 41 Kb of the gene is presented in table 3.7. Significant association was observed between the T allele of marker rs927544 and ADHD (Table 3.7: $\chi^2 = 5.56$, p = 0.023, OR = 1.5). A trend towards association was also found with marker rs2770296 ($\chi^2 = 3.13$, p = 0.093, OR = 1.37). Stratifying by sex of transmitting parent revealed a significant association with paternal transmission of allele T of marker rs977003 and allele G of marker rs9316233 (Table 3.15: $\chi^2 = 4.24$, p = 0.053, OR = 1.79 and $\chi^2 = 4.26$, p = 0.054, OR = 1.87 respectively).

Allele T of marker rs927544 was also found to be associated with several sub groups. These include an increase in transmission of alleles of paternal origin (Table 3.15: $\chi^2 = 3.57$, p = 0.077, OR = 1.65), maternal origin (Table 3.21: $\chi^2 = 4.26$, p = 0.054, OR = 1.87), a negative family history of the disorder (Table 3.27: $\chi^2 = 4.26$, p = 0.054, OR = 1.87) and ADHD probands with co-morbid ODD (Table 3.67: $\chi^2 = 5.23$, p = 0.0139, OR = 1.76). In addition this allele was observed to be overtransmitted to ADHD probands with the hyperactive subtype. However, due to the small number of children in this group (n =11) the results are not reliable (Table 3.59: T: 7 NT: 1; $\chi^2 = 4.5$, p = 0.07, OR = 7). Allele T of marker rs2770296 show a trend towards association with probands with the inattentive ADHD subtype (Table 3.51: $\chi^2 = 3.52$, p = 0.093, OR = 2.28). The T allele of marker rs1745837 was significantly overtransmitted to ADHD probands with comorbid ODD (Table 3.67: $\chi^2 = 4.67$, p = 0.0436, OR = 2) and allele T of marker rs2770296 exhibited a trend towards significant association with this subgroup ($\chi^2 = 3.81$, p = 0.067, OR = 1.68). TDT analysis for a positive family history of ADHD (Table 3.35), combined type ADHD (Table 3.43), and probands with comorbid CD (Table 3.75) showed no evidence of association between 5-HT2A and ADHD.

LD measures show a block of relatively high LD (D' = 0.57-0.98) encompassing markers rs2770296, rs1928040 and rs927544 (Figure 3.9). Markers rs977003, rs1745837, rs9316233 and rs2770296 were not found to be in strong LD with each other (D' = 0-31).

Examination of haplotypes found no increased transmission of haplotypes to ADHD probands (Table 3.85). However, there was a trend towards increased transmission of a haplotype consisting of alleles C and T of markers rs1928040 and rs927544 respectively ($\chi^2 = 3.23$, $p = 0.0723$).

3.5.7. MAO-A

TDT analysis conducted on four MAO-A variants showed significantly higher transmission of the 941G allele (Table 3.8: $\chi^2 = 5.1$, $p = 0.03$, OR = 1.7) to ADHD cases as compared to the lower activity 941T allele. Stratifying the sample for family history of ADHD (positive or negative) revealed an increased transmission of the more active 941G allele from parents with no history of ADHD (Table 3.28: $\chi^2 = 5.67$, $p = 0.0241$, OR = 2). Splitting the sample into ADHD subtypes showed an association between the 941G allele and the inattentive subtype (Table 3.52: $\chi^2 = 7.36$, $p = 0.0123$, OR = 10) and a trend towards association with the combined subtype (Table 3.44: $\chi^2 = 3.42$, $p = 0.088$, OR = 1.8). In addition the CA repeat showed a trend towards association with the hyperactive subtype (Table 3.60: LRS = 11.67, df = 6, $p = 0.069$). TDT analysis for a positive family history of ADHD (Table 3.36) and of probands with comorbid ODD and CD (Tables 3.68 and 3.76 respectively) showed no evidence of association between MAO-A and ADHD.

LD, measured as D', among the MAO-A markers was significant between all examined markers (Table 3.78) with values ranging from between 0.63 and 0.91.

Haplotype analysis using a two-marker window revealed a significantly increased transmission of a haplotype containing the shorter 3 allele of the 30bp VNTR (allele 1) and allele 6 of the CA repeat (famhap global statistic = 24.86, $p = 0.05$). This association was further enhanced when a three-marker window (including the 941G/T SNP) was analysed. A haplotype comprising allele 1 of the 30bp VNTR, allele 6 of the CA repeat and the 941G allele (allele 2) was preferentially transmitted to ADHD probands (famhap global statistic = 34.54, $p = 0.01$).

3.5.8. MAO-B

TDT and ETDT analysis on both of the examined MAO-B markers are presented in table 3.9. The CA repeat showed association with the inattentive subtype (Table 3.53: LRS = 13.3, df = 6, p = 0.038). This variant also exhibited association with ADHD probands with comorbid CD (Table 3.77: LRS = 11.23, df = 5, p = 0.046). In addition, a trend toward association of the C allele of SNP rs1799836 to ADHD hyperactive type was observed (Table 3.61: $\chi^2 = 5$, p = 0.063). Due to the lack of transmission of the other allele (T = 0, NT = 5) and the small sample size (n=11) this result is not reliable and the odds ratio could not be calculated. TDT analysis of family history of ADHD (Tables 3.29 and 3.37 respectively), combined ADHD subtype (Table 3.45), and probands with comorbid ODD (Table 3.69) showed no evidence of association between MAO-B and ADHD.

D' analysis showed weak evidence for LD between the two markers (Table 3.79: D' = 0.28).

Haplotype analysis revealed no significant transmission of haplotypes to ADHD probands (Table 3.87).

3.5.9. TDT analyses results for serotonergic candidate genes (n = 178)

For all tables below: significant ($p<.05$) transmissions are highlighted in dark orange and bold print. Trends towards significance ($0.094<p>0.05$) are highlighted in light orange.

Table 3.1 TPH1

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4537731	G (0.55)	46	37	0.97	0.38	1.24
	A (0.45)	37	46			
rs1800532	A (0.45)	54	74	3.13	0.093	1.37
	C (0.55)	74	54			
rs10488683	A (0.56)	73	76	0.06	0.87	1.04
	G (0.44)	76	73			
rs211102	G (0.83)	38	42	0.2	0.738	1.1
	A (0.17)	42	38			

Table 3.2 TPH2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4570625	G (0.82)	44	49	0.268	0.679	1.11
	T (0.18)	49	44			
rs10748186	C (0.53)	62	54	0.55	0.516	1.15
	T (0.47)	54	62			
rs2129575	G (0.78)	58	48	0.943	0.382	1.21
	T (0.22)	48	58			
rs1386488	A (0.81)	50	34	3.05	0.101	1.47
	C (0.19)	34	50			
rs10879345	C (0.45)	76	72	0.11	0.805	1.1
	T (0.55)	72	76			
rs1843809	**A (0.85)**	**54**	**22**	**13.47**	**0.0003**	**2.45**
	C (0.15)	**22**	**54**			
rs1386496	T (0.80)	59	46	1.61	0.242	1.28
	C (0.20)	46	59			
rs1386494	G (0.82)	48	32	3.2	0.093	1.5
	A (0.18)	32	48			
rs1386493	C (0.78)	61	43	3.4	0.095	1.4
	T (0.22)	43	61			

rs1023990	C (0.25)	60	59	0.008	1	1
	T (0.75)	59	60			
rs1386497	**A (0.73)**	**74**	**51**	**4.2**	**0.048**	**1.45**
	C (0.27)	**51**	**74**			
rs1487278	C (0.26)	67	78	0.83	0.41	1.16
	T (0.74)	78	67			
rs1487275	G (0.29)	54	68	1.6	0.24	1.26
	T (0.71)	68	54			
rs1487279	A (0.70)	76	60	1.88	0.20	1.26
	T (0.30)	60	76			

Table 3.3 TPH2 (UK Results) (n = 108)

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs1386488	A(0.77)	23	19	0.38	0.644	1.2
	C(0.23)	19	23			
rs1843809	A(0.79)	21	20	0.024	1	1.05
	C(0.21)	20	21			
rs1386496	T(0.78)	18	22	0.4	0.636	0.81
	C(0.22)	22	18			
rs1386493	C(0.62)	23	26	0.184	0.775	0.89
	T(0.38)	26	23			

Table 3.45-HTT

Marker	Allele/Freq	T	NT	χ^2	p	OR
Promoter	Long (0.54)	82	88	0.21	0.703	1.07
	Short (0.46)	88	82			
VNTR	12 Repeat (0.55)	74	64	0.72	0.444	1.15
	10 Repeat (0.45)	64	74			
rs1872924	T (0.81)	28	31	0.15	0.795	1.11
	C (0.19)	31	28			
rs4325622	T (0.57)	79	65	1.36	0.279	1.21
	C (0.43)	65	79			
3'UTR	G (0.44)	68	81	1.13	0.326	1.19
	T (0.56)	81	68			

Table 3.5 VMAT2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs2283135	G(0.90)	18	22	0.4	0.636	1.22
	C(0.10)	22	18			
rs363387	G(0.10)	22	18	0.4	0.636	1.22
	T(0.90)	18	22			
rs363220	T (0.82)	36	49	1.98	0.193	1.36
	C (0.18)	49	36			
rs4752045	G (0.62)	66	61	0.19	0.723	1.08
	C (0.38)	61	66			
rs363226	C (0.71)	75	53	3.78	0.063	1.42
	G (0.29)	53	75			
rs363279	T (0.89)	24	28	0.86	0.678	1.33
	C (0.11)	28	24			

Table 3.6 5-HT1B

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs1228814	A(0.44)	82	94	0.82	0.407	1.15
	C(0.56)	94	82			
T-261G (rs1157585)	T(0.58)	87	82	0.15	0.758	1.06
	G(0.42)	82	87			
A-161T (rs130058)	A(0.71)	74	65	0.58	0.497	1.14
	T(0.29)	65	74			
G861C (rs6296)	G(0.71)	84	66	2.16	0.165	1.27
	C(0.29)	66	84			
rs6297	A(0.80)	56	46	1	0.373	1.22
	G(0.20)	46	56			

Table 3.7 5-HT2A

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs977003	T(0.54)	71	65	0.26	0.668	1.09
	G(0.46)	65	71			
rs1745837	T(0.70)	55	53	0.03	0.923	1.03
	C(0.30)	53	55			
rs9316233	C(0.83)	40	52	1.56	0.251	1.3
	G(0.17)	52	40			
rs2770296	T(0.68)	74	54	3.13	0.093	1.37
	C(0.32)	74	54			
rs1928040	T(0.48)	71	61	0.76	0.43	1.62
	C(0.52)	61	71			
rs927544	**T(0.66)**	**79**	**52**	**5.56**	**0.023**	**1.5**
	C(0.34)	**52**	**79**			

Table 3.8 MAO-A

Marker	Allele	T	NT	χ^2/LRS	p	OR
30 bp VNTR[a]	3	39	48	0.9	0.39	1.23
	3a, 4, 5	48	39			
CA(n)[b]	1	7	9	10.8 df=7	0.15	-
	2	2	0			
	3	28	21			
	4	2	9			
	5	4	4			
	6	15	18			
	7	42	34			
	8	6	11			
941G/T	**T**	**26**	**45**	**5.1**	**0.03**	**1.73**
	G	**45**	**26**			
rs979605	A	35	31	0.2	0.71	1.13
	G	31	35			

Table 3.9 MAO-B

Marker	Allele	T	NT	χ^2/LRS	p	OR
rs1799836	T	47	47	0	1	1
	C	47	47			
CA(n)[c] rs3838196	1	0	0	5.8 df=7	0.56	-
	2	5	10			
	3	37	27			
	4	23	23			
	5	25	34			
	6	17	17			
	7	8	5			
	8	1	0			
	9	0	0			
	10	1	1			
	11	0	0			
	12	0	0			

Significant ($p<.05$) transmissions are highlighted in dark orange and bold print. Trends towards significance ($0.094<p>0.05$) are highlighted in light orange.

[a] Due to the functional roles the alleles of the MAO-A 30 bp VNTR were grouped into two classes (3 allele vs. all longer alleles [3a, 4 and 5]) and named allele 1 and 2 respectively. Alleles 3 and 4 however, in concordance with other studies, constituted over 95% of all observed alleles.

[b] Eight alleles were detected, which were named according to fragment length from 1 (longest) to 8 (shortest).

[c] Twelve alleles were detected, which were named according to fragment length from 1 (longest) to 12 (shortest). Only eight of these alleles were found to be transmitted from heterozygous mothers.

3.5.9.1 TDT results from paternal transmissions only (n = 170)

Table 3.10 TPH1

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4537731	G (0.55)	15	12	0.33	0.70	1.25
	A (0.45)	12	15			
A-218C	A (0.45)	21	29	1.28	0.322	1.38
	C (0.55)	29	21			
rs10488683	A (0.56)	25	31	0.64	0.50	1.24
	G (0.44)	31	25			
rs211102	G (0.83)	18	18	0	0	1
	A (0.17)	18	18			

Table 3.11 TPH2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4570625	G (0.82)	17	21	0.421	0.627	1.24
	T (0.18)	21	17			
rs10748186	C (0.53)	30	21	1.58	0.262	1.43
	T (0.47)	21	30			
rs2129575	G (0.78)	25	22	0.191	0.771	1.14
	T (0.22)	22	25			
rs1386488	A (0.81)	26	15	2.95	0.117	1.73
	C (0.19)	15	26			
rs10879345	C (0.45)	29	28	0.017	1	1.03
	T (0.55)	28	29			
rs1843809	**A (0.85)**	**26**	**7**	**10.94**	**0.00132**	**3.71**
	C (0.15)	**7**	**26**			
rs1386496	T (0.80)	26	20	0.728	0.461	1.3
	C (0.20)	20	26			
rs1386494	G (0.82)	23	17	0.9	0.43	1.35
	A (0.18)	17	23			
rs1386493	**C (0.78)**	**32**	**17**	**4.59**	**0.044**	**1.88**
	T (0.22)	**17**	**32**			
rs1023990	C (0.25)	28	20	1.33	0.312	1.4
	T (0.75)	20	28			
rs1386497	A (0.73)	28	20	1.33	0.312	1.4
	C (0.27)	20	28			
rs1487278	C (0.26)	34	25	1.37	0.298	1.36
	T (0.74)	25	34			

rs1487275	G (0.29)	24	28	0.307	0.678	1.16
	T (0.71)	28	24			
rs1487279	**A (0.70)**	**33**	**17**	**5.12**	**0.032**	**1.94**
	T (0.30)	**17**	**33**			

Table 3.12 5-HTT

Marker	Allele/Freq	T	NT	χ^2	p	OR
Promoter	Long (0.54)	26	35	1.33	0.306	1.35
	Short (0.46)	35	26			
VNTR	12 Repeat (0.55)	24	21	0.2	0.766	1.14
	10 Repeat (0.45)	21	24			
rs1872924	T (0.81)	10	18	2.28	0.185	1.8
	C (0.19)	18	10			
rs4325622	T (0.57)	31	27	0.28	0.694	1.15
	C (0.43)	27	31			
3'UTR	G (0.44)	22	32	1.85	0.22	1.45
	T (0.56)	32	22			

Table 3.13 VMAT2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs2283135	G(0.90)	5	10	1.67	0.302	2
	C(0.10)	10	5			
rs363387	G(0.10)	10	5	1.67	0.302	2
	T(0.90)	5	10			
rs363220	T(0.82)	16	21	0.67	0.511	1.31
	C(0.18)	21	16			
rs4752045	G(0.62)	28	25	0.17	0.784	1.12
	C(0.38)	25	28			
rs363226	C(0.71)	33	25	1.1	0.358	1.32
	G(0.29)	25	33			
rs363279	T(0.89)	11	10	0.05	1	1.1
	C(0.11)	10	11			

Table 3.14 5-HT1B

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs1228814	A(0.44)	27	38	1.86	0.215	1.41
	C(0.56)	38	27			
T-261G (rs1157585)	T(0.58)	38	31	0.71	0.47	1.23
	G(0.42)	31	38			
A-161T (rs130058)	A(0.71)	33	25	1.1	0.358	1.32
	T(0.29)	25	33			
G861C (rs6296)	G(0.71)	36	23	2.4	0.117	1.5
	C(0.29)	23	36			
rs6297	A(0.80)	24	15	2.1	0.2	1.6
	G(0.20)	15	24			

Table 3.15 5-HT2A

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs977003	T(0.54)	34	19	4.24	0.053	1.79
	G(0.46)	19	34			
rs1745837	T(0.70)	23	15	1.68	0.256	1.53
	C(0.30)	15	23			
rs9316233	C(0.83)	16	30	4.26	0.054	1.87
	G(0.17)	30	16			
rs2770296	T(0.68)	30	18	3	0.111	1.67
	C(0.32)	18	30			
rs1928040	T(0.48)	25	20	0.56	0.551	1.25
	C(0.52)	20	25			
rs927544	T(0.66)	39	24	3.57	0.077	1.63
	C(0.34)	24	39			

Significant ($p<.05$) transmissions are highlighted in dark orange and bold print. Trends towards significance ($0.094<p>0.05$) are highlighted in light orange.

3.5.9.2. TDT results for maternal transmissions only (n = 178)

Table 3.16 TPH1

Marker	Allele/Freq	T	NT	χ^2	p	OR
RS4537731	G (0.55)	18	11	1.69	0.26	1.64
	A (0.45)	11	18			
A-218C	A (0.45)	24	36	2	0.15	1.5
	C (0.55)	36	24			
rs10488683	A (0.56)	30	27	0.16	0.79	1.11
	G (0.44)	27	30			
rs211102	G (0.83)	13	17	2.4	0.58	1.5
	A (0.17)	17	13			

Table 3.17 TPH2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4570625	G (0.82)	18	19	0.027	1	0.947
	T (0.18)	19	18			
rs10748186	C (0.53)	22	22	0	1	1
	T (0.47)	22	22			
rs2129575	G (0.78)	24	17	1.195	0.349	1.4
	T (0.22)	17	24			
rs1386488	A (0.81)	22	17	0.641	0.522	1.3
	C (0.19)	17	22			
rs10879345	C (0.45)	30	27	0.157	0.791	1.1
	T (0.55)	27	30			
rs1843809	**A (0.85)**	**25**	**12**	**4.57**	**0.047**	**2.1**
	C (0.15)	**12**	**25**			
rs1386496	T (0.80)	27	20	1.04	0.382	1.35
	C (0.20)	20	27			
rs1386494	G (0.82)	25	15	2.5	0.154	1.67
	A (0.18)	15	25			
rs1386493	C (0.78)	26	23	0.183	0.775	1.13
	T (0.22)	23	26			
rs1023990	C (0.25)	23	30	0.924	0.41	1.3
	T (0.75)	30	23			
rs1386497	A (0.73)	35	20	4.09	0.058	1.75
	C (0.27)	20	35			
rs1487278	**C (0.26)**	**16**	**36**	**7.69**	**0.0078**	**2.25**
	T (0.74)	**36**	**16**			

rs1487275	G (0.29)	16	26	2.38	0.164	1.63
	T (0.71)	26	16			
rs1487279	A (0.70)	25	26	0.019	1	0.96
	T (0.30)	26	25			

Table 3.18 5-HTT

Marker	Allele/Freq	T	NT	χ^2	p	OR
Promoter	Long(0.54)	32	33	0.015	1	1.03
	Short(0.46)	33	32			
VNTR	12 Repeat (0.55)	33	26	0.83	0.435	1.27
	10 Repeat (0.45)	26	33			
rs1872924	T (0.81)	16	11	0.93	0.442	1.45
	C (0.19)	11	16			
rs4325622	T (0.57)	34	24	1.72	0.237	1.42
	C (0.43)	24	34			
3'UTR	G (0.44)	30	33	0.14	0.801	1.1
	T (0.56)	33	30			

Table 3.19 VMAT2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs2283135	G(0.90)	11	10	0.05	1	1.1
	C(0.10)	10	11			
rs363387	G(0.10)	11	10	0.05	1	1.1
	T(0.90)	10	11			
rs363220	T(0.82)	16	24	1.6	0.268	1.5
	C(0.18)	24	16			
rs4752045	G(0.62)	26	24	0.08	0.888	1.08
	C(0.38)	24	26			
rs363226	C(0.71)	34	20	3.63	0.076	1.7
	G(0.29)	20	34			
rs363279	T(0.89)	12	17	0.86	0.458	1.42
	C(0.11)	17	12			

Table 3.20 5-HT1B

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs1228814	A(0.44)	31	32	0.02	1	1.03
	C(0.56)	32	31			
T-261G (rs1157585)	T(0.58)	37	36	0.01	1	1.03
	G(0.42)	36	37			
A-161T (rs130058)	A(0.71)	29	26	0.16	0.788	1.12
	T(0.29)	26	29			
G861C (rs6296)	G(0.71)	29	23	0.69	0.488	1.26
	C(0.29)	23	29			
rs6297	A(0.80)	23	22	0.02	1	1.04
	G(0.20)	22	23			

Table 3.21 5-HT2A

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs977003	T(0.54)	18	27	1.8	0.233	1.5
	G(0.46)	27	18			
rs1745837	T(0.70)	20	26	0.78	0.461	1.3
	C(0.30)	26	20			
rs9316233	C(0.83)	17	15	0.13	0.860	1.13
	G(0.17)	15	17			
rs2770296	T(0.68)	25	17	1.52	0.280	1.47
	C(0.32)	17	25			
rs1928040	T(0.48)	27	22	0.51	0.568	1.23
	C(0.52)	22	27			
rs927544	**T(0.66)**	**30**	**16**	**4.26**	**0.054**	**1.87**
	C(0.34)	**16**	**30**			

Significant ($p<.05$) transmissions are highlighted in dark orange and bold print. Trends towards significance ($0.094<p>0.05$) are highlighted in light orange.

3.5.9.3 TDT results for transmissions from parents with no family history of ADHD (n = 94)

Table 3.22 TPH1

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4537731	G (0.55)	29	20	1.65	0.25	1.45
	A (0.45)	20	29			
rs1800532	A (0.45)	36	52	2.91	0.109	1.44
	C (0.55)	52	36			
rs10488683	A (0.56)	45	55	1	0.368	1.22
	G (0.44)	55	45			
rs211102	G (0.83)	22	31	1.53	0.272	1.4
	A (0.17)	31	22			

Table 3.23 TPH2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4570625	G (0.82)	28	28	0	1	1
	T (0.18)	28	28			
rs10748186	C (0.53)	37	39	0.05	0.909	1.05
	T (0.47)	39	37			
rs2129575	G (0.78)	38	28	1.5	0.268	1.36
	T (0.22)	28	38			
rs1386488	A (0.81)	35	24	2.05	0.193	1.46
	C (0.19)	24	35			
rs10879345	C (0.45)	43	49	0.39	0.602	1.14
	T (0.55)	49	43			
rs1843809	**A (0.85)**	**37**	**14**	**10.37**	**0.002**	**2.64**
	C (0.15)	**14**	**37**			
rs1386496	T (0.80)	42	31	1.66	0.242	1.35
	C (0.20)	31	42			
rs1386494	G (0.82)	35	24	2.05	0.193	1.46
	A (0.18)	24	35			
rs1386493	C (0.78)	45	29	3.46	0.081	1.55
	T (0.22)	29	45			
rs1023990	C (0.25)	37	44	0.604	0.51	1.2
	T (0.75)	44	37			
rs1386497	A (0.73)	50	35	2.65	0.128	1.43
	C (0.27)	35	50			
rs1487278	C (0.26)	41	52	1.3	0.3	1.23
	T (0.74)	52	41			

rs1487275	G (0.29)	36	46	1.22	0.32	1.27
	T (0.71)	46	36			
rs1487279	A (0.70)	49	39	1.14	0.337	1.26
	T (0.30)	39	49			

Table 3.24 5-HTT

Marker	Allele/Freq	T	NT	χ^2	p	OR
Promoter	Long (0.54)	52	58	0.33	0.634	1.15
	Short (0.46)	58	52			
VNTR	12 Repeat (0.55)	48	36	1.71	0.23	1.33
	10 Repeat (0.45)	36	48			
rs1872924	T (0.81)	23	19	0.38	0.644	1.21
	C (0.19)	19	23			
rs4325622	T (0.57)	54	44	1.02	0.363	1.23
	C (0.43)	44	54			
3'UTR	G (0.44)	46	50	0.17	0.76	1.09
	T (0.56)	50	46			

Table 3.25 VMAT2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs2283135	G(0.90)	15	18	0.27	0.728	1.2
	C(0.10)	18	15			
rs363387	G(0.10)	18	14	0.5	0.597	1.28
	T(0.90)	14	18			
rs363220	T(0.82)	27	29	0.07	0.894	1.07
	C(0.18)	29	27			
rs4752045	G(0.62)	42	41	0.01	1	1.02
	C(0.38)	41	42			
rs363226	C(0.71)	47	32	2.8	0.115	1.47
	G(0.29)	32	47			
rs363279	T(0.89)	16	19	0.28	0.736	1.18
	C(0.11)	19	16			

Table 3.26 5-HT1B

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs1228814	A(0.44)	58	60	0.03	0.927	1.03
	C(0.56)	60	58			
T-261G (rs1157585)	T(0.58)	55	55	0	1	1
	G(0.42)	55	55			
A-161T (rs130058)	A(0.71)	48	37	1.42	0.278	1.30
	T(0.29)	37	48			
G861C (rs6296)	G(0.71)	52	35	3.32	0.085	1.49
	C(0.29)	35	52			
rs6297	A(0.80)	38	32	0.51	0.55	1.19
	G(0.20)	32	38			

Table 3.27 5-HT2A

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs977003	T(0.54)	40	43	0.18	0.826	1.07
	G(0.46)	43	40			
rs1745837	T(0.70)	38	38	0	1	1
	C(0.30)	38	38			
rs9316233	C(0.83)	25	30	0.45	0.59	1.2
	G(0.17)	30	25			
rs2770296	T(0.68)	44	31	2.25	0.165	1.41
	C(0.32)	31	44			
rs1928040	T(0.48)	43	37	0.45	0.576	1.16
	C(0.52)	37	43			
rs927544	**T(0.66)**	**52**	**30**	**5.9**	**0.019**	**1.73**
	C(0.34)	**30**	**52**			

Table 3.28 MAO-A

Marker	Allele	T	NT	χ^2/LRS	p	OR
30 bp VNTR	3	30	31	0.016	1	1.03
	3a, 4, 5	31	30			
CA(n)	1	4	4	5.05 df = 7	0.653	-
	2	1	0			
	3	19	15			
	4	2	8			
	5	3	4			
	6	12	14			
	7	31	26			
	8	4	7			
941G/T	**T**	**17**	**34**	**5.67**	**0.0241**	**2**
	G	**34**	**17**			
rs979605	A	26	22	0.33	0.665	1.18
	G	22	26			

Table 3.29 MAO-B

Marker	Allele	T	NT	χ^2/LRS	p	OR
rs1799836	T	41	34	0.65	0.489	1.2
	C	34	41			
CA(n)	1	0	0	9.23 df = 6	0.161	-
	2	4	7			
	3	29	21			
	4	17	17			
	5	19	30			
	6	14	11			
	7	6	3			
	8	0	0			
	9	0	0			
	10	1	1			
	11	0	0			
	12	0	0			

Significant ($p<.05$) transmissions are highlighted in dark orange and bold print.

Trends towards significance ($0.094<p>0.05$) are highlighted in light orange.

3.5.9.4 TDT results for transmissions from parents with a positive history of ADHD (n = 79)

Table 3.30 TPH1

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4537731	G (0.55)	13	9	0.73	0.52	1.44
	A (0.45)	9	13			
rs1800532	A (0.45)	14	19	0.75	0.487	1.37
	C (0.55)	19	14			
rs10488683	A (0.56)	23	16	1.26	0.337	1.44
	G (0.44)	16	23			
rs211102	G (0.83)	13	10	0.39	0.68	1.3
	A (0.17)	10	13			

Table 3.31 TPH2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4570625	G (0.82)	9	15	1.5	0.307	1.67
	T (0.18)	15	9			
rs10748186	**C (0.53)**	**21**	**7**	**7**	**0.0125**	**3**
	T (0.47)	**7**	**21**			
rs2129575	G (0.78)	14	15	0.03	1	1.07
	T (0.22)	15	14			
rs1386488	A (0.81)	12	9	0.43	0.664	1.33
	C (0.19)	9	12			
rs10879345	**C (0.45)**	**24**	**11**	**4.83**	**0.041**	**2.18**
	T (0.55)	**11**	**24**			
rs1843809	A (0.85)	13	5	3.56	0.096	2.6
	C (0.15)	5	13			
rs1386496	T (0.80)	13	13	0	1	1
	C (0.20)	13	13			
rs1386494	G (0.82)	11	5	2.25	0.21	2.2
	A (0.18)	5	11			
rs1386493	C (0.78)	14	14	0	1	1
	T (0.22)	14	14			
rs1023990	C (0.25)	20	11	2.62	0.15	1.8
	T (0.75)	11	20			
rs1386497	A (0.73)	15	11	0.62	0.557	1.36
	C (0.27)	11	15			
rs1487278	C (0.26)	18	17	0.03	1	1.06
	T (0.74)	17	18			

rs1487275	G (0.29)	15	20	0.71	0.5	1.33
	T (0.71)	20	15			
rs1487279	A (0.70)	19	15	0.47	0.608	1.26
	T (0.30)	15	19			

Table 3.32 5-HTT

Marker	Allele/Freq	T	NT	χ^2	p	OR
Promoter	Long (0.54)	16	20	0.44	0.618	1.25
	Short (0.46)	20	16			
VNTR	12 Repeat (0.55)	18	18	0	1	1
	10 Repeat (0.45)	18	18			
rs1872924	T (0.81)	4	11	3.26	0.118	2.75
	C (0.19)	11	4			
rs4325622	T (0.57)	17	13	0.53	0.585	1.31
	C (0.43)	13	17			
3'UTR	G (0.44)	15	23	1.68	0.256	1.53
	T (0.56)	23	15			

Table 3.33 VMAT2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs2283135	G(0.90)	2	4	0.67	0.688	2
	C(0.10)	4	2			
rs363387	G(0.10)	4	2	0.67	0.688	2
	T(0.90)	2	4			
rs363220	T(0.82)	8	16	2.67	0.152	2
	C(0.18)	16	8			
rs4752045	G(0.62)	17	15	0.125	0.86	1.13
	C(0.38)	15	17			
rs363226	C(0.71)	22	16	0.95	0.418	1.38
	G(0.29)	16	22			
rs363279	T(0.89)	6	8	0.29	0.791	1.33
	C(0.11)	8	6			

Table 3.34 5-HT1B

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs1228814	A(0.44)	17	29	3.13	0.104	1.71
	C(0.56)	20	17			
T-261G (rs1157585)	T(0.58)	29	21	1.28	0.322	1.38
	G(0.42)	21	29			
A-161T (rs130058)	A(0.71)	20	20	0	1	1
	T(0.29)	20	20			
G861C (rs6296)	G(0.71)	17	17	0	1	1
	C(0.29)	17	17			
rs6297	A(0.80)	13	11	0.17	0.839	1.18
	G(0.20)	11	13			

Table 3.35 5-HT2A

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs977003	T(0.54)	21	13	1.88	0.229	1.62
	G(0.46)	13	21			
rs1745837	T(0.70)	12	9	0.43	0.664	1.33
	C(0.30)	9	12			
rs9316233	C(0.83)	7	15	2.91	0.134	2.14
	G(0.17)	15	7			
rs2770296	T(0.68)	22	12	2.94	0.121	1.83
	C(0.32)	12	22			
rs1928040	T(0.48)	17	15	0.125	0.86	1.13
	C(0.52)	15	17			
rs927544	T(0.66)	21	12	2.45	0.163	1.75
	C(0.34)	12	21			

Table 3.36 MAO-A

Marker	Allele	T	NT	χ^2/LRS	p	OR
30 bp VNTR	3	8	14	1.64	0.286	1.75
	3a, 4, 5	14	8			
CA(n)	1	3	4	3.87 df = 7	0.794	-
	2	1	0			
	3	8	6			
	4	0	0			
	5	1	0			
	6	3	4			
	7	9	9			
	8	2	4			
941G/T	T	7	10	0.529	0.629	1.42
	G	10	7			
rs979605	A	8	7	0.06	1	1.14
	G	7	8			

Table 3.37 MAO-B

Marker	Allele	T	NT	χ^2/LRS	p	OR
rs1799836	T	6	11	1.47	0.332	1.83
	C	11	6			
CA(n)	1	0	0	3.25 df = 6	0.776	-
	2	1	3			
	3	6	6			
	4	6	5			
	5	6	3			
	6	3	6			
	7	2	2			
	8	1	0			
	9	0	0			
	10	0	0			
	11	0	0			
	12	0	0			

Significant ($p<.05$) transmissions are highlighted in dark orange and bold print.

3.5.9.5 TDT results for transmissions to probands with combined type ADHD (n = 116)

Table 3.38 TPH1

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4537731	G (0.55)	25	26	0.02	1	1.04
	A (0.45)	26	25			
rs1800532	A (0.45)	34	43	1.05	0.362	1.26
	C (0.55)	43	34			
rs10488683	A (0.56)	49	46	0.09	0.838	1.06
	G (0.44)	46	49			
rs211102	G (0.83)	20	25	0.56	0.551	1.25
	A (0.17)	25	20			

Table 3.39 TPH2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4570625	G (0.82)	25	35	1.67	0.245	1.4
	T (0.18)	35	25			
rs10748186	C (0.53)	38	34	0.22	0.724	1.12
	T (0.47)	34	38			
rs2129575	G (0.78)	33	29	0.26	0.70	1.14
	T (0.22)	29	33			
rs1386488	A (0.81)	33	22	2.2	0.18	1.5
	C (0.19)	22	33			
rs10879345	C (0.45)	39	32	0.69	0.48	1.22
	T (0.55)	32	39			
rs1843809	**A (0.85)**	**36**	**14**	**9.68**	**0.0026**	**2.57**
	C (0.15)	**14**	**36**			
rs1386496	T (0.80)	33	29	0.26	0.70	1.14
	C (0.20)	29	33			
rs1386494	G (0.82)	29	23	0.69	0.49	1.26
	A (0.18)	23	29			
rs1386493	C (0.78)	37	32	0.36	0.63	1.16
	T (0.22)	32	37			
rs1023990	C (0.25)	41	35	0.47	0.57	1.17
	T (0.75)	35	41			
rs1386497	A (0.73)	50	36	2.28	0.16	1.39
	C (0.27)	36	50			
rs1487278	C (0.26)	46	45	0.01	1	1.02
	T (0.74)	45	46			

rs1487275	G (0.29)	37	38	0.02	1	1.02
	T (0.71)	38	37			
rs1487279	A (0.70)	48	38	1.16	0.33	1.26
	T (0.30)	38	48			

Table 3.40 5-HTT

Marker	Allele/Freq	T	NT	χ^2	p	OR
Promoter	Long (0.54)	52	57	0.23	0.702	1.11
	Short (0.46)	57	52			
VNTR	12 Repeat (0.55)	48	43	0.27	0.675	1.12
	10 Repeat (0.45)	43	48			
rs1872924	T (0.81)	15	21	1	0.405	1.4
	C (0.19)	21	15			
rs4325622	T (0.57)	52	39	1.86	0.208	1.33
	C (0.43)	39	52			
3'UTR	G (0.44)	39	51	1.6	0.246	1.31
	T (0.56)	51	39			

Table 3.41 VMAT2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs2283135	G(0.90)	9	12	0.43	0.664	1.33
	C(0.10)	12	9			
rs363387	G(0.10)	13	9	0.73	0.523	1.44
	T(0.90)	9	13			
rs363220	T(0.82)	30	33	0.14	0.801	1.1
	C(0.18)	33	30			
rs4752045	G(0.62)	42	35	0.64	0.494	1.2
	C(0.38)	35	42			
rs363226	C(0.71)	45	38	0.59	0.51	1.18
	G(0.29)	38	45			
rs363279	T(0.89)	17	15	0.125	0.86	1.13
	C(0.11)	15	17			

Table 3.42 5-HT1B

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs1228814	A(0.44)	46	63	2.65	0.125	1.37
	C(0.56)	63	46			
T-261G (rs1157585)	T(0.58)	56	47	0.79	0.431	1.19
	G(0.42)	47	56			
A-161T (rs130058)	A(0.71)	50	36	2.28	0.161	1.39
	T(0.29)	36	50			
G861C (rs6296)	G(0.71)	49	44	0.27	0.679	1.11
	C(0.29)	44	49			
rs6297	A(0.80)	40	31	1.14	0.342	1.29
	G(0.20)	31	40			

Table 3.43 5-HT2A

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs977003	T(0.54)	53	44	0.83	0.417	1.2
	G(0.46)	44	53			
rs1745837	T(0.70)	37	36	0.013	1	1.03
	C(0.30)	36	37			
rs9316233	C(0.83)	23	31	1.18	0.341	1.35
	G(0.17)	31	23			
rs2770296	T(0.68)	41	35	0.47	0.567	1.17
	C(0.32)	35	41			
rs1928040	T(0.48)	42	42	0	1	1
	C(0.52)	42	42			
rs927544	T(0.66)	47	38	0.95	0.386	1.24
	C(0.34)	38	47			

Table 3.44 MAO-A

Marker	Allele	T	NT	χ^2/LRS	p	OR
30 bp VNTR	3	23	25	0.08	0.885	1.09
	3a, 4, 5	25	23			
CA(n)	1	5	5	0.942 df = 6	0.987	-
	2	0	0			
	3	17	14			
	4	1	2			
	5	3	3			
	6	10	15			
	7	25	24			
	8	5	5			
941G/T	T	15	27	3.42	0.088	1.8
	G	27	15			
rs979605	A	22	16	0.95	0.418	1.38
	G	16	22			

Table 3.45 MAO-B

Marker	Allele	T	NT	χ^2/LRS	p	OR
rs1799836	T	33	31	0.06	0.901	1.06
	C	31	33			
CA(n)	1	0	0	6.81 df = 7	0.448	-
	2	4	3			
	3	22	18			
	4	15	14			
	5	16	24			
	6	9	12			
	7	6	3			
	8	1	0			
	9	0	0			
	10	1	0			
	11	0	0			
	12	0	0			

Significant ($p<.05$) transmissions are highlighted in dark orange and bold print. Trends towards significance ($0.094<p>0.05$) are highlighted in light orange.

3.5.9.6 TDT results for transmissions to probands with inattentive type ADHD (n = 21)

Table 3.46 TPH1

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4537731	G (0.55)	9	4	1.92	0.27	2.25
	A (0.45)	4	9			
rs1800532	**A (0.45)**	**5**	**15**	**5**	**0.041**	**3**
	C (0.55)	**15**	**5**			
rs10488683	A (0.56)	7	13	1.8	0.263	1.86
	G (0.44)	13	7			
rs211102	G (0.83)	3	10	3.77	0.09	3.33
	A (0.17)	10	3			

Table 3.47 TPH2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4570625	G (0.82)	11	3	4.57	0.057	3.67
	T (0.18)	3	11			
rs10748186	C (0.53)	5	7	0.33	0.77	1.4
	T (0.47)	7	5			
rs2129575	**G (0.78)**	**12**	**3**	**5.4**	**0.035**	**4**
	T (0.22)	**3**	**12**			
rs1386488	A (0.81)	4	6	0.4	0.75	1.5
	C (0.19)	6	4			
rs10879345	C (0.45)	5	10	1.67	0.30	2
	T (0.55)	10	5			
rs1843809	A (0.85)	11	4	3.26	0.12	2.75
	C (0.15)	4	11			
rs1386496	T (0.80)	7	4	0.81	0.55	1.75
	C (0.20)	4	7			
rs1386494	G (0.82)	6	7	0.07	1	1.16
	A (0.18)	7	6			
rs1386493	C (0.78)	7	5	0.33	0.77	1.4
	T (0.22)	5	7			
rs1023990	C (0.25)	6	9	0.6	0.61	1.5
	T (0.75)	9	6			
rs1386497	A (0.73)	10	5	1.67	0.30	2
	C (0.27)	5	10			
rs1487278	C (0.26)	7	14	2.33	0.19	2
	T (0.74)	14	7			

rs1487275	G (0.29)	8	18	3.85	0.075	2.25
	T (0.71)	18	8			
rs1487279	A (0.70)	13	8	1.19	0.38	1.63
	T (0.30)	8	13			

Table 3.48 5-HTT

Marker	Allele/Freq	T	NT	χ^2	p	OR
Promoter	Long (0.54)	10	7	0.53	0.629	1.43
	Short (0.46)	7	10			
VNTR	12 Repeat (0.55)	6	8	0.29	0.791	1.33
	10 Repeat (0.45)	8	6			
rs1872924	T (0.81)	4	2	0.67	0.688	2
	C (0.19)	2	4			
rs4325622	T (0.57)	8	10	0.22	0.815	1.25
	C (0.43)	10	8			
3'UTR	G (0.44)	13	6	2.57	0.167	2.17
	T (0.56)	6	13			

Table 3.49 VMAT2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs2283135	G(0.90)	3	5	0.5	0.727	1.67
	C(0.10)	5	3			
rs363387	G(0.10)	5	3	0.5	0.727	1.67
	T(0.90)	3	5			
rs363220	**T(0.82)**	**1**	**8**	**5.44**	**0.039**	**8**
	C(0.18)	**8**	**1**			
rs4752045	G(0.62)	5	9	1.14	0.424	1.8
	C(0.38)	9	5			
rs363226	C(0.71)	10	7	0.53	0.629	1.43
	G(0.29)	7	10			
rs363279	T(0.89)	5	3	0.5	0.727	1.67
	C(0.11)	3	5			

Table 3.50 5-HT1B

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs1228814	A(0.44)	9	8	0.05	1	1.12
	C(0.56)	8	9			
T-261G (rs1157585)	T(0.58)	4	11	3.27	0.118	2.75
	G(0.42)	11	4			
A-161T (rs130058)	A(0.71)	5	10	1.67	0.302	2
	T(0.29)	10	5			
G861C (rs6296)	G(0.71)	10	6	1	0.454	1.67
	C(0.29)	6	10			
rs6297	A(0.80)	5	5	0	1	1
	G(0.20)	5	5			

Table 3.51 5-HT2A

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs977003	T(0.54)	6	4	0.4	0.754	1.5
	G(0.46)	4	6			
rs1745837	T(0.70)	8	6	0.28	0.791	1.33
	C(0.30)	6	8			
rs9316233	C(0.83)	8	8	0	1	1
	G(0.17)	8	8			
rs2770296	T(0.68)	16	7	3.52	0.093	2.28
	C(0.32)	7	16			
rs1928040	T(0.48)	15	8	2.13	0.21	1.87
	C(0.52)	8	15			
rs927544	T(0.66)	12	6	2	0.238	2
	C(0.34)	6	12			

Table 3.52 MAO-A

Marker	Allele	T	NT	χ^2/LRS	p	OR
30 bp VNTR	3	8	7	0.06	1	1.14
	3a, 4, 5	7	8			
CA(n)	1	0	1	6.84 df = 7	0.445	-
	2	1	0			
	3	6	3			
	4	1	1			
	5	1	0			
	6	0	1			
	7	4	7			
	8	1	1			
941G/T	**T**	**1**	**10**	**7.36**	**0.012**	**10**
	G	**10**	**1**			
rs979605	A	7	4	0.82	0.549	1.75
	G	4	7			

Table 3.53 MAO-B

Marker	Allele	T	NT	χ^2/LRS	p	OR
rs1799836	T	4	3	0.143	1	1.33
	C	3	4			
CA(n)	1	0	0	13.3 df = 6	0.038	-
	2	1	4			
	3	4	4			
	4	2	1			
	5	1	4			
	6	4	0			
	7	2	0			
	8	0	0			
	9	0	0			
	10	0	1			
	11	0	0			
	12	0	0			

Significant ($p<.05$) transmissions are highlighted in dark orange and bold print. Trends towards significance ($0.094<p>0.055$) are highlighted in light orange.

3.5.9.7. TDT results for transmissions to probands with hyperactive-impulsive type ADHD (n = 11)

Table 3.54 TPH1

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4537731	G (0.55)	4	2	0.67	0.68	2
	A (0.45)	2	4			
rs1800532	A (0.45)	2	6	2	0.29	3
	C (0.55)	6	2			
rs10488683	A (0.56)	2	8	3.6	0.109	4
	G (0.44)	8	2			
rs211102	G (0.83)	2	1	0.33	1	2
	A (0.17)	1	2			

Table 3.55 TPH2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4570625	G (0.82)	1	2	0.22	1	2
	T (0.18)	2	1			
rs10748186	C (0.53)	4	1	1.8	0.38	4
	T (0.47)	1	4			
rs2129575	G (0.78)	4	4	0	1	1
	T (0.22)	4	4			
rs1386488	A (0.81)	1	1	0	1	1
	C (0.19)	1	1			
rs10879345	C (0.45)	3	1	1	0.63	3
	T (0.55)	1	3			
*rs1843809	A (0.85)	0	2	2	0.5	-
	C (0.15)	2	0			
rs1386496	T (0.80)	4	2	0.67	0.68	2
	C (0.20)	2	4			
rs1386494	G (0.82)	2	2	0	1	1
	A (0.18)	2	2			
rs1386493	C (0.78)	5	2	1.29	0.45	2.5
	T (0.22)	2	5			
rs1023990	C (0.25)	3	3	0	1	1
	T (0.75)	3	3			
rs1386497	A (0.73)	4	4	0	1	1
	C (0.27)	4	4			
rs1487278	C (0.26)	5	2	1.29	0.45	2.5
	T (0.74)	2	5			

rs1487275	G (0.29)	2	3	0.2	1	1.5
	T (0.71)	3	2			
rs1487279	A (0.70)	3	4	0.14	1	1.33
	T (0.30)	4	3			

Table 3.56 5-HTT

Marker	Allele/Freq	T	NT	χ^2	p	OR
Promoter	Long (0.54)	6	5	0.1	1	1.2
	Short (0.46)	5	6			
VNTR	12 Repeat (0.55)	5	8	0.7	0.581	1.6
	10 Repeat (0.45)	8	5			
rs1872924	T (0.81)	1	1	0	1	1
	C (0.19)	1	1			
rs4325622	T (0.57)	3	4	0.14	1	1.33
	C (0.43)	4	3			
3'UTR	G (0.44)	4	7	0.82	0.549	1.75
	T (0.56)	7	4			

Table 3.57 VMAT2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs2283135	G(0.90)	2	1	0.33	1	2
	C(0.10)	1	2			
rs363387	G(0.10)	1	1	0	1	1
	T(0.90)	1	1			
rs363220	T(0.82)	2	2	0	1	1
	C(0.18)	2	2			
rs4752045	G(0.62)	7	2	2.78	0.18	3.5
	C(0.38)	2	7			
rs363226	C(0.71)	6	3	1	0.508	2
	G(0.29)	3	6			
*rs363279	T(0.89)	0	1	1	1	-
	C(0.11)	1	0			

Table 3.58 5-HT1B

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs1228814	A(0.44)	5	8	0.69	0.581	1.6
	C(0.56)	8	5			
T-261G (rs1157585)	T(0.58)	8	4	1.33	0.388	2
	G(0.42)	4	8			
A-161T (rs130058)	A(0.71)	6	4	0.4	0.754	1.5
	T(0.29)	4	6			
G861C (rs6296)	G(0.71)	6	4	0.4	0.754	1.5
	C(0.29)	4	6			
rs6297	A(0.80)	4	3	0.14	1	1.33
	G(0.20)	3	4			

Table 3.59 5-HT2A

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs977003	T(0.54)	2	6	2	0.289	3
	G(0.46)	6	2			
rs1745837	T(0.70)	4	3	0.14	1	1.33
	C(0.30)	3	4			
rs9316233	C(0.83)	3	3	0	1	1
	G(0.17)	3	3			
rs2770296	T(0.68)	4	1	1.8	0.375	4
	C(0.32)	1	4			
rs1928040	T(0.48)	5	1	2.67	0.219	5
	C(0.52)	1	5			
rs927544	T(0.66)	7	1	4.5	0.07	7
	C(0.34)	1	7			

Table 3.60 MAO-A

Marker	Allele	T	NT	χ^2/LRS	p	OR
30 bp VNTR	3	2	5	1.28	0.453	2.5
	3a, 4, 5	5	2			
CA(n)	1	1	2	11.67 df = 6	0.069	
	2	0	0			
	3	1	0			
	4	1	1			
	5	0	1			
	6	0	3			
	7	6	0			
	8	0	1			
941G/T	T	1	2	0.33	1	2
	G	2	1			
rs979605	A	2	2	0	1	1
	G	2	2			

Table 3.61 MAO-B

Marker	Allele	T	NT	χ^2/LRS	p	OR
*rs1799836	T	0	5	5	0.063	-
	C	5	0			
CA(n)	1	0	0	2.634 df = 4	0.621	-
	2	0	0			
	3	2	2			
	4	2	2			
	5	3	1			
	6	1	2			
	7	0	1			
	8	0	0			
	9	0	0			
	10	0	0			
	11	0	0			
	12	0	0			

Trends towards significance (0.094<p>0.055) are highlighted in light orange.

*Result unreliable.

3.5.9.8. TDT results for transmissions to probands with comorbid ODD (n = 85)

Table 3.62 TPH1

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4537731	G (0.55)	19	15	0.47	0.61	1.26
	A (0.45)	15	19			
rs1800532	A (0.45)	26	35	1.32	0.30	1.35
	C (0.55)	35	26			
rs10488683	A (0.56)	30	29	0	1	0
	G (0.44)	29	30			
rs211102	G (0.83)	18	16	0.12	0.86	1.12
	A (0.17)	16	18			

Table 3.63 TPH2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4570625	G (0.82)	9	16	1.96	0.23	1.78
	T (0.18)	16	9			
rs10748186	C (0.53)	16	16	0	1	1
	T (0.47)	16	16			
rs2129575	G (0.78)	22	20	0.09	0.88	1.1
	T (0.22)	20	22			
rs1386488	A (0.81)	21	15	1	0.41	1.4
	C (0.19)	15	21			
rs10879345	C (0.45)	24	24	0	1	1
	T (0.55)	24	24			
rs1843809	**A (0.85)**	**25**	**10**	**6.43**	**0.016**	**2.5**
	C (0.15)	**10**	**25**			
rs1386496	T (0.80)	25	19	0.82	0.45	1.32
	C (0.20)	19	25			
rs1386494	G (0.82)	22	12	2.94	0.12	1.83
	A (0.18)	12	22			
rs1386493	C (0.78)	25	16	1.98	0.21	1.56
	T (0.22)	16	25			
rs1023990	C (0.25)	23	25	0.08	0.89	1.09
	T (0.75)	25	23			
rs1386497	A (0.73)	36	21	3.95	0.063	1.71
	C (0.27)	21	36			
rs1487278	C (0.26)	32	36	0.23	0.72	1.12
	T (0.74)	36	32			

rs1487275	G (0.29)	23	27	0.32	0.67	1.17
	T (0.71)	27	23			
rs1487279	A (0.70)	32	25	0.86	0.43	1.28
	T (0.30)	25	32			

Table 3.64 5-HTT

Marker	Allele/Freq	T	NT	χ^2	p	OR
Promoter	Long (0.54)	43	34	1.05	0.362	1.26
	Short (0.46)	34	43			
VNTR	12 Repeat (0.55)	27	31	0.27	0.694	1.15
	10 Repeat (0.45)	31	27			
rs1872924	T (0.81)	7	13	1.8	0.263	1.86
	C (0.19)	13	7			
rs4325622	T (0.57)	37	35	0.05	0.906	1.06
	C (0.43)	35	37			
3'UTR	G (0.44)	29	46	3.85	0.064	1.6
	T (0.56)	46	29			

Table 3.65 VMAT2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs2283135	G(0.90)	8	5	0.69	0.581	1.6
	C(0.10)	5	8			
rs363387	G(0.10)	6	8	0.28	0.791	1.33
	T(0.90)	8	6			
rs363220	T(0.82)	21	20	0.02	1	1.05
	C(0.18)	20	21			
rs4752045	G(0.62)	29	30	0.02	1	1.03
	C(0.38)	30	29			
rs363226	C(0.71)	33	24	1.42	0.289	1.37
	G(0.29)	24	33			
rs363279	T(0.89)	16	10	1.38	0.327	1.6
	C(0.11)	10	16			

Table 3.66 5-HT1B

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs1228814	**A(0.44)**	**31**	**51**	**4.88**	**0.035**	**1.65**
	C(0.56)	**51**	**31**			
T-261G (rs1157585)	**T(0.58)**	**50**	**31**	**4.56**	**0.048**	**1.6**
	G(0.42)	**31**	**50**			
A-161T (rs130058)	A(0.71)	39	29	1.47	0.275	1.34
	T(0.29)	29	39			
G861C (rs6296)	G(0.71)	30	43	2.31	0.16	1.43
	C(0.29)	43	30			
rs6297	A(0.80)	30	22	1.23	0.332	1.36
	G(0.20)	22	30			

Table 3.67 5-HT2A

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs977003	T(0.54)	30	28	0.07	0.896	1.07
	G(0.46)	28	30			
rs1745837	**T(0.70)**	**28**	**14**	**4.67**	**0.0436**	**2**
	C(0.30)	**14**	**28**			
rs9316233	C(0.83)	18	23	0.61	0.53	1.28
	G(0.17)	23	18			
rs2770296	T(0.68)	37	22	3.81	0.067	1.68
	C(0.32)	22	37			
rs1928040	T(0.48)	29	22	0.96	0.401	1.32
	C(0.52)	22	29			
rs927544	**T(0.66)**	**44**	**25**	**5.23**	**0.0139**	**1.76**
	C(0.34)	**25**	**44**			

Table 3.68 MAO-A

Marker	Allele	T	NT	χ^2/LRS	p	OR
30 bp VNTR	3	18	24	0.86	0.441	1.33
	3a, 4, 5	24	18			
CA(n)	1	4	4	6.75 df = 7	0.455	-
	2	1	0			
	3	14	11			
	4	2	7			
	5	3	2			
	6	7	13			
	7	23	18			
	8	3	4			
941G/T	T	14	22	1.78	0.243	1.57
	G	22	14			
rs979605	A	19	14	0.76	0.487	1.36
	G	14	19			

Table 3.69 MAO-B

Marker	Allele	T	NT	χ^2/LRS	p	OR
rs1799836	T	21	23	0.09	0.88	1.09
	C	23	21			
CA(n)	1	0	0	6.53 df = 7	0.479	-
	2	2	4			
	3	19	13			
	4	6	12			
	5	14	13			
	6	8	7			
	7	2	3			
	8	1	0			
	9	0	0			
	10	1	1			
	11	0	0			
	12	0	0			

Significant ($p<.05$) transmissions are highlighted in dark orange and bold print. Trends towards significance ($0.094<p>0.05$) are highlighted in light orange.

3.5.9.9. TDT results for transmissions to probands with comorbid Conduct Disorder (n = 30)

Table 3.70 TPH1

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4537731	G (0.55)	12	4	4	0.077	3
	A (0.45)	4	12			
rs1800532	A (0.45)	7	10	0.53	0.629	1.43
	C (0.55)	10	7			
rs10488683	A (0.56)	12	16	0.57	0.572	1.33
	G (0.44)	16	12			
rs211102	G (0.83)	7	5	0.33	0.77	1.4
	A (0.17)	5	7			

Table 3.71 TPH2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4570625	G (0.82)	7	8	0.067	1	1.14
	T (0.18)	8	7			
rs10748186	C (0.53)	14	11	0.36	0.69	1.27
	T (0.47)	11	14			
rs2129575	G (0.78)	10	8	0.22	0.82	1.25
	T (0.22)	8	10			
rs1386488	A (0.81)	9	4	1.92	0.27	2.25
	C (0.19)	4	9			
rs10879345	C (0.45)	14	12	0.15	0.84	1.16
	T (0.55)	12	14			
rs1843809	A (0.85)	7	6	0.08	1	1.17
	C (0.15)	6	7			
rs1386496	T (0.80)	10	7	0.53	0.629	1.43
	C (0.20)	7	10			
rs1386494	G (0.82)	9	6	0.6	0.607	1.5
	A (0.18)	6	9			
rs1386493	C (0.78)	7	9	0.25	0.804	1.28
	T (0.22)	9	7			
rs1023990	C (0.25)	7	13	1.8	0.263	1.86
	T (0.75)	13	7			
rs1386497	A (0.73)	10	9	0.05	1	1.11
	C (0.27)	9	10			
rs1487278	C (0.26)	8	9	0.06	1	1.12
	T (0.74)	9	8			

rs1487275	G (0.29)	7	8	0.06	1	1.14
	T (0.71)	8	7			
rs1487279	A (0.70)	13	12	0.04	1	1.08
	T (0.30)	12	13			

Table 3.72 5-HTT

Marker	Allele/Freq	T	NT	χ^2	p	OR
Promoter	Long (0.54)	18	13	0.81	0.473	1.38
	Short (0.46)	13	18			
VNTR	12 Repeat (0.55)	23	12	3.46	0.089	1.92
	10 Repeat (0.45)	12	23			
rs1872924	T (0.81)	6	9	0.6	0.607	1.5
	C (0.19)	9	6			
rs4325622	T (0.57)	16	8	2.67	0.152	2
	C (0.43)	8	16			
3'UTR	G (0.44)	13	11	0.17	0.839	1.18
	T (0.56)	11	13			

Table 3.73 VMAT2

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs2283135	G(0.90)	1	4	1.8	0.375	4
	C(0.10)	4	1			
rs363387	G(0.10)	4	1	1.8	0.375	4
	T(0.90)	1	4			
rs363220	T(0.82)	5	8	0.69	0.581	1.6
	C(0.18)	8	5			
rs4752045	G(0.62)	16	8	2.67	0.152	2
	C(0.38)	8	16			
rs363226	C(0.71)	10	9	0.05	1	1.11
	G(0.29)	9	10			
rs363279	T(0.89)	3	5	0.5	0.727	1.67
	C(0.11)	5	3			

Table 3.74 5-HT1B

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs1228814	A(0.44)	15	22	1.32	0.324	1.47
	C(0.56)	22	15			
T-261G (rs1157585)	T(0.58)	17	16	0.03	1	1.06
	G(0.42)	16	17			
A-161T (rs130058)	A(0.71)	16	12	0.57	0.572	1.33
	T(0.29)	12	16			
G861C (rs6296)	G(0.71)	13	8	1.19	0.383	1.63
	C(0.29)	8	13			
rs6297	A(0.80)	12	11	0.04	1	1.1
	(0.20)	11	12			

Table 3.75 5-HT2A

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs977003	T(0.54)	14	17	0.29	0.72	1.21
	G(0.46)	17	14			
rs1745837	T(0.70)	9	8	0.05	1	1.12
	C(0.30)	8	9			
rs9316233	C(0.83)	6	6	0	1	1
	G(0.17)	6	6			
rs2770296	T(0.68)	11	10	0.04	1	1.1
	C(0.32)	10	11			
rs1928040	T(0.48)	10	9	0.05	1	1.11
	C(0.52)	9	10			
rs927544	T(0.66)	10	8	0.22	0.815	1.25
	C(0.34)	8	10			

Table 3.76 MAO-A

Marker	Allele	T	NT	χ^2/LRS	p	OR
30 bp VNTR	3	9	8	0.05	1	1.13
	3a, 4, 5	8	9			
CA(n)	1	1	3	7.19 df = 6	0.303	-
	2	1	0			
	3	5	0			
	4	0	0			
	5	0	2			
	6	1	1			
	7	5	6			
	8	1	2			
941G/T	T	5	11	2.25	0.21	2.2
	G	11	5			
rs979605	A	11	4	3.27	0.118	2.75
	G	4	11			

Table 3.77 MAO-B

Marker	Allele	T	NT	χ^2/LRS	p	OR
rs1799836	T	12	11	0.04	1	1.09
	C	11	12			
CA(n)	1	0	0	11.25 df = 5	0.046	-
	2	3	1			
	3	3	3			
	4	10	2			
	5	3	10			
	6	0	3			
	7	1	1			
	8	0	0			
	9	0	0			
	10	0	0			
	11	0	0			
	12	0	0			

Trends towards significance ($0.094 < p > 0.055$) are highlighted in light orange.

3.5.10 Linkage Disequilibrium measures

Red blocks indicate strong linkage disequilibrium. D' values are included except where there is complete LD (D'=1), then the block is empty. Lighter colours (white-pink) indicate weak LD. Red blocks indicate strong LD. Black lines mark the boundaries for LD blocks which were defined by the "solid spine of LD" analysis available in Haploview. Linkage disequilibrium measurement used throughout this thesis is expressed as D' which ranges between 0-1. The D' values determined using the program Haploview. D' values are included except where there is complete LD (D'=1), then the block is empty.

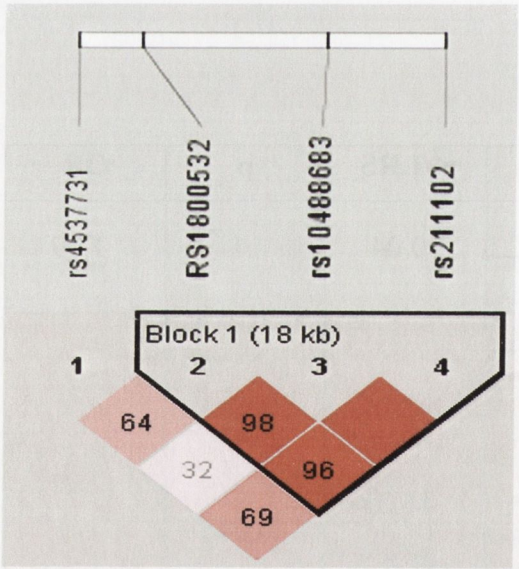


Figure 3.3 LD measures of the TPH1 gene

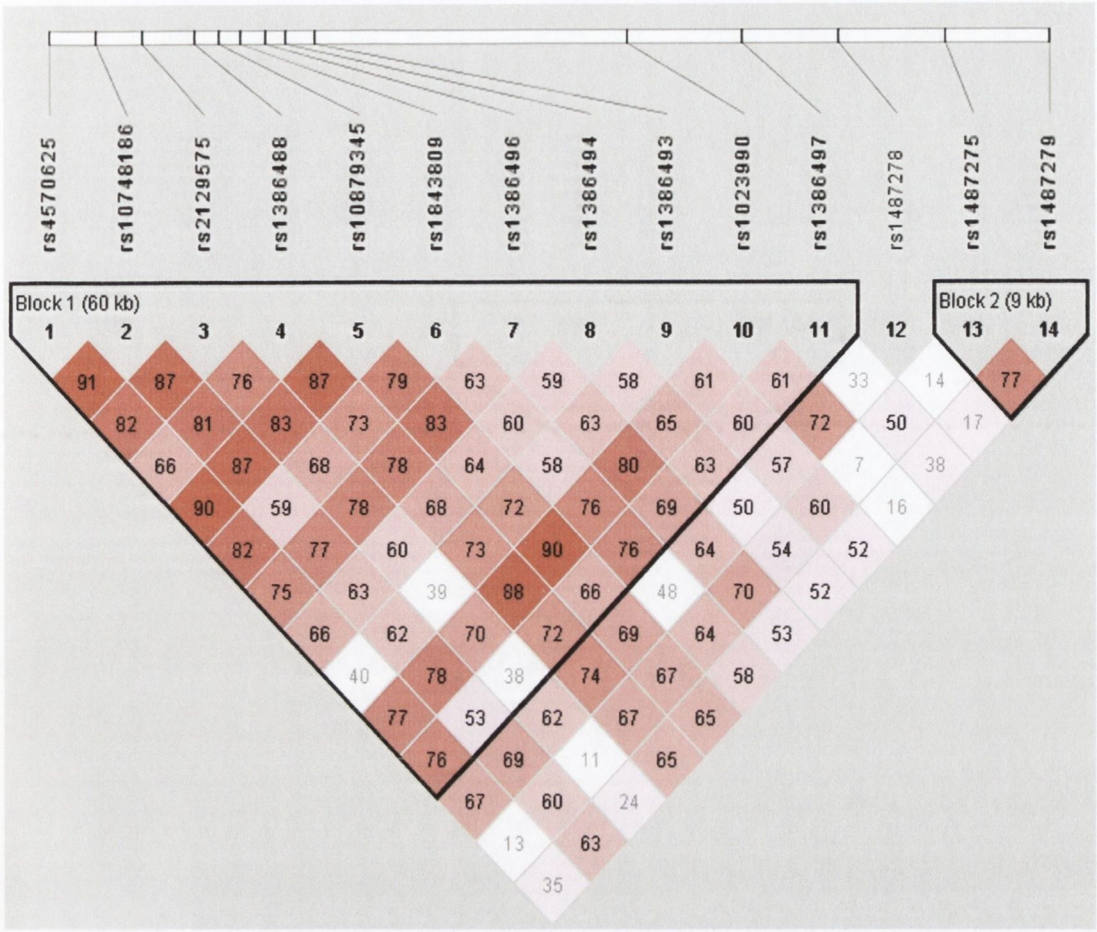


Figure 3.4 **LD measures of the TPH2 gene**

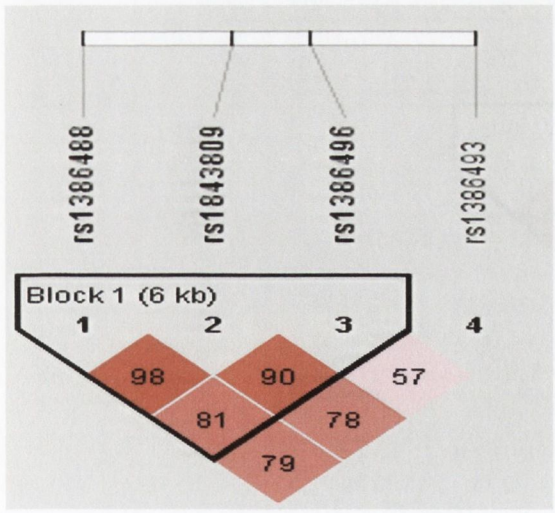


Figure 3.5 **LD measures of the TPH2 gene in a sample of ADHD probands from the United Kingdom**

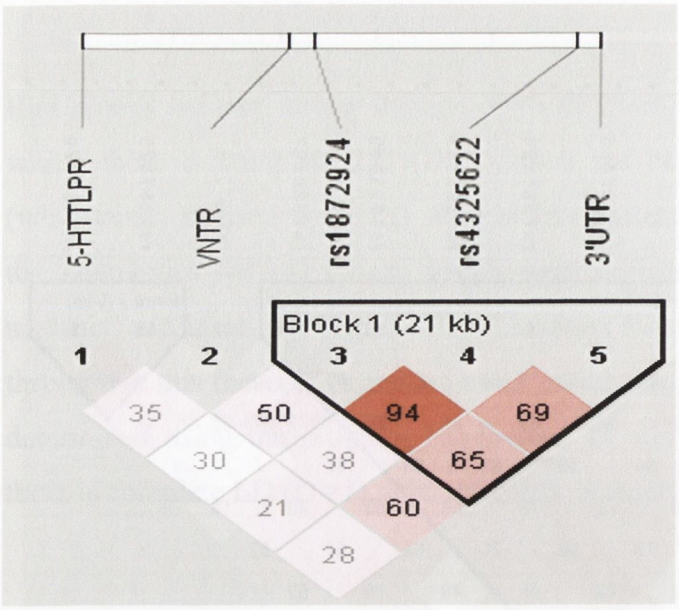


Figure 3.6 LD measures of the 5-HTT gene

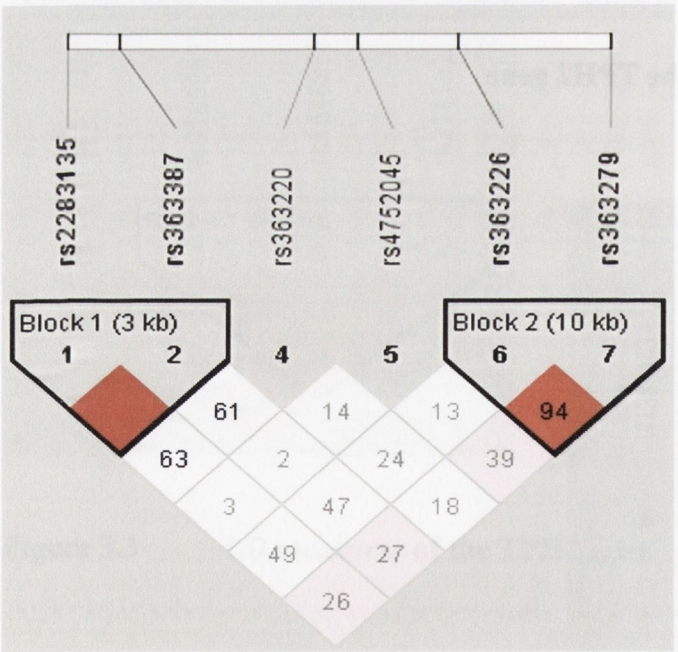


Figure 3.7 LD measures of the VMAT2 gene

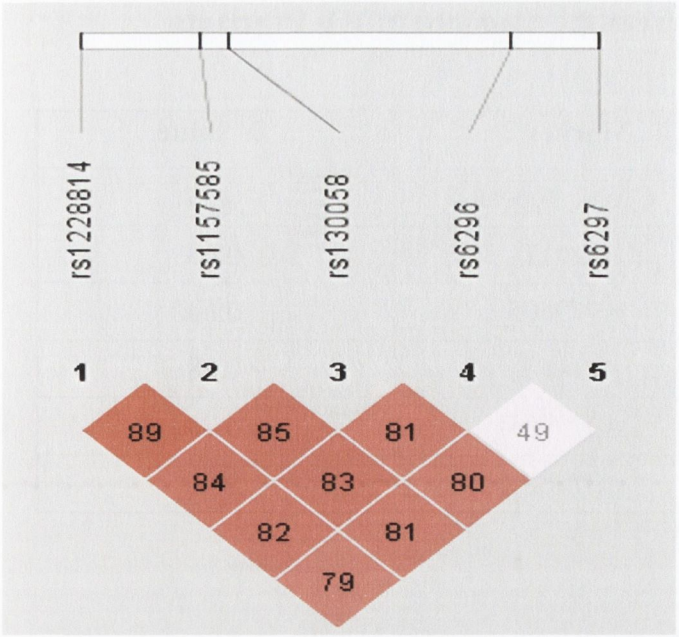


Figure 3.8 LD measures of the 5-HT1B gene

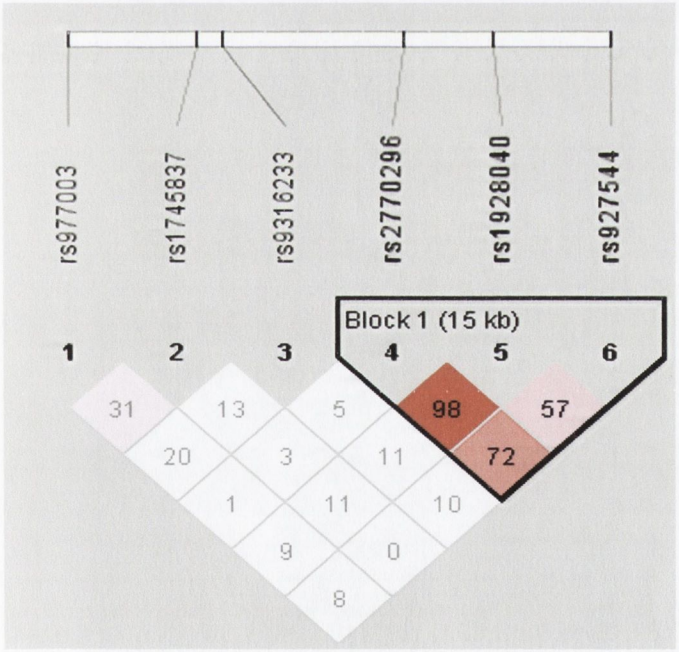


Figure 3.9 LD measures of the 5HT2A gene

Table 3.78 LD analysis between the examined MAO-A variants

Marker 1	Marker 2	D' value
30 bp VNTR	CA(n) Repeat	0.73
30 bp VNTR	G941T	0.63
30 bp VNTR	rs979605	0.64
CA(n) Repeat	G941T	0.68
CA(n) Repeat	rs979605	0.75
G941T	rs979605	0.91

Table 3.79 LD analysis between the two examined MAO-B variants

Marker 1	Marker 2	D' value
rs1799836	CA(n) Repeat	0.28

3.5.11 Transmission of Haplotypes

Table 3.80 Selected haplotype analyses of TPH1 gene

Markers	Alleles	Expected	Observed	χ^2	p value	Global χ^2	Global p value
rs4537731-rs1800532	A-A	91.22	102.43	4.79	0.0286	6.105 (3df)	0.1066
rs1800532-rs10488683	A-A	126.71	134.99	1.91	0.167	2.25 (3df)	0.5222
rs10488683-rs211102	A-G	192.18	193.89	0.07	0.7913	0.195 (2df)	0.9071
rs4537731-rs1800532- rs10488683	A-A-A	95.01	105.3	3.87	0.0492	5.506 (6df)	0.4807
rs1800532-rs10488683-rs211102	A-A-G	127.73	136.08	1.95	0.1626	2.316 (4df)	0.6779
All Four Markers	A-C-A-G	92.95	102.95	3.76	0.0525	10.32 (8df)	0.2433

Table 3.81 Selected haplotype analyses of the TPH2 gene

Markers	Alleles	Expected	Observed	χ^2	p value	Global χ^2	Global p value
rs10879345-rs1843809	T-A	134.35	119.61	6.72	0.0095	16.92 (3df)	0.0007
rs1843809-rs1386496	A-T	284.21	269.64	8.37	0.0038	13.15 (3df)	0.0043
rs1386496-rs1386494	T-G	277.63	261.8	8.84	0.0029	12.74 (3df)	0.0052
rs1386488-rs10879345-rs1843809	A-T-A	105.43	93.467	5.19	0.0227	19.26 (7df)	0.0074
rs10879345-rs1843809-rs1386496	T-A-T	102.98	92.539	3.87	0.0492	22.28 (7df)	0.0023
rs1843809-rs1386496-rs1386494	**A-T-G**	**271.81**	**250.8**	**16.86**	**0.0000**	**24.96 (7df)**	**0.0008**
rs1386488-rs10879345-rs1843809-rs1386496	A-T-A-T	97.62	86.45	4.77	0.0290	25.36 (13df)	0.0207
rs10879345-rs1843809-rs1386496-rs1386494	T-A-T-G	99.27	87.603	5.23	0.0222	36.103 (13df)	0.0006

rs1843809-rs1386496-rs1386494-rs1386493	A-T-G-C	252.24	232.28	13.13	0.0003	32.925 (13df)	0.0017
rs10879345-rs1843809- rs1386496-rs1386494-rs1386493	T-A-T-G-C	89.82	77.29	6.4	0.0114	47.16 (22df)	0.0014
rs1386488-rs10879345-rs1843809-rs1386496-rs1386494- rs1386493	A-T-A-T-G-C	88.75	76.62	6.09	0.0136	61.41 (36df)	0.0052
rs10879345-rs1843809-rs1386496-rs1386494-rs1386493- rs1023990	T-A-T-G-C-C	79.66	68.25	5.56	0.0184	64.58 (36df)	0.0024
rs1843809-rs1386496-rs1386494-rs1386493-rs1023990- rs1386497	A-T-G-C-T-A	161.82	143.34	9.54	0.0020	*	*
rs10879345-rs1843809-rs1386496-rs1386494	T-A-T-G	99.27	87.603	5.23	0.0222	36.103 (13df)	0.0006
rs1843809-rs1386496-rs1386494-rs1386493	A-T-G-C	252.24	232.28	13.13	0.0003	32.925 (13df)	0.0017
rs10879345-rs1843809- rs1386496-rs1386494-rs1386493	T-A-T-G-C	89.82	77.29	6.4	0.0114	47.16 (22df)	0.0014
rs1386496-rs1386494-rs1386493-rs1023990-rs1386497	T-G-C-T-A	163.87	149.18	5.76	0.0164	45.97 (25df)	0.0065
rs1386488-rs10879345-rs1843809-rs1386496-rs1386494- rs1386493	A-T-A-T-G-C	88.75	76.62	6.09	0.0136	61.41 (36df)	0.0052
rs1843809-rs1386496-rs1386494-rs1386493-rs1023990- rs1386497	A-T-G-C-T-A	161.82	143.34	9.54	0.0020	*	*
rs10879345-rs1843809-rs1386496-rs1386494-rs1386493-rs023990-rs1386497	T-A-T-G-C-T-A	155.72	138.52	8.376	0.0038	86.62 (53df)	0.0024

* indicates that the global test could not be calculated.

Table 3.82 Selected haplotype analyses of the 5-HTT gene

Markers	Alleles	Observed	Expected	χ^2	p value	Global χ^2	Global p value
5-HTTLPR-VNTR	Short-12R	117.36	110.12	1.545	0.2139	1.75 (3df)	0.6259
VNTR-rs1872924	10R-T	129.24	133.87	0.69	0.4062	1.25 (3df)	0.7410
rs1872924-rs4325622	T-T	118.94	114.38	0.724	0.3948	1.9 (3df)	0.5934
rs4325622-3'UTR	T-T	163.93	156.33	1.495	0.2214	2.81 (3df)	0.4219
5-HTTLPR-VNTR- rs1872924	Short-12R-T	94.537	91.357	0.363	0.5468	3.24 (7df)	0.8619
VNTR- rs1872924- rs4325622	12R-T-T	100.7	98.941	0.109	0.7413	2.54 (6df)	0.8640
rs1872924- rs4325622-3'UTR	T-T-T	99.69	98.03	0.095	0.7579	5.19 (6df)	0.5197
5-HTTLPR-VNTR- rs1872924-rs4325622	Short-12R-T-T	20.325	17.056	2.031	0.1541	*	*
VNTR- rs1872924- rs4325622-3'UTR	12R-T-T-T	13.817	13.32	0.048	0.8266	12.04 (12df)	0.4425
All five markers	Short-12R-T-T-T	31.46	32	0.023	0.8795	*	*

Short = Short promoter variant, 12R = 12 repeat variant of intron 2 VNTR, 10R = 10 Repeat variant of intron 2 VNTR.
* indicates that the global test could not be calculated

Table 3.83 Haplotype analyses of the VMAT2 gene

Markers	Alleles	Observed	Expected	χ^2	p value	Global χ^2	Global p value
rs2283135-rs363387	G-G	322	320.13	0.34	0.559	0.34 (1df)	0.5598
rs363387-rs363220	G-C	64.75	59.30	1.45	0.229	2.13 (3df)	0.5465
rs363220-rs4552045	C-G	37.09	29.73	4.68	0.030	5.06 (3df)	0.1675
rs4552045-rs363226	G-G	165.83	158.12	1.76	0.185	3.05 (3df)	0.3843
rs363226-rs363279	G-T	209.17	203.02	1.06	0.303	2.84 (2df)	0.2421
rs2283135-rs363387-rs363220	G-G-C	65.27	59.87	1.43	0.232	1.95 (3df)	0.5839
rs363387-rs363220-rs4552045	G-C-G	36.98	30.45	3.91	0.048	5.14 (6df)	0.5255
rs363220- rs4552045-rs363226	C-G-C	32.33	26.12	3.9	0.048	8.39 (7df)	0.2995
rs4552045-rs363226-rs363279	G-C-T	151.56	145.34	1.14	0.286	3.05 (5df)	0.6923
All Six Markers	G-G-C-G-G-T	28.94	23.42	3.59	0.058	*	*

* indicates that the global test could not be calculated

Table 3.84 Haplotype analyses of the 5-HT1B gene

Markers	Alleles	Observed	Expected	χ^2	p value	Global χ^2	Global p value
rs1228814-rs1157585	A-T	202.96	193.11	2.12	0.1454	6.32 (3df)	0.097
rs1157585-rs130058	T-A	207.69	199.5	1.5	0.2207	2.71 (3df)	0.4385
rs130058-rs6296	A-G	166.24	159.12	1.07	0.3009	2.03 (3df)	0.5662
rs6296-rs6297	G-A	196.14	193.77	0.126	0.7226	9.33 (3df)	0.0252
rs1228814-rs1157585- rs130058	A-T-A	198.54	186.65	3.09	0.0788	8.52 (7df)	0.2887
rs1157585- rs130058- rs6296	T-A-G	114.18	106.36	1.64	0.2003	5.26 (7df)	0.6288
rs130058- rs6296- rs6297	A-G-A	108.71	105.44	0.284	0.5941	*	*
rs1228814-rs1157585- rs130058-rs6296	A-T-A-G	108.48	99.92	2.05	0.1522	13.05 (14df)	0.5228
rs1157585- rs130058- rs6296-rs6297	T-A-G-A	58.11	54.96	0.446	0.5042	19.42 (12df)	0.079
All Five Markers	A-T-A-G-A	54.44	51.12	0.507	0.4764	27.06 (23df)	0.2534

* indicates that the global test could not be calculated

Table 3.85 Haplotype analyses of the 5-HT2A gene

Markers	Alleles	Observed	Expected	χ^2	p value	Global χ^2	Global p value
rs977003-rs1745837	G-T	123.56	122.16	0.08	0.7773	0.54 (3df)	0.9102
rs1745837-rs9316233	T-C	198.43	204.85	1.16	0.2815	2.43 (3df)	0.4884
rs9316233-rs2770296	C-T	190.98	184.58	1.03	0.3102	5.82 (3df)	0.1207
rs2770296-rs1928040	T-C	152.95	144.94	1.66	0.1976	4.24 (3df)	0.237
rs1928040-rs927544	C-T	135.75	125.06	3.23	0.0723	7.63 (3df)	0.0542
rs977003-rs1745837- rs9316233	G-T-C	114.23	111.81	0.177	0.674	10.42 (7df)	0.1662
rs1745837- rs9316233- rs2770296	T-C-T	146.66	140.84	0.911	0.3398	8.14 (7df)	0.3201
rs9316233-rs2770296- rs1928040	C-T-C	132.32	126.93	0.763	0.3824	7.06 (6df)	0.315
rs2770296-rs1928040- rs927544	T-C-T	135.66	126.47	2.27	0.1319	11.99 (6df)	0.0623
rs977003-rs1745837- rs9316233-rs2770296	G-T-C-T	81.22	73.92	2.18	0.1394	19.47 (15df)	0.1934
rs1745837- rs9316233- rs2770296--rs1928040	T-C-T-C	104.49	98.62	1.05	0.3055	10.29 (12df)	0.5899
rs9316233- rs2770296-- rs1928040-rs927544	C-T-C-T	124.04	114.78	2.28	0.1311	15.86 (11df)	0.146
All Six Markers	G-T-C-T-C-T	56.16	51.24	1.31	0.2524	41.91 (36df)	0.2298

Table 3.86 Selected haplotype analysis of the MAO-A gene

Markers	Alleles	Transmitted	Not transmitted	Famhap global statistic	p value
VNTR-CA(n)	3 allele-6 Repeat	24	11	24.86	0.05
VNTR-CA(n)- G941T	3allele-6Repeat-G	14	5	34.54	0.01
All four markers	3 allele-6 Repeat-G-A	12	10	18.33	0.174

Table 3.87 Haplotype analysis of the MAO-B gene

Markers	Alleles	Transmitted	Not transmitted	Famhap global statistic	p value
rs1799836-Ca(n)	T-3Repeat	15	14	13	0.511

For all tables above: Haplotype p-values represent the p-value for the transmission of indicated variants of the haplotype. The global p-value represents the overall significance for the haplotype using all possible variants. All haplotypes presented have a frequency >5%. Significant ($p<.05$) transmissions are highlighted in dark orange. Trends towards significance ($0.094<p>0.055$) are highlighted in light orange.

LRS = Likelihood Ratio Score (LRS) value, which is equivalent to the Extended TDT (ETDT) chi square of Sham and Curtis.

df = degrees of freedom.

3.6. Mutation Screening of Exons 4 and 5 of the TPH2 gene

TDT and haplotype analysis highlighted the over-transmission of allele A of marker rs1843809 of the TPH2 gene. This marker lies between exons 4 and 5 and at the time of undertaking this work no SNPs or other variants had been reported in these exons. It was therefore decided to screen these exons by sequencing fifteen probands homozygous for the A allele, 15 heterozygous probands and the two probands that were homozygous for the C allele of the marker. Samples were prepared (chapter 2, section 2.2.5) and sent to MWG Biotech for sequencing of both the forward and reverse strands. Sequences were viewed and compared using the CodonCode Aligner program (www.codoncode.com/aligner/download.htm).

3.6.1. Results

No novel mutations were detected in either exon 4 or 5. Due to the low frequency of probands homozygous for the C allele it is possible that there may be a rare mutation(s) in these exons but were not discovered in this analyses. Further mutation screening in other population samples is recommended before these exons are discounted.

3.7. Discussion

The purpose of this study was to identify and map the extent of association between ADHD and eight serotonergic candidate genes. These genes were chosen because of their direct control over the levels of available serotonin in the synapses as serotonin concentration is thought to be lower in the brains of children with ADHD. TPH2 is the rate-limiting enzyme in serotonin biosynthesis in the brain, 5-HTT, and VMAT2 control the amount of serotonin in the synaptic cleft, serotonin receptors 5-HT1B, and 5-HT2A are involved in receiving serotonin signals and modulating the release of serotonin, MAO-A and B are involved in the degradation of serotonin.

Frontal cortex serotonin has been shown to play a significant role in the modulation of attention and response control (Ruotsalainen et al, 1997). Developmental faults in these areas may lead to ADHD symptoms such as lack of inhibition and self-control. In addition, the right prefrontal cortex has been shown to be smaller in children with ADHD (Castellanos et al, 1996). Animal model studies show important evidence for the involvement of serotonin in ADHD behaviours, for example the 5-HT1B knockout mouse model. This mouse model displays an increased locomotor response to cocaine acquisition and alcohol intake, as well as hyperactive and aggressive behaviour (Brunner et al, 1997). The hyperlocomotive effect of the 5-HT1B agonist RU24969 was found to be absent in mice lacking the receptor. Biochemical, pharmacological and animal model studies have significantly contributed to understanding the aetiology of ADHD. However, identifying underlying genetic variation that contributes risk would enable functional studies to focus on individual proteins or biological systems.

3.7.1. TDT Analysis of Candidate Genes

3.7.1.1 TPH2

Analysis of 14 TPH2 markers showed a very strong association between ADHD and marker rs1843809 ($p = 0.0003$, OR = 2.45) and a marginal association with marker rs1386497 ($p = 0.048$, OR = 1.45). A trend toward association was also seen with markers rs1386494 and rs1386493. A haplotype made up of the markers rs1843809, rs1386496 and rs1386494 is highly significantly associated with the disorder ($p = 0.00000$, Global $p = 0.0008$) indicating the importance of this region and the surrounding sequences in the aetiology of ADHD.

A highly significant association between the C allele of rs1843809 and ADHD was reported in Phase I of the IMAGE sample (Brookes et al, 2006). However, this finding is with the opposite allele to what is reported in this thesis. Surprisingly, the initial analysis of the unpublished Phase II IMAGE data (Dr. Richard Anney, personal communication) is reported as showing association with the A allele which, if true, confirms the original findings in the Irish sample reported in this thesis. Zill et al (2004) reported association between TPH2 polymorphisms and major depression (MD). Interestingly, they observed that the rs1843809 A allele, which is most significantly associated with ADHD in the Irish sample, is also the allele associated with MD. In addition, the second TPH2 variant associated with MD, rs1386494, is located in intron 5 of the gene which is covered by the haplotype associated with ADHD. An increase in transmission of allele G of marker rs1386494 to ADHD probands was also observed in this thesis, though this did not reach significance.

The TPH2 markers that were observed to be associated with ADHD in the Irish sample map to intron 5 of the gene. It is unlikely therefore that these markers would have a profound effect on the gene expression or protein structure as introns are spliced out during mRNA processing. Having said this, a recent study suggests the potential effect of intronic sequences on gene expression (Kleinjan and van Heyningen, 2005). The associated intronic variant (rs1843809) may be in linkage disequilibrium with another variant that predisposes to ADHD. This presumptive

causative variant would likely be located in the control or the coding regions of the gene. TPH2 is a member of an aromatic amino acid hydroxylase family that includes phenylalanine hydroxylase (PH), and tyrosine hydroxylase (TH). These enzymes are composed of an amino-terminal regulatory domain and a carboxyl-terminal catalytic domain. The carboxyl-terminal of these enzymes is predicted to form two thirds of the protein. A conserved five amino acid (aa) domain (Val, Pro, Trp, Phe, Pro) is present in all these enzymes, which serves to delineate the boundaries between the regulatory and catalytic domains. DNA and amino acid sequences map this motif to aa 105-109 and aa 151-156 for TPH1 and TPH2 respectively. Deletion of the first 106 amino acids in TPH1 had no effect on enzyme activity but deletion of the first 116 amino acids (including those between 105-109) was found to abolish the enzyme activity demonstrating the importance of the region between residues 106 and 116 for TPH1 function (Kumer et al, 1997). It is plausible that DNA modification at the corresponding region in TPH2 (including aa 151-156) may influence the activity of the TPH2 enzyme consequently affecting the level of serotonin synthesis. Low levels of serotonin are thought to be correlated with ADHD. DNA sequence analysis shows that this motif maps to exon 4 of the TPH2 gene (Veenstra-Vander Weele et al, 2003).

The TPH2 markers associated with ADHD are intronic (mapped to intron 5) and found to be within a haplotype block encompassing exons 4 and 5 of the TPH2 gene. This could suggest that DNA variations within these exons and/or their boundaries may have profound effect on the function of TPH2. However, sequence analysis of the entire exons 4 and 5 and their boundaries in 32 ADHD probands revealed no evidence any DNA abnormalities in this region at least in this sample.

The carboxyl-terminal catalytic domain is also essential for protein function; a recent study (Zhang et al, 2004) demonstrated that the C1473G substitution in mice at the Tph2 gene results in Pro447Arg change. This resulted in a 50% and 70% reduction in the levels of 5-HT synthesis in the frontal cortex and striatum respectively for the mice homozygous for the 1473G allele (Arg^{447}) compared to mice homozygous for the 1473C allele (Pro^{447}). This further emphasizes the importance of the TPH2 gene in the aetiology of ADHD and suggests the significance of further fine mapping toward the 3' end of the gene which was not densely mapped in this investigation.

3.7.1.2 5-HTT

The serotonin transporter gene (5-HTT) is the most extensively analysed serotonergic candidate gene in relation to the aetiology of ADHD. However reported results have so far been conflicting. It was reported that the long allele of the promoter variant (L/L) 5-HTTLPR is more active than the short (S/S) and will therefore remove serotonin from the synapse at a higher rate. This makes 5-HTTLPR an excellent candidate for hyposerotonergic hypothesis of ADHD. Neither the long nor short variant of 5-HTTLPR, however, was found to be associated with ADHD in this sample (Table 3.4: $\chi^2 = 0.21$, $p = 0.703$, OR = 1.07). This is in keeping with results from several other groups (Chapter 1, Table 1.2). Although the results are controversial 5-HTT should not be discarded as a potential candidate gene for ADHD.

3.7.1.3 5-HT2A

TDT analysis was carried out on six markers covering the 5-HT2A gene. A significant association was found with marker rs927544 and ADHD. A pooled analysis by Hawi et al (2002), including the Irish sample used in this thesis, found no overall association between the 5-HT2A marker His452Tyr and ADHD. However, association was observed between the 452His allele and ADHD in the Irish sample alone. Guimaraes et al (2007) also found association with this allele but found that preferential transmission was to boys and not to girls. The A allele of the 5-HT2A marker -1438A/G has been linked to functional remission in adolescent boys with ADHD (Li et al, 2006). This marker is in strong LD with marker rs927544 which was observed to be associated with ADHD probands in this sample. If this association proves true through replication work future research on this gene may examine the area of remission of ADHD in adolescents in large samples from different populations.

3.7.1.4. 5-HT1B

The results of the 5-HT1B gene association analysis are different to those reported by Hawi et al (2002) which analysed a subset of the sample used in this thesis. Subsequent to the analysis of 5-HT1B undertaken in this thesis the original sample size was increased; re-diagnosed using CAPA and individuals no longer willing to participate in the research were removed. This has resulted in a drop in the significance level of the association with the G861C marker of 5-HT1B. As the sample used in this thesis is more stringently diagnosed than the original and has been extended with the addition of 98 new families, it is likely that these results are more accurate than those reported for the Irish sample by Hawi et al (2002). The original reported association may have been a false positive finding or represent a slightly different clinical phenotype for which the G861 variant was associated. For example markers rs1228814 and rs1157585 of 5-HT1B both show overtransmission of alleles to ADHD probands with co-morbid ODD which could be classified as a different clinical phenotype to the broad ADHD phenotype.

3.7.1.5. MAO-A

For the MAO-A gene, TDT analysis was conducted on four markers that were either previously analysed for association with ADHD in earlier studies (Jiang et al, 2001; Payton et al, 2001; Manor et al, 2002; Lawson et al, 2003) or have been shown to directly influence enzyme activity (Hotamisligil and Breakefield, 1991). A significant association between ADHD and the more active 941G allele was observed in the current investigation. The increased frequency of the MAO-A 941G allele in ADHD can be explained on the basis that elevated MAO-A activity would result in a higher turnover and therefore decreased levels of serotonin. This fits well with the proposed hyposerotonergic hypothesis of ADHD as peripheral measures of blood serotonin as well as central 5-HT function have been reported to be reduced in children with ADHD (Spivak et al., 1999; Kruesi et al., 1990). Also, there is some evidence that serotonin agonists such as fluoxetine might be effective in the pharmacological treatment of ADHD (Gainetdinov et al., 1999). The higher activity variant of MAO-A (941G) generating a higher serotonin turnover would be likely to exacerbate serotonin deficiency in ADHD and thereby increase the susceptibility to the disorder.

The present study failed to confirm previous positive linkage/association results of ADHD with the longer, higher activity alleles of the MAO-A 30-bp VNTR (Manor et al., 2002) and the MAO-A CA(n) microsatellite (Jiang et al, 2001; Payton et al, 2001). An explanation for these divergent findings could be population-specific genetic heterogeneity. For example a Chinese study reported strong evidence for association of ADHD with the DXS7 marker which is closely linked to the MAO-A gene (Jiang et al., 2000). This finding however could not be confirmed in an Irish study (Lowe et al., 2001). Additionally, applying a TDT design in the investigation of x-chromosomal markers such as MAO-A and MAO-B restricts the use of informative transmissions to heterozygous mothers only. This substantially decreases the statistical power and also increases the risk of false negative results. In this case, as suggested by Manor et al (2002) a case-control-based approach might be of additional value. The findings of the present study indicate that the higher activity variant of the MAO-A gene (941G) might be a risk factor in the development of ADHD. The significant association between MAO-A and ADHD in the Irish sample has recently been supported by the IMAGE data in which a significant association was also reported with MAO-A and ADHD (Brookes et al, 2006).

In keeping with the published literature, no evidence of association between MAO-B and ADHD was observed. This may indicate the absence of a direct role of this gene in the development of ADHD or that the gene may be of a very minor effect which could not be detected in the current investigation.

3.7.2. Parent of origin effect

Paternal over transmission of risk alleles to ADHD children has been reported for several genes including those of serotonergic system (Kent et al, 2005; Hawi et al, 2005 and Goos et al, 2007). In this chapter, preferential paternal over-transmission of alleles from the TPH2 and 5-HT2A genes to ADHD cases was observed (Tables 3.11 and 3.15 respectively). The strongest evidence is with the marker rs1843809 of the TPH2 gene (paternal transmissions: OR = 3.71). This finding could not be

replicated in the UK ADHD sample. However, it is important to emphasize that the UK sample is smaller in size (consisting of 108 families, 59 of which are full trios) and thus has limited power to detect the association reported in the Irish sample.

Although genomic imprinting (a situation where certain genes are expressed in a parent of origin specific manner) may contribute to the paternal over transmission in ADHD, no empirical evidence has yet been reported to support this assumption. DNA and mRNA analyses showed that the 102C allele of the 5-HT2A marker 102T/C (rs6313) is expressed at lower levels than 102T in the peripheral lymphocytes of healthy heterozygous individuals (Fukuda et al, 2006). In addition the group reported mono allelic expression at this marker (Fukuda et al, 2006). Although the marker 102T/C (rs6313) was not examined in this investigation it is in strong LD with marker rs927544, which is significantly associated with ADHD in this sample (Table 3.7), and marker rs2770296 which exhibited a trend toward association with the disorder. This however does not explain whether the association reflects imprinting operating on 5HT2A.

3.7.3. Family history of ADHD

ADHD is a complex polygenic disorder that is the result of multiple genetic risk factors acting in combination with lifestyle and environmental factors. ADHD tends to "run in families", but the inheritance model does not fit simple patterns as seen with Mendelian diseases. Furthermore, sporadic ADHD is not uncommon in the general population where families with no history of ADHD are found to have children with the disorder. It can be argued that sporadic cases are more likely to be due to environmental risk factors, with the familial cases more likely to be due to genetic risk factors. Therefore, analysis of the data was based on parental history of ADHD. Results showed that significant over transmission of alleles are from parents with no history of ADHD. This is particularly true in the case of the most significantly associated markers of TPH2 (rs1843809), 5-HT2A (rs927544) and MAO-A (914 G/T). However, two TPH2 markers (rs107418186 and rs10879345), not associated with ADHD in the total sample, showed association with ADHD when the analyses are conducted by parental history of ADHD. It should be noted that classification of a family history of the disorder was based on a retrospective

questionnaire (WURS) based on the parent's opinions of their own behaviour as children. These types of measures are known to be susceptible to bias and can sometimes result in misdiagnosis (McCann et al, 2000).

It is believed that families with a history of ADHD are more genetically loaded and would therefore be more likely to pass genetic risk factors to their offspring. Nevertheless, this does not seem to be the case in the serotonergic genes found to be associated with ADHD in this sample. This situation can be partially explained on the basis that the sample size is reduced after dividing into family history positive and negative which may result in false positive and negative findings. Alternatively, the issue may be much more complex than a simple pattern of Mendelian inheritance. Research in this area might need to include comparing behavioural measures, such as dimensions of conduct disturbance, learning and academic problems, of children with and without a family history of the disorder to confirm observations that these do vary between these subtypes as reported by August and Stewart (1983) (section 3.4.2).

3.7.4. ADHD subtype

There has been an ongoing debate as to whether the inattentive and combined subtypes of ADHD should be classified as two distinct and unrelated disorders. An in depth review by Milich et al (2001) reported that the two subtypes have no defining features in common and as such would not share a common core deficit. The authors concluded that the inattentive group is a valid disorder but is not a member of the ADHD category. In response to this theory Lahey (2001) commented that the empirical evidence does not, as yet, support or reject the proposal but future research will be important in resolving this issue.

Apart from a few reports (Holmes et al, 2002; Kirley et al, 2004; Lowe et al, 2004) the area of sub-phenotypic analysis has not been extensively reported in ADHD. However, in the current investigation, analyses have been conducted to further explore this research area. Family-based analysis of the four examined TPH1 markers showed no association with ADHD. However, the C allele of the marker A-218C (rs1800532) is significantly associated with the inattentive subtype of

ADHD (χ^2 = 5, p = 0.041, OR = 3). This result needs to be replicated in larger sample as the number of individuals in this sample with the inattentive subtype is small (n = 21).

For the TPH2 gene, only marker rs1843809 retains significant association with the combined type subgroup. In addition, three markers (rs4570625, rs2129575, and rs1487275) that were not associated with the ADHD in the entire sample show a trend towards association with inattentive type ADHD (Table 3.47). It is important to mention that marker rs4570625 has been shown to bias amygdala activity in people that possess the T allele of the marker (either homozygous or heterozygous) (Gutknecht et al, 2006) though the exact role of this SNP is unclear. Plessen et al (2006) examined amygdala morphology in ADHD combined type probands and reported evidence to suggest that there is atypical connectivity between the amygdala and the prefrontal cortex in these individuals. The authors suggest this may contribute to behavioural disinhibition as the connectivity of the prefrontal regions with the amygdala regulates a range of emotional, memory and attentional processes. This would be in keeping with the excess transmission of the G allele of marker rs4570625 to the ADHD inattentive group identified in this study.

Markers from the VMAT2, MAO-A and MAO-B genes were also observed to be associated with the inattentive subtype (Tables 3.49, 3.52 and 3.53 respectively). Markers rs363220 of VMAT2 and the CA repeat of MAO-B showed no association with the entire sample or the combined subtype but showed a significant association with the inattentive subtype (χ^2 = 5.44, p = 0.0391, OR = 8 and LRS = 13.3, df = 6, p = 0.038 respectively). Furthermore, the association observed between the MAO-A marker 941G/T and the total sample (Table 3.8) was greatly enhanced when the inattentive subtype was considered separately (χ^2 = 7.36, p = 0.012, OR = 10). These results are interesting and suggest that without subdivision or refinement of the broad DSM-IV phenotype it is possible that valuable information might be lost. These findings need to be treated cautiously as the number of transmissions is small given the sample size. Further studies, preferably on larger inattentive type samples, are required to examine the ADHD sub-phenotypic relation to the serotonergic system genes.

3.7.5. Comorbid disorders

The absence of, or failure to, replicate molecular genetic association studies between ADHD and good candidate genes can be partially attributed to the fact that ADHD is a clinically and genetically heterogenic disorder. It has been suggested that molecular studies may yield a better success by examining more heritable subtypes of ADHD phenotype. The ADHD phenotype comorbid with conduct problems may represent such a phenotype. It has been reported that the outcome of ADHD will be more severe when the disorder is comorbid with conduct disorder. In addition, ADHD individuals with co-morbid oppositional defiant disorder (ADD-ODD) have been found to be more disturbed than those with ADHD or ODD alone, they have an earlier age of onset and more severe aggressive, antisocial, academic, and social impairment (Carlson et al, 1997). In addition, Kadesjo et al (2003) reported that children with ADHD and ODD had consistently higher rates of ADHD symptoms compared to those with ADHD alone. It is possible that the markers found to be associated with ADHD-ODD may be associated with the symptoms of ADHD more closely related to ODD (i.e. hyperactive-impulsive symptoms). The ODD symptoms may purely be a marker of ADHD severity and thus the strength of a genetic component. Holmes et al (2002) demonstrated that the association of DRD4 VNTR is much stronger and was detected in a smaller sample of ADHD comorbid with conduct problems (ODD and CD). This finding was repeated in a study by Kirley et al (2004) which used the same sample as the one used for this thesis.

In the present study, several markers that are not associated with ADHD exhibited association/trends toward association when the sample was divided into ADHD individuals with co-morbid ODD. This is particularly true in the case of 5HT1B. Analysis of ADHD-ODD probands showed significant associations with markers rs1228814 and T-261G (rs1157585) (Table 3.66: $\chi^2 = 4.88$, $p = 0.035$, OR = 1.65 and $\chi^2 = 4.56$, $p = 0.048$, OR = 1.6 respectively). In addition, similar findings were also observed with the 5-HT2A gene. The T allele of marker rs1745837 was significantly over transmitted to ADHD probands with comorbid ODD (Table 3.67: $\chi^2 = 4.67$, $p = 0.0436$, OR = 2).

Although a smaller number of the current ADHD sample are comorbid for CD (n = 30) a trend toward association the marker rs10488683 of the TPH1 gene was observed (Table 3.70: $\chi 2 = 4$, $p = 0.077$, OR = 3). Similarly, a trend towards association between the 12 repeat allele of the intron 2 VNTR of 5HTT and ADHD probands with co-morbid CD (ADHD-CD) was also detected (Table 3.72: $\chi 2 = 3.46$, $p = 0.089$, OR = 1.92). In addition association was observed between the CA repeat of the MAO-B gene and ADHD-CD individuals (LRS = 11.25, df = 5, $p = 0.046$).

Although these results are preliminary and require independent replication, their importance should not be underestimated. The area of sub-phenotypic analysis has not been extensively reported in relation to ADHD. This is presumably due to the small total sample sizes which become even smaller when sub group analysis is performed. However the results of this thesis suggest that without subdivision or refinement of the broad DSM-IV phenotype, for example ADHD-ODD individuals, it is possible that valuable information will be lost. In addition, variants that are classified as "not associated" with the disorder may in fact be associated with a sub group of the disorder. The small size of the 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 (TPH2 and MAO-A markers) supports the hypothesis that a more refined phenotype may be successful in the determination of true susceptibility variants for ADHD, particularly for those with co-morbid ODD.

3.8. Concluding remark

There is a large and varied range of biological evidence supporting the role of serotonin in the development of ADHD. The biochemical findings of decreased concentration of serotonin in the blood of ADHD children, animal model studies, pharmacological research, and the recent molecular genetic studies (section 3.1) are indicative of a significant role for serotonin in the aetiology of ADHD. Unfortunately the exact role of serotonin in this process is not clear-cut and the mechanism of action has not yet been worked out.

Administration of fluoxetine (a selective serotonin re-uptake inhibitor) was found to attenuate the hyperactive phenotype produced by the knocking out the dopamine transporter gene (DAT-KO) in mice, whereas it had no effect on wild type animals (Gainetdinov et al, 1999). This action was mediated by an increased extracellular concentration of serotonin (5-HT), presumably due to blockade of the serotonin transporter. The serotonin transporter (5-HTT) has been widely investigated at a molecular level for its relation to ADHD. The results however have been ambiguous. While several genetic studies support the association of the more active allele (LL) of the promoter insertion deletion variant, others do not. It is still uncertain to what extent 5-HTT is involved in ADHD and what the exact mechanism is through which this gene contributes to the development of ADHD. The results of the current study do not support the association between 5-HTT and ADHD. However, the hyperactive effect observed in the DAT-KO mice was profoundly reduced when the mice were treated with 5-hydroxytryptophan or with the dietary 5-HT precursor, L-tryptophan. Both these compounds are converted to serotonin via the catalyst TPH2 in the brain. The resulting calming effect may be due to an increase in the production of serotonin after an increase in dietary tryptophan. Consequently it follows that any variant in the TPH2 gene that causes a reduction in production of the TPH2 protein will result in a decrease in serotonin in the brain and the resulting hyperactive phenotype. In support of this, a recent study by Scheuch et al (2007) reported that a polymorphism in the putative promoter region of the gene results in a 22% decrease in TPH2 transcriptional activity in primary serotonergic neurons. The TPH2 gene was found to be significantly

associated with ADHD in this thesis though further analysis is required to unambiguously confirm this association.

Pharmacological evidence has shown that administration of the 5-HT1B agonist, RU24969 in mice results in an increase in locomotion which was absent in the mice that had the receptor knocked out. This indicates that an increase in locomotion is mediated by the 5-HT1B receptor. 5-HT1B knock out mice were also reported to exhibit more aggressive and impulsive behaviour than wild type (Saudou et al, 1994). These findings suggest that 5-HT1B plays an important role in controlling hyperactive and impulsive traits, behaviours which are characteristic of the ADHD phenotype. The findings of this thesis provide support for the involvement of the 5-HT1B receptor in the aetiology of the ADHD-ODD subtype.

Apart from the MAO-A variant G941T, there is no direct evidence to suggest that the serotonergic associated markers analysed in this thesis are themselves functional. It is possible that the associated markers are in LD with, as yet unidentified, functional markers. Replication of these findings by independent samples, preferably from different ethic groups, is essential to firmly implicate these genes in ADHD. Future work should aim to clarify the role of putatively associated markers, such as TPH2 marker rs1843809, in the regulation of protein activity and/or function.

The serotonin pathway is a complicated one and it is possible that the effects of a small number of genes, each of minor effect, together contribute to the hyposerotonemia of ADHD. It is also possible that not all variants are required for the development of ADHD, and that some of these may be population specific. Furthermore, several lines of evidence suggest that interaction with, and balance between dopaminergic and serotonergic neurotransmission is important in the mediation of the symptoms of ADHD. Finally, the role of the serotonin system in the development of ADHD remains an interesting puzzle to be resolved in the field of ADHD.

Chapter 4

Sub-phenotype and quantitative trait analysis of the TPH1, TPH2, and 5-HTT genes

4.1 Sleep disorders in ADHD and the TPH1 gene

Sleep disturbances in children with ADHD have been widely reported and were included in the diagnostic criteria in DSM-III, though they are not included in DSM-IV. Sleep can be affected by environmental, medical, and genetic factors as well as biological influences. Approximately one third of medication-free children with ADHD experience chronic sleep-onset insomnia (initial insomnia) which is defined as a persistent disability to fall asleep at a desired time in the evening. This may intensify daytime mood, behavioural and/or cognitive problems (Sadeh et al, 2003). Sleep-related disorders can impair working memory, sustained attention and working memory which are also characteristics of ADHD. Therefore sleep disorders, in theory, may intensify, or imitate clinical indicators of ADHD in individuals with or without the disorder (van der Heijden et al, 2005).

Thunstrom (2002) reported that severe sleep problems in infancy are associated with subsequent development of ADHD. One in four of the children with severe sleep disorders in infancy later qualified for a diagnosis of ADHD. This, of course, may be cause or effect. Kaplan et al (1987) found that non-medicated children had more difficulty falling asleep, more night time awakenings, and more early morning awakenings. Van der Heijden et al (2007) examined chronic sleep onset insomnia in 105 medication free children aged between 6-12 years old in a double-blind randomized trial. Patients were administered either 3-6 mg of melatonin or a placebo. Sleep onset was advanced by 26.9 ± 47.8 minutes with melatonin but was delayed by 10.5 ± 37.4 minutes with the placebo ($p< 0.0001$). There was also an advance of dim light melatonin onset of 44.4 ± 67.9 minutes with melatonin and a delay of 12.8 ± 60 minutes with the placebo. Dim light melatonin onset (DLMO) is the circadian rhythm clock time at which the endogenous melatonin levels start to rise in the subjective evening and is considered the most reliable phase marker of the biological clock rhythm (Klerman et al, 2002). The same group (Van der Heijden, 2005) reported that medication-free children with ADHD and sleep-onset insomnia have a delayed evening increase in endogenous melatonin levels. Melatonin onset normally precedes sleep onset by approximately two hours. Excretion of melatonin peaks between 1 a.m. and 3 a.m. and then decreases to barely detectable levels from about 9 a.m. onwards (Lack and Wright, 2007).

Melatonin has been disputed as being the causative factor of sleep onset in humans but a study by Scheer et al (2005) has reported that patients with spinal cord injury that interrupts the pathway of melatonin production, resulting in no night-time melatonin production, have a significantly decreased sleep quality when compared to spinal cord injury patients with normal night levels of melatonin. It is therefore plausible to conclude that any interruption in the production of melatonin can affect sleep quality. Serotonin is the precursor for melatonin and is highly synthesised in the pineal gland (Figure 4.1). Tryptophan hydroxylase 1 is the rate-limiting enzyme for serotonin and melatonin synthesis in peripheral tissues including the pineal gland. The pineal gland is considered to be peripheral tissue as it is separated from the brain by the blood-brain barrier. A study by Sugden (2003) reported that Tph1 mRNA was 105-fold more abundant in the rat pineal gland than TPH2. Synthesis of serotonin in the pineal gland is therefore wholly dependent on TPH1. DL-6-Fluorotryptophan (6-FT) is a competitive inhibitor of TPH1 and was reported to increase wakeful time and decrease paradoxical sleep time in rats (Sugden and Fletcher, 1981). TPH1 dysfunction may therefore contribute to sleep disturbances by influencing the level of serotonin synthesis, consequently affecting the production of melatonin. As a result, a decrease in melatonin production will lead to sleep disturbances as mentioned above.

4.1.1 Aim

Here I undertake a hypothesis driven approach to investigate Tryptophan Hydroxylase 1 (TPH1) in relation to sleep disorders in children with ADHD.

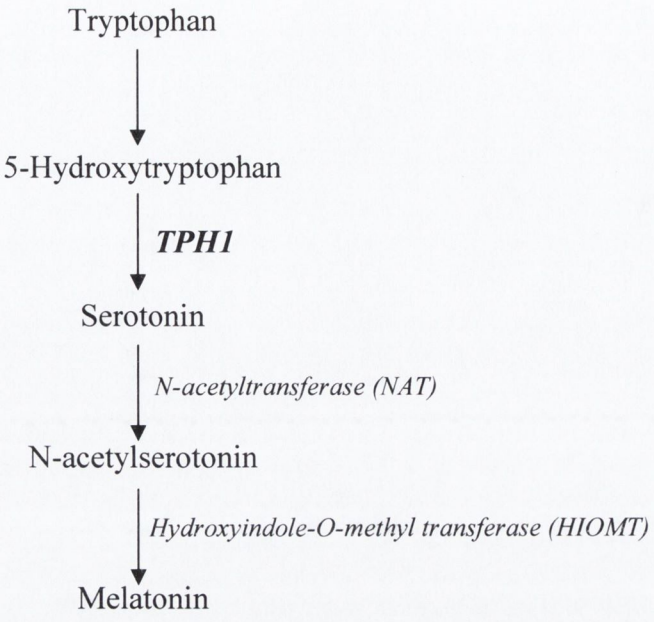


Figure 4.1 The biosynthesis of melatonin (from Panzer, 1998)

4.1.2 Materials and methods

Analysis was performed on families with children with reported sleep disturbance from both the Irish sample used in this thesis (chapter 2, section 2.1.1.) and the STAR sample combined (n = 83). Families in the STAR sample were recruited as part of a naturalistic, prospective study of stimulant response in ADHD (chapter 2, section 2.1.2.). Transmission of alleles from parents to probands with sleep disturbances was tested using the TDT (section 2.3.2.). Haplotype analysis was performed using the program TRANSMIT (chapter 2, section 2.3.5.). All markers were tested for Hardy-Weinberg equilibrium. Sleep disturbance was rated in both samples using the Child and Adolescent Psychiatric Assessment (CAPA). None of the children were taking medication at the time of assessment as stimulant medication can cause sleep problems in affected children. ADHD probands that were classified as having a sleep disorder were, for the majority, further classified as having initial insomnia (63%), middle insomnia (32%), or early morning wakening (4%). Initial insomnia is characterised by long sleep-onset latency (SOL) and is a serious problem as it can lead to exhaustion in parents, morning sleepiness in children and poor tolerance of stimulant medication (Weiss et al, 2006).

4.1.3 Results

4.1.3.1 TDT Testing

The observed genotype frequencies did not differ from those expected according to Hardy-Weinberg equilibrium for all examined markers. TDT analysis showed no association between any of the four markers and sleep disorders in ADHD (Table 4.1). Marker rs4537731 proved difficult to genotype for both samples. Two attempts were made on the STAR sample but both failed. It was therefore decided to forego genotyping of this marker for the STAR sample.

Table 4.1 TDT results for TPH1 markers (n = 83)

Marker	Allele/Freq	T	NT	χ^2	p	OR
rs4537731	G (0.55)	17	13	0.3	0.584	1.31
	A (0.45)	13	17			
rs1800532	A (0.45)	23	24	0	1	1
	C (0.55)	24	23			
rs10488683	A (0.56)	27	26	0	1	1
	G (0.44)	26	27			
rs211102	G (0.83)	16	8	2.67	0.152	2
	A (0.17)	8	16			

4.1.3.2 Transmission of Haplotypes

Haplotype transmissions were tested using the TRANSMIT programme. There were no significant haplotype transmissions to affected offspring with sleep disturbance (Table 4.2).

Table 4.2 Haplotype transmission data

Markers	Alleles	Observed Transmission	Expected Transmission	χ^2	Haplotype p value
rs4537731-rs1800532	G-C	68.18	67.79	0.01	0.9203
rs1800532-rs10488683	C-G	66.45	61.91	1.13	0.2878
rs10488683-rs211102	G-G	41.08	37.19	1.15	0.2835
rs4537731-rs1800532-rs10488683	G-C-G	40.79	38.14	0.71	0.3994
rs1800532-rs10488683-rs211101	C-G-G	40.84	35.98	1.82	0.1773
All four markers	G-C-G-G	34.92	31.79	0.83	0.3623

4.1.4. Discussion

Association studies between TPH1 and ADHD have been conflicting with one study reporting a positive association (Li et al, 2003) and one reporting a lack of association (Tang et al, 2003). The identification of the second form of TPH, TPH2, which is expressed in the brain, has led research into serotonin and psychiatric disorders away from TPH1. This is not surprising given that TPH is mainly expressed in the pineal gland and in peripheral areas. Nevertheless, there have been positive associations found between TPH1 and manic depression, anger-related traits and anxiety (Bellivier et al, 1998; Rujescu et al, 2002; Sun et al, 2004) which indicates a role for TPH1 in some aspects of psychiatric disorders. Initial TDT analysis did not find any association between ADHD and TPH1 in this study (chapter 3). As TPH1 is involved in melatonin synthesis a hypothesis-driven candidate gene approach to examine the relationship between potential TPH1 risk variants and sleep disorders in ADHD was undertaken.

While this study did not find any excess transmission of alleles to ADHD probands with reported sleep problems it does not rule out the involvement of the TPH1 gene in the aetiology of dyssomnia. The strengths of this study were that all children were assessed in both samples using the CAPA instrument which limits inconsistent diagnostic criteria and procedures. In addition, all probands were known to have been medication free at the time of assessment. There are however some limits to this study. Firstly, assessment of probands, with respect to their sleep, was carried out using subjective measures rated by parents. A previous study has shown that these measures can be biased as assessing sleep in another individual is difficult (Means et al, 2003) and parents may be comparing the child to their other children. Subjective measures have also frequently found sleep problems in children but few have been found when using objective measures (e.g. Polysomnography, actigraphy) (Corkum et al, 1998). Polysomnography is considered to be the most accurate measure of sleep architecture. However, this method usually requires a stay in a laboratory setting which itself is known to change sleep patterns. Assessment of children with ADHD in these settings would therefore be difficult and may not give an accurate assessment of the problem. Secondly, both samples have small numbers of children with reported sleep disorders and not all specify the type of problem encountered (i.e. initial insomnia,

middle insomnia, early morning wakening). However, the majority (96%) of the probands had either initial or middle insomnia. Thirdly, there is no control group to compare the affected children to.

A study of sleep disorders in children with ADHD (Corkum et al, 1999) found that dyssomnias were related to confounding factors such as comorbid ODD or anxiety disorders, rather than to ADHD alone. Analysis of comorbid status of the probands in this study reported that approximately 56% and 57% of the children had comorbid ODD in the Irish and STAR samples respectively. In addition, 32% of children with reported sleep disorders in the STAR sample also had a comorbid anxiety disorder (in all, 16% had both comorbid ODD and anxiety disorder). Corkum et al (1999) reported that, in their sample, the only association between ADHD and sleep disorders was between an increase in sleep-related involuntary movements and the combined subtype of ADHD. Unfortunately sleep-related involuntary movements were not recorded for either sample used in this study. However, approximately 90% of the both samples with a reported sleep disorder have combined type ADHD. Future analysis in this area might include re-assessing these children to see if they have sleep-related involuntary movements to check for association with the combined subtype, though this would mean recruiting more children with both hyperactive-impulsive and inattentive subtypes that have a sleep disorder as controls. It would appear that the relationship between ADHD and sleep disorders therefore partly depends on the type of sleep problem (i.e. sleep related involuntary movements), and confounding factors such as ODD and AD. This however does not rule out the involvement of genetic factors in the aetiology of sleep disorders in ADHD.

4.2 Quantitative trait analysis of parent ratings of impulsivity for two serotonergic genes: TPH2 and 5-HTT

ADHD is a heterogeneous disorder with considerable variations across behavioural dimensions. Family, twin and adoption studies have shown that genetic factors play a major role in the aetiology of the disorder (section 1.2.1). In addition molecular genetic studies have associated a number of genes with ADHD, including genes of the serotonergic system such as the serotonin transporter (5-HTT). However, there have been difficulties in replicating results between groups. This may be due to the complicated connection between the causal genes involved in the aetiology of ADHD and the phenotypic expression of these genes at a behavioural level.

This thesis presents evidence for the association of the TPH2 gene and ADHD. TDT analysis of 14 TPH2 polymorphisms shows a very strong association between ADHD and the marker rs1843809 and a marginal association with the marker rs1386497 (section 3.5.2). The TPH2 gene would seem to be a strong candidate gene for involvement in the aetiology of ADHD due to its position as the rate-limiting catalyst in the serotonin biosynthesis pathway. Any functional polymorphism in the coding region could have major implications for serotonin production in the brain. A reduction in serotonergic functioning has been found in children with ADHD (Coleman, 1971; Krusei et al, 1990; Spivak et al, 1999) and relatively low serotonin platelet levels have been reported in children with ADHD (Rapoport et al, 1974). In addition, a reduction in serotonin levels has been linked to impulsive behaviour, a characteristic behaviour of ADHD (Halperin et al, 1997). Evidence for the theory that serotonin is significantly involved in control of impulsive behaviour has been accumulating. Soubrie (1986) analysed the influence of drugs that increase and decrease serotonergic neurotransmission and observed that behavioural disinhibition, which can be directly related to impulsivity, is associated with decreases in brain serotonin levels. A study by Linnoila et al (1993) reported that low levels of the 5-HT metabolite 5-HIAA in the cerebrospinal fluid (CSF) are associated with impaired impulse control.

Dependence upon a categorical classification of ADHD as either unaffected or affected for association analysis may result in valuable information being lost. ADHD is known to encompass separate, but associated, aspects of hyperactivity-impulsivity and inattention. Subtype classification of ADHD has proven to be useful in identifying genes associated with the inattentive subtype in this thesis but has proven problematic for the hyperactive-impulsive subtype as the sample size is limited (n = 11). Combining the hyperactive-impulsive subtype with the combined subtype will only serve to increase the degree of hyperactive-impulsive traits in the combined group. In addition, subtype analysis does not take into account the full variance of behavioural traits within the population. For example ADHD probands classified as inattentive may actually have up to four or five symptoms of hyperactivity/impulsivity therefore do not meet the threshold criteria for the combined type subgroup. Using an approach that makes use of continuously distributed quantitative traits (e.g. impulsive behaviour) can provide the power to detect a relationship between specific characteristics of ADHD behaviour and variation in genetic risk. For example, the DAT1 9-repeat allele has been reported to possibly associate with the impulsivity phenotype of ADHD (Kim et al, 2006).

4.2.1. Impulsivity in ADHD

The term impulsivity is used to describe a wide range of behaviours. One definition is as follows: "impulsivity encompasses a range of actions which are poorly conceived, prematurely expressed, unduly risky or inappropriate to the situation and that often result in undesirable consequences" (Daruna and Barnes, 1993). Impulsive behaviour has been shown to be an associated feature in several psychiatric conditions including personality, substance abuse, bipolar, attention deficit, and conduct disorders (Moeller et al, 2001). Impulsivity can manifest itself as impatience, talking excessively, having difficulty awaiting turns in group situations, and frequently interrupting others in social, academic, or occupational settings. Impulsive behaviour can lead to an increase in accidents and engagement in potentially dangerous activities without consideration of possible consequences. A recent study, for example, reported that ADHD children were 1.7 times more likely to have an accident than controls (Swenson et al, 2004).

Impulsive behaviour is considered to be a core symptom of ADHD by the DSM-IV (American Psychiatric Association, 1994). According to the DSM-IV description of ADHD, impulsivity may be present if an individual:

- 'Has difficulty waiting turn in games or in group situations'
- 'Often blurts out the answers to questions'
- 'Often interrupts or intrudes on others'
- 'Often talks excessively'

As described in the introduction, a reduction in serotonin levels in the brain has been associated with ADHD. A decrease in serotonin levels is also considered to affect impulsive behaviour. The synthesis of serotonin in the brain depends on the availability of free tryptophan. Depletion of tryptophan levels appears to, consistently, lead to increases in aggressive tendencies, impulsivity and decreases in cooperativeness (Coccaro and Kavoussi, 1997; Doland et al, 2001; Walderhaug et al, 2002; Walderhaug et al, 2007). Manipulation of serotonin levels can be achieved through depletion or enhancement of dietary tryptophan. This process is carried out using the acute tryptophan depletion (ATD) method whereby subjects ingest a combination of amino acids devoid of tryptophan. Any subsequent behavioural changes could be seen as a direct result of lower serotonin function in the central nervous system (CNS). Studies have been carried out on nonclinical samples to assess the effect of tryptophan depletion on personality and behaviour. Walderhaug et al. (2002) studied the effect of tryptophan depletion on behavioural measures of impulsivity using the continuous performance test on 24 healthy young males. They found that rapid lowering of tryptophan levels results in an increase in impulsiveness and a decrease in discriminating ability in normal subjects. Positron Emission Topography (PET) scans have also suggested that acute tryptophan depletion reduces serotonin synthesis in frontal brain regions that are crucial for attentional performance (Nishizawa et al, 1997; Rubia et al, 2005; Talbot and Cooper, 2006). An intron-8 polymorphism (rs1386483) of the TPH2 gene was found to be associated with response inhibition in unselected college students ($n = 199$) (Stoltenberg et al, 2006). Poor response inhibition is a characteristic of impulsive behaviour. Individuals homozygous for the T allele had the longest Stop Signal reaction time (i.e. greater impulsivity, $p = 0.01$). The Stop Signal test measures the ability to inhibit a response that has already been initiated (Logan et

al, 1984). The authors suggest that individuals with a T/T genotype may have reduced TPH2 function and correspondingly low central serotonin levels.

The serotonin transporter has been reported to be associated with ADHD in some groups but the results have not consistently been replicated and no association was observed between 5-HTT and ADHD in this thesis (section 3.5.3). The gene has been implicated in impulsive behaviour and as such analyses using quantitative behavioural measures of impulsivity may emphasize a role for 5-HTT in impulsive behaviour in ADHD individuals. Analyses of the 5-HTT gene using ATD have also implicated the gene in impulsive behaviour in non-ADHD individuals. Lee et al (2003) have reported an association between the serotonin transporter promoter variant 5-HTTLPR and impulsive subjects in a Korean sample. This polymorphism has been found to be associated with ADHD in several studies (Chapter 1, Table 1.2). There were significant differences in allelic frequencies of the short allele (5-HTTLPR-S) and the long allele (5-HTTLPR-L) between impulsive (IS) and non-impulsive (NIS) males (IS vs. NIS: 69.4 vs. 77.8%; and 30.6 vs. 22.2% respectively). Interestingly, the long allele has been found to be associated with ADHD in several studies (Table 1.2). Oades et al (2002) explored the relationship between the density and affinity of the serotonin transporter to clinical (Conners parent questionnaire) and experimental (Stop signal reaction time) measures of impulsivity. Decreased receptor affinity for serotonin was found to be associated with difficulty in inhibiting response in the stop signal test. The authors concluded that low affinity of serotonin for the 5-HTT could be related to cognitive impulsivity (distractibility).

4.2.2 Aim

The aim of this study was to examine a quantitative behavioural trait in relation to the Tryptophan Hydroxylase 2 (TPH2) and Serotonin Transporter (5-HTT) genes. ADHD symptoms of impulsivity were examined as a continuous trait to ascertain the relationship of this behavioural trait with variation in the above genes, also shown to be associated with ADHD.

4.2.3 Materials and Methods

Symptom dimensions were derived from CAPA scores. There are four specific questions relating to impulsive tendencies:

1. Often talks excessively
2. Difficulty awaiting turn in games or group situations
3. Often blurts out answers before questions have been completed
4. Often interrupts or intrudes on others (e.g. butts into others' conversations)

Parents rated their children for each of the symptoms with a 0, 2, or 3 depending on the lack of or severity of the symptoms (0 = not at all, 2 = pretty much and 3 = very much). The total scores for these symptoms were tallied by adding up all the ratings. For example a child that scores a three on all four questions will have a maximum impulsivity score of 12. Kruskal-Wallis testing, a non-parametric test, (chapter 2, section 2.3.6.) was used to evaluate if allelic variants of the serotonin transporter and TPH2 genes were associated with different impulsivity ratings. It was necessary to use a non-parametric form of testing to allow for differences in sample sizes and standard deviations. All calculations were performed using SPSS (version 15).

4.2.4. Results

4.2.4.1. Investigation of the Association of Allelic Variants with Impulsivity

For the TPH2 gene statistically significant differences in the measure of impulsivity was observed for three markers, according to genotype (Table 4.3). The box plot revealed that the CC homozygotes (11) of the rs1023990, rs1487278 and rs10879345 variants have the lowest impulsivity scores, with CT and TT individuals (12 and 22) having higher impulsive scores (Figures 4.2, 4.3 and 4.4), Kruskal-Wallis testing indicated that these differences in impulsivity ratings are statistically significant for these markers (Table 4.2: rs1023990: $\chi^2 = 8.664$, p-value = 0.013; rs1487278: $\chi^2 = 8.469$, p-value = 0.014; rs10879345: $\chi^2 = 8.936$, p-value = 0.011). There was no significant difference between the remaining markers of TPH2 (Table 4.3) and impulsivity ratings.

Kruskal-Wallis testing on the serotonin transporter variants showed no significant difference between allelic genotypes and impulsivity scores (Table 4.4).

Table 4.3 Kruskal-Wallis Test results for differing allelic variants and the associated impulsivity ratings. Statistically different ratings (highlighted in bold and orange) were associated with allelic variants in the rs1023990 and rs1487278 variants. As there were less than 5 individuals with a 22 genotype in the rs1843809, rs1386488, rs1386494 and rs1386496 tests, a chi-square with k-1 (the number of groups -1) degrees of freedom (df) was used to approximate the significance level for the test.

TPH2 Variant	Kruskal Wallis χ^2	d.f.	p value
rs4570625	1.442	2	0.486
rs10748186	1.640	2	0.440
rs2129575	2.746	2	0.253
rs1386488	0.194	2	0.907
rs10879345	**8.936**	**2**	**0.011**
rs1843809	1.830	2	0.400
rs1386496	4.151	2	0.125
rs1386494	3.402	2	0.183
rs1386493	2.753	2	0.253
rs1023990	**8.664**	**2**	**0.013**
rs1386497	0.366	2	0.833
rs1487278	**8.469**	**2**	**0.014**
rs1487275	2.338	2	0.311
rs1487279	2.743	2	0.254

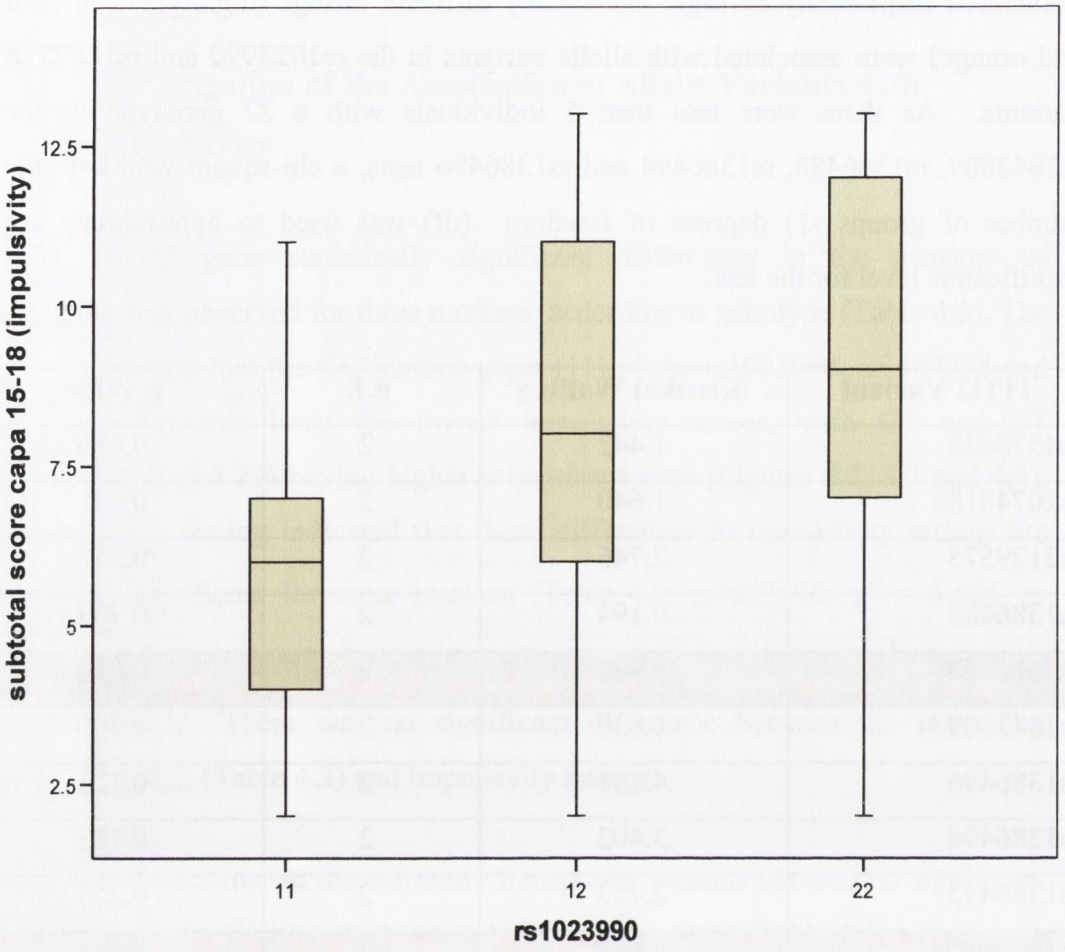


Figure 4.2 Box plot of rs1023990 allelic variants and associated impulsivity ratings. CC homozygotes are represented by 11, and show the lowest impulsivity scores. TT homozygotes are represented by 22 and show the highest impulsivity scores, while heterozygous individuals (12) show intermediate to high impulsivity scores. C is the minor allele for this variant (0.25).

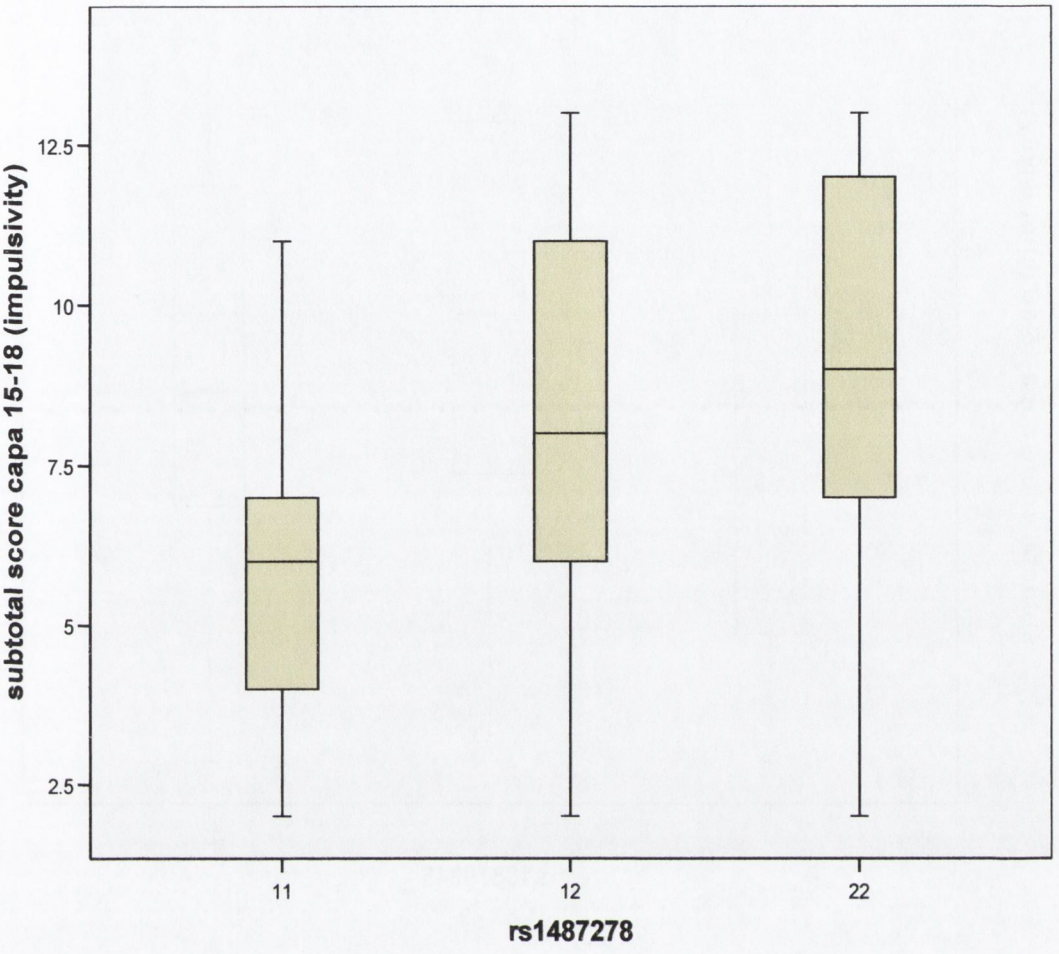


Figure 4.3 Box plot of rs1487278 allelic variants and their associated impulsivity ratings. CC homozygotes are represented by 11, and show the lowest impulsivity scores. TT homozygotes are represented by 22, and show the highest impulsivity scores, while heterozygous individuals (12) show intermediate to high impulsivity scores. C is the minor allele for this variant (0.26).

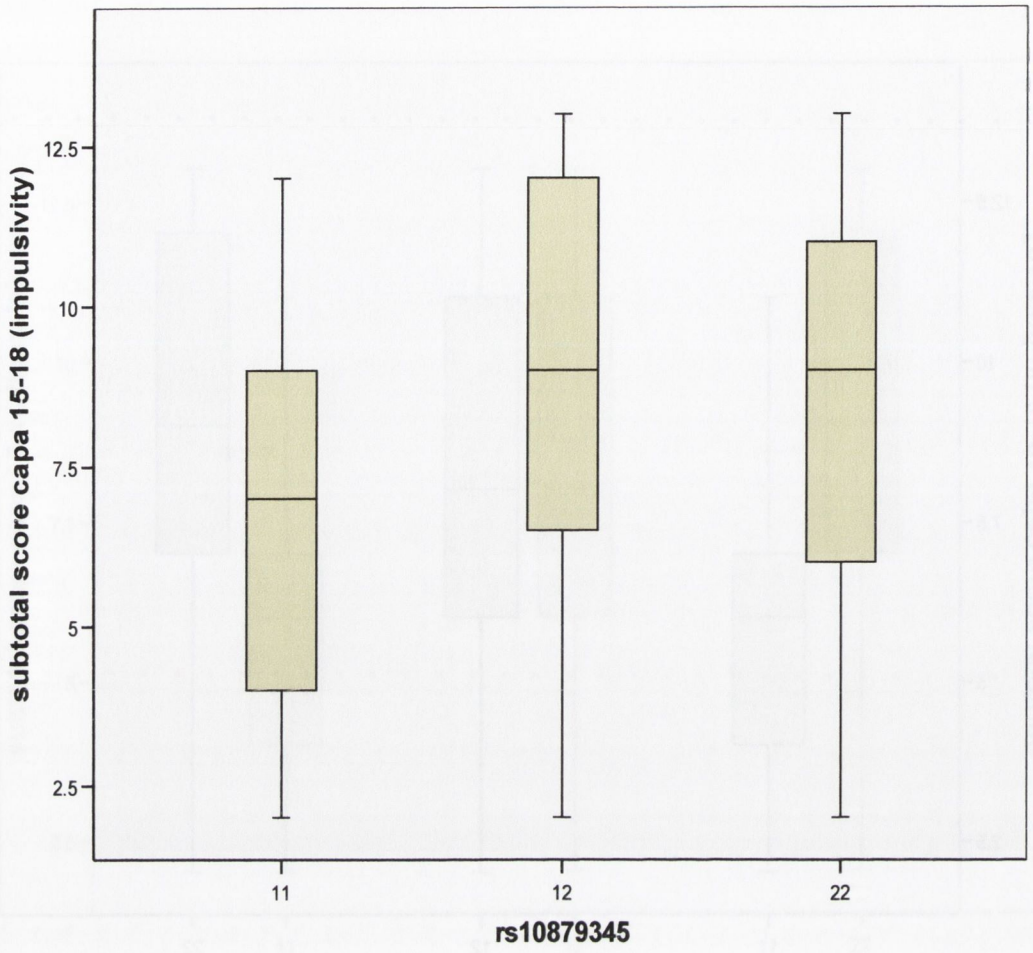


Figure 4.4 Box plot of rs10879345 allelic variants and associated impulsivity ratings. CC homozygotes are represented by 11, and show the lowest impulsivity scores. TT homozygotes are represented by 22. TT and CT individuals show the highest median impulsivity scores.

Table 4.4 Kruskal-Wallis test results for differing allelic variants of the 5-HTT gene and the associated impulsivity ratings.

5-HTT variant	Kruskal Wallis χ^2	df	p value
5-HTTLPR	0.008	2	0.996
Intron 2 VNTR	0.497	2	0.920
rs1872924	0.497	2	0.780
rs4325622	0.149	2	0.928
3'UTR	0.413	2	0.813

4.2.5 Discussion

This study was undertaken to investigate the impulsive behavioural aspect of the ADHD phenotype to determine if this phenotypic trait has a distinct association with particular genetic risk factors for the disorder.

The TPH2 markers, rs1023990, rs1487278 and rs10879345, were found to be associated with parent reported impulsive behaviour. However, previous TDT analysis found no excess transmission of alleles of these markers to children diagnosed with combined type or hyperactive-impulsive type ADHD (chapter 3, Tables 3.35 and 3.37 respectively). These findings provide evidence for a trait specific relationship with these markers and ADHD. The marker rs1843809 was observed to be the most significantly associated marker with ADHD in this study (Chapter 3, Table 3.2: $\chi^2 = 13.47$, $p = 0.0003$, OR = 2.45). Nevertheless, Kruskal-Wallis testing did not show any significant difference between genotypes of this marker and impulsivity scores. It is possible that rs1843809 is associated with clinical aspects of ADHD and not related to specific ADHD symptoms alone. It is also possible that the rating scale used in this study is poorly correlated with an underlying distribution of genetic liability.

Lim et al (2007) recently reported that two SNPS in exons 7 and 9 (rs7305115 and rs4290270), of the TPH2 gene, had significant allelic expression imbalance (AEI) in 19/27 samples (1.2 to 2.5 fold) of frozen sections of rostral pons. According to the authors this may imply the presence of cis-acting variants that differentially affect TPH2 mRNA levels in the human pons. AEI was reported to be tightly associated with the minor allele of an exon 7 SNP (rs7305115) in 17/18 subjects, possibly reflecting a gain-of-function for this allele. Markers rs1023990, rs10879345 and rs1487278 are in strong LD ($D' = 0.78$) with this marker which could indicate that a loss of TPH2 function resulting from this allelic imbalance could result in the impulsive symptoms rated for the children in this study.

For the serotonin transporter, Kruskal-Wallis analysis did not return any significant differences between allelic variants impulsivity scores. The failure to find a clear association between a specific behavioural dimension of the ADHD phenotype with the 5-HTT gene potentially offers an understanding of the variability of

findings within the literature for the 5-HTTLPR variant. It is possible that the gene is associated with another behavioural dimension such as inattention. If inattention rather than impulsivity is the driving trait in the relationship between ADHD and the 5-HTT gene, sample variation in the level of inattention across samples would likely affect outcomes and may explain the lack of association found between this gene and ADHD in general and impulsive measures in this sample.

There are some limitations to this study. The power of the sample may not have been enough to detect associations when phenotypic measurement is taken into consideration. In addition, impulsivity measures were not based on objective measures such as neuropsychological tests, witnessed by an individual unrelated to any of the families in the study. This may be important as scores may be biased by parental responses especially as testing was only carried out at one time-point and thus can lead to measurement error. Studies investigating clinical and personal characteristics of impulsivity found that rapid-response impulsivity type neuropsychological tests, such as the continuous performance test (CPT), best predict impulsive measures (Swann et al, 2002; Dougherty et al, 2003). The CPT requires participants to make a rapid evaluation of stimuli in order to decide whether or not to respond. Correct responses to target stimuli ("hits") are measured against responses to non-target stimuli ("false alarms"). Responding to non-target stimuli has been linked to impulsivity (Swann et al, 2002; Dougherty et al, 2003; Walderhaug et al, 2007). Future work in this area might examine the TPH2 markers in relation to results on the CPT. In addition, as ADHD is thought to represent an extreme of a set of behavioural traits quantitatively distributed in the population it would also be interesting to compare these results with matched controls to see if these risk alleles correlate with impulsivity symptoms in the general population.

In conclusion, the data suggests that alleles of polymorphisms in TPH2 may be associated with measures of impulsivity in this sample but this result will need to be replicated in other population samples before any firm conclusions can be made.

Chapter 5

Evolutionary-based haplotype analysis of the TPH2 gene in the Irish and IMAGE samples

5.1 Introduction

The Tryptophan Hydroxylase gene (TPH) is a strong candidate gene for involvement in the aetiology of ADHD. The position of TPH as the rate-limiting catalyst in the serotonin biosynthesis pathway means that any functional polymorphism in the coding region could have major implications in serotonin production in the brain. A reduction in serotonergic functioning has been found in children with ADHD (Coleman, 1971; Krusei et al, 1990; Spivak et al, 1999) and relatively low serotonin platelet levels have been reported in children with ADHD (Rapoport et al, 1974). In addition, impulsive behaviour is frequently associated with ADHD, which is also associated with a reduction in serotonergic function (Halperin et al, 1997). Until relatively recently the TPH2 gene remained undiscovered. In 2003, Walther and Bader (2003) reported the presence of a second, neuronal, form of TPH, now known as TPH2, and found it to be wholly expressed in the brain (Chapter 3, section 3.3.1.2). It is reasonable to hypothesise that genetic abnormalities in this gene may influence the concentration of brain serotonin levels and contribute to the development of many psychiatric disorders including ADHD.

At the time of initial analysis of the TPH2 gene for this thesis there were very few published association studies on TPH2 and none in relation to ADHD. Subsequently two papers have reported association between ADHD and TPH2 (Walitza et al, 2006; Brookes et al, 2006). However, the results from Brookes et al (2006) study on the TPH2 gene have presented a conundrum. A SNP (rs1843809) found to be highly significantly associated with ADHD in the Irish sample was also found to be associated with ADHD in the IMAGE phase I sample, but the association was with the opposite allele. In an attempt to uncover possible reasons behind this contradictory finding this chapter describes an examination of the patterns of haplotype associations in the Irish sample and the individual population samples that make up the IMAGE sample, taking an evolutionary perspective.

5.2. Population Analysis

5.2.1. Introduction

TDT analysis of the TPH2 gene in this thesis found significant association with the A allele of marker rs1843809. In contrast to this, the IMAGE study found association with the C allele of this marker in the Phase I sample. Re-genotyping of 48 individuals from both samples (96 in total) was first performed to potentially explain the inconsistent results but both samples were found to be genotyped correctly. While the finding of association with opposite alleles is not unique it does raise the question of whether the result is a spurious association or evidence of confirmation of association between the TPH2 gene and ADHD. One theory is that such findings, also referred to as "flip-flop" associations, may be due to multi-locus effects. Lin et al (2007) examined single-locus association under four models of multi-locus effects and observed that the direction of allelic association could flip when the target risk allele is positively associated with an associated allele at a different locus. They stated that the likelihood of "flip-flop" associations depends on allele frequency and is only seen when the two loci act in unison to influence risk of the disease. An additional theory is that flip-flop findings could be due to potential differences in the genetic architecture of the sampled populations. Linkage Disequilibrium (LD) patterns also differ between populations and could lead to the contradictory patterns of association observed when non-functional markers are tested.

5.2.2. Association testing: TDT versus Cladogram Analysis

The TDT for single SNPs requires that at least one of the parents should be heterozygous in order to test for departure from Mendel's first law. Therefore, when both parents and child are heterozygous for a bi-allelic marker it is not possible to determine transmissions/non-transmissions from individual parents. To overcome this problem TDT analysis using haplotypes may resolve some of the limitations of TDT analysis using a single SNP. Haplotypes incorporate more information about the underlying polymorphisms than the genotypes for individual

SNPs do, and they are considered a more informative format of data in association analysis (Tang, 2005). However, while this method will increase the power of the TDT it can also limit the power of the test as there may be a larger number of haplotypes relative to the number of alleles of the individual SNPs. This will require an increase in the degrees of freedom of the statistical test. In addition, if a haplotype occurs in only one or two individuals it could lead to statistical problems due to small sample sizes which will make it difficult to estimate their possible effect on susceptibility to a disorder (Bardel et al, 2005). Testing specific haplotypes or groups of haplotypes, known as cladogram analysis, has been suggested as a solution to this problem.

Templeton et al (1987) were first to suggest focusing on particular haplotypes or groups of haplotypes (cladograms) and using the evolutionary relationships between the haplotypes to aid in the discovery of risk variants for a disorder. Grouping haplotypes together will reduce the number of degrees of freedom required for the TDT and will increase the number of individuals in different haplotype groups thereby increasing sample size. The assumption is that haplotypes associated with a disorder should cluster together, will contain many of the same polymorphisms, and might therefore induce similar effects on disease predisposition (Daly et al, 2001).

There are two approaches to grouping haplotypes; (1) a phenetic approach based on similarity among haplotypes and (2) a cladistic approach that is based on the evolutionary relationship of haplotypes. In this study, a cladistic approach has been used. The evolutionary history of a sample of haplotypes can be represented by a simple coalescent process. Coalescence refers to mapping allelic variations backwards through evolutionary history, via mutations, to the ancestral alleles. Coalescent theory can be used to make predictions regarding ages and frequencies of alleles, selection, and rates of mutation (Hillis et al, 1996). A series of mutations can change a single ancestral haplotype into a diversity of haplotypes. The history of these haplotypes can be represented in a cladogram or haplotype tree (see Figure 5.1, (a), (b), and (c)).

If a functional polymorphism is not genotyped in an association study, it will be embedded in the evolutionary history of the haplotypes that make up the cladogram, provided it is in the region of the gene covered by the haplotypes (Templeton AR, 1996). Using Figure 5.1 (c) as an example, if a deleterious mutation occurred after the evolution of haplotype C-C-A-G-A, then this mutation will be embedded in the clade represented by haplotypes C-C-A-G-C and C-C-C-A-C. The key advantage of the cladistic analysis approach is that it directs the search for polymorphisms that have a direct impact on liability for a disorder through the pattern of association found over related haplotypes making haplotype analysis more sensitive than a series of single SNP association tests (Seltman et al, 2003). In this respect, cladistic analyses of the Irish sample and the population samples from the IMAGE study, where positive associations with TPH2 have been found, could clarify whether the same, or related, haplotypes are consistently implicated in ADHD aetiology. This in turn could indicate which haplotypes should be further characterized in the search for a susceptibility polymorphism.

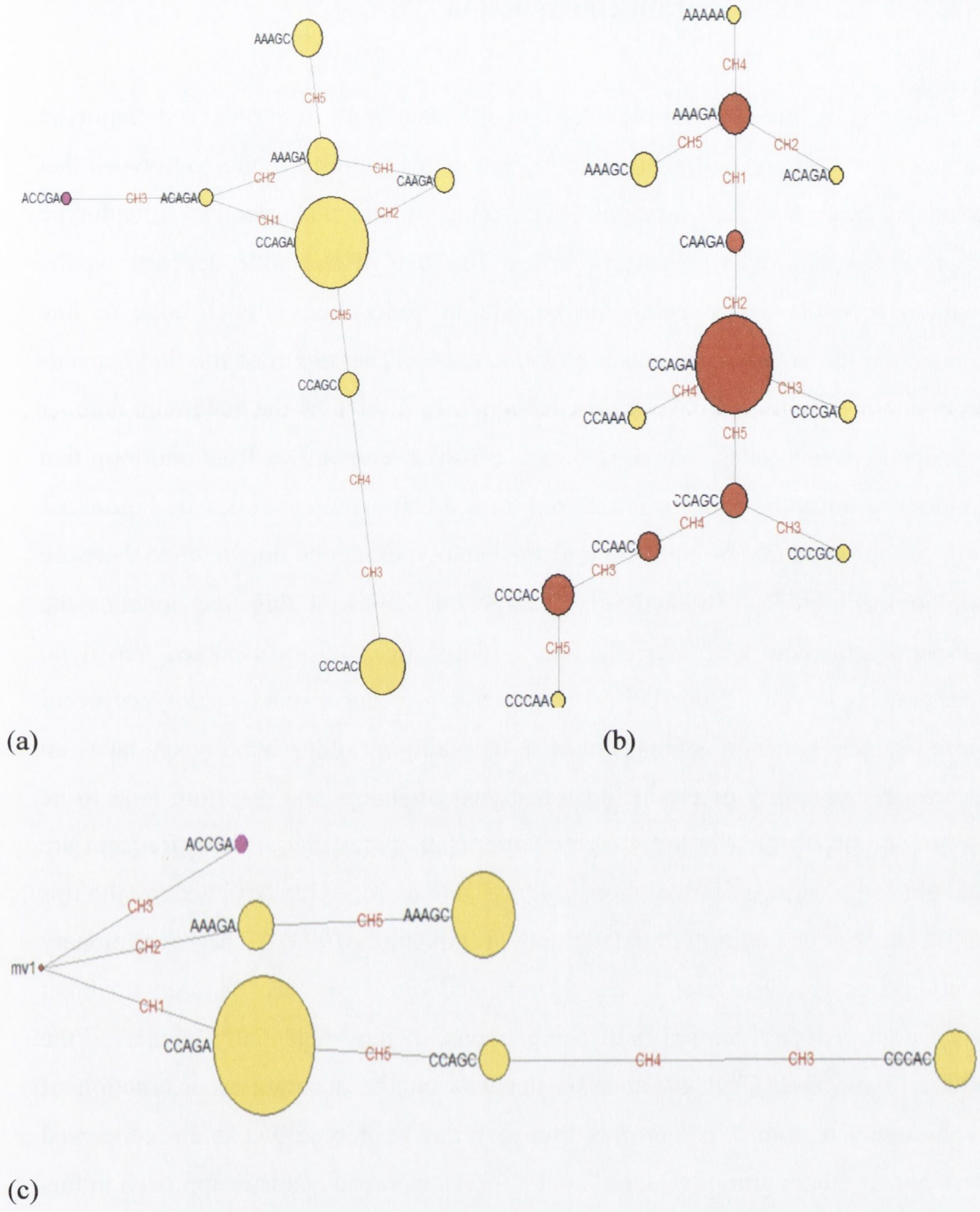


Figure 5.1 **(a)** An example of a haplotype network. **(b)** A haplotype tree showing how more frequent haplotypes (red circles) tend to occupy central positions of the tree while less frequent, presumably younger, haplotypes (yellow circles) occupy the tips of the tree. **(c)** An example of a rooted haplotype tree.
The circle (referred to as a node) size reflects the frequency (%) of the haplotype in a population. The letters CH represent a mutation e.g. CH1 means a change at SNP one in the haplotype. Haplotype A-C-C-G-A (pink) is the ancestral haplotype.

5.2.3. Cladogram construction

For any population it is possible to construct a cladogram from observed haplotype data. The cladogram will contain nodes and edges connecting the haplotypes that differ by one or more mutations. Each node of the cladogram is a haplotype (represented as circles of varying sizes. The size of the node depends on the frequency of the haplotype in the population under study). Each edge or line connecting the nodes represents a mutational step. The length of the line depends on the number of mutations between the nodes. In this study, the maximum number of steps between nodes is two. If such a construct contains at least one loop that connects a subset of nodes it is referred to as a haplotype network (see Figure 5.1 (a)). A haplotype tree or cladogram of the haplotypes can be drawn when there are no loops (Figure 5.1 (b) and (c)) (Templeton, 2005). If this tree contains the ancestral sequence it is referred to as a rooted tree and an unrooted tree if no ancestral haplotype included. Under neutral coalescence, older haplotypes occur more frequently than younger ones. In addition, older haplotypes have an increased probability of producing mutational offshoots and therefore tend to be more centrally located in a network or tree. Haplotypes that are less frequent are thought to be younger in evolutionary terms and are more likely to occupy the tips of networks or trees (Crandall and Templeton, 1993). The evolutionary relationships between haplotypes, as indicated by a tree, can be used to direct analyses to reduce the number of comparisons, thereby potentially increasing the power of the test. Cladistic analysis depends on the accurate reconstruction of evolutionary relationships and the discovery that haplotype blocks are conserved even across ethnic groups (Gabriel et al, 2002) supports a cladistic approach in this form of association analysis.

5.2.4. ET-TDT

Seltman et al (2001) designed a cladistic-based analysis, the evolutionary tree transmission disequilibrium test (ET-TDT), that utilises parent-child trios and both quantitative and qualitative measures. ET-TDT has the ability to guide the search for mutations that directly affect liability to a disease/disorder and was found to increase interpretability and sometimes power over a TDT-based analysis. This

method was the first to incorporate haplotype uncertainty into the test statistic which must be taken into account when using family data. This problem was overcome by treating the phase as "missing data" and estimating the frequency distribution of the haplotypes by likelihood based methods. According to the authors, the result is the probability that the observed multilocus genotype resolves into each pair of haplotypes consistent with the genotype. These probabilities are used to weight the likelihood contribution for each consistent configuration (Seltman et al, 2003). The more family members genotyped the greater the restrictions on the haplotypes consistent with multilocus genotypes and hence the greater the power to detect significant associations between haplotypes and phenotypes. The same group subsequently developed the eHap software (Evolutionary-based haplotype analysis package, Seltman et al, 2003) which incorporates the ET-TDT test. The eHap software is designed to analyse multilocus data as haplotypes and determine whether there is an association between haplotypes and phenotypes (Seltman et al, 2003). This program was used to analyse the data for this study.

While a number of SNPs in the TPH2 gene where analysed in this thesis and the IMAGE study, only five were common to both studies. These SNPs were used to make direct comparisons of haplotypes between the populations in the samples. As the data contains population samples of European ancestry it allows evaluation of the degree of similarity of local LD structure at TPH2 among the different European samples and also allows for comparison of these results with samples from Israel. This permits determination of whether this LD structure is a contributing factor to the observed differences in the reported associations between the TPH2 marker rs1843809 and ADHD.

5.2.5. Aim

To conduct an evolutionary-based association analysis using haplotype data from the Irish population and from the International Multicentre ADHD Genetics (IMAGE) study to explain the conflicting findings between the two samples and narrow down the region of association between the TPH2 gene and ADHD for future functional studies on the gene.

5.3. Materials and Methods

For this analysis, the Irish sample used throughout this thesis will be referred to as Ireland and the Irish sample from the IMAGE study will be referred to as Dublin IMAGE. The IMAGE study is a multi-centre study involving eleven centres from Europe and Israel (Chapter 2, section 2.1.4) including two centres from the UK (London), two centres from Germany (Essen and Goettingen), two from Israel (Tel Aviv and Jerusalem), one from Switzerland (Zurich), one from Belgium, one from the Netherlands (Rotterdam) and one from Spain.

There were five SNPs common to both the IMAGE study and this study (rs4570625, rs2129575, rs1843809, rs1386493, and rs1487275. All five markers are polymorphic in all populations) and these were used to analyse the haplotype structure of TPH2. The resulting haplotype system spans approximately 78kb of the gene from the promoter region to intron 8. Markers were used to construct LD maps and graphic representations of the LD structures were created by Haploview (http://www.broad.mit.edu/mpg/ haploview/).

The Evolutionary-based Haplotype analysis Package (eHAP) was used to analyse specific groups/clades of haplotypes for association with ADHD (Seltman et al, 2001 and 2003. This program is freely available at: http://wpicr.wpic. pitt.edu/wpiccompgen/ (see Appendix III for input files). For each individual in a sample, eHAP produces a list of all haplotype pairs that are consistent with the individual's multilocus genotype and that of any family members that are also genotyped. The program produces a distance matrix in terms of weighted differences between haplotypes and uses this to graphically connect similar haplotypes (haplotypes differing by a single mutation or by user-defined criterion). For this analysis, eHAP assesses the association between ADHD status and haplotype transmission and uses the evolutionary relationships among the haplotypes to structure tests of significance while taking haplotype uncertainty into account.

Median-joining networks of TPH2 haplotypes were constructed with the program Network 4.201 (http://www.fluxus-engineering.com) using the haplotype frequencies computed by eHap. Although the eHap constructs visual cladograms in

the output file, Network produces better visual tree images. Network produces haplotype networks where possible therefore loops were broken according to the cladogram output from eHap.

5.4. Results

5.4.1 Haplotype frequency and LD structure comparison between populations

Across all populations, the haplotypes found had broadly similar frequencies (see Table 5.1). This suggests that the LD structure is very similar across all populations. This is further supported by the LD block structure (see Figures 5.2 (a)-(l)). These results rule out the possibility that differences in LD values between the population samples are the cause of the contradictory findings between the IMAGE and Irish samples.

Table 5.1 Haplotype structures and relative frequencies.

Marker 1 = rs4570625
2 = rs2129575
3 = rs1843809
4 = rs1386493
5 = rs1487275.

Haplotype	Marker					No. of populations haplotype occurs in	Average Freq (%)	Range (%)
	1	2	3	4	5			
h1	C	C	A	G	A	12	55	49.8 - 68
h2	C	C	C	A	C	12	12	8 – 16.5
h3	A	A	A	G	C	12	9	5.9 – 16.2
h4	A	A	A	G	A	12	7	6.3 – 12.9
h5	C	C	A	G	C	6	4	2.1 - 6
h6	C	C	A	A	C	1	4	0 - 4

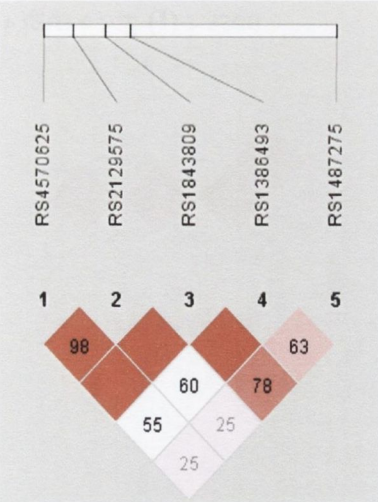


Figure 5.2 (a) London (1)

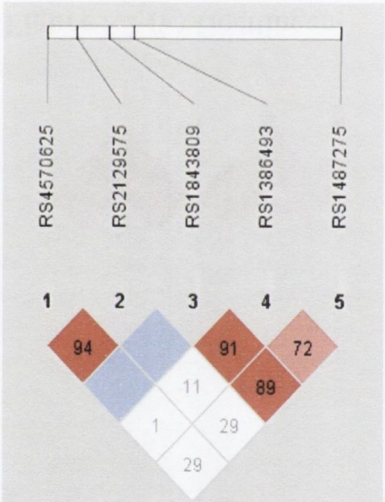


Figure 5.2 (b) Jerusalem

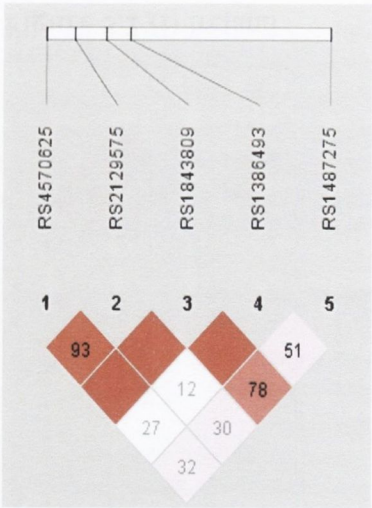


Figure 5.2 (c) Tel Aviv

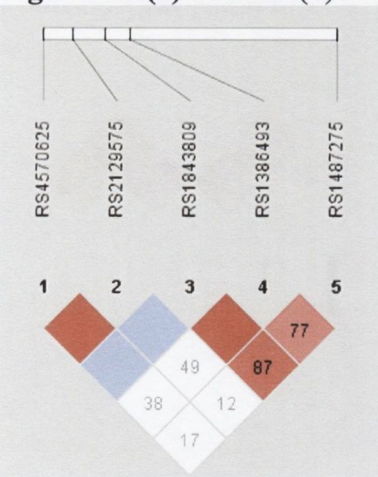


Figure 5.2 (d) Belgium

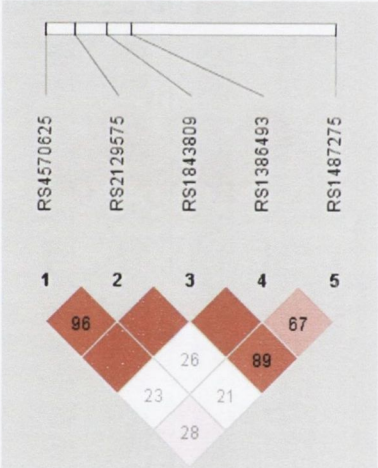


Figure 5.2 (e) London (2)

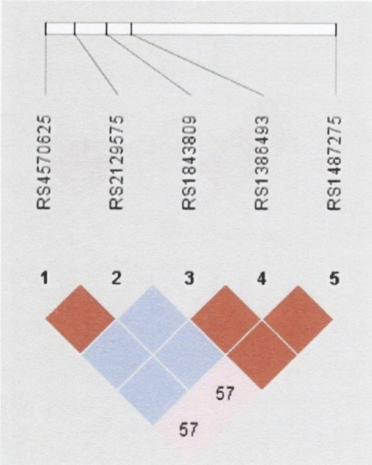


Figure 5.2 (f) Zurich

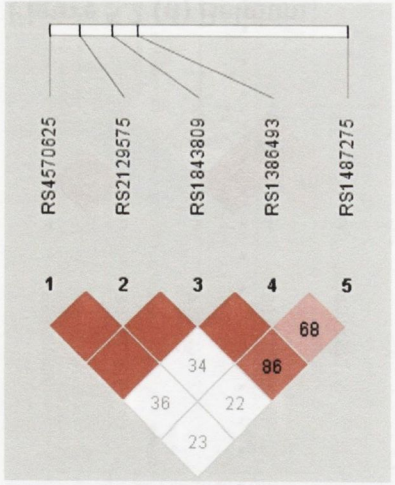


Figure 5.2 (g) Dublin IMAGE

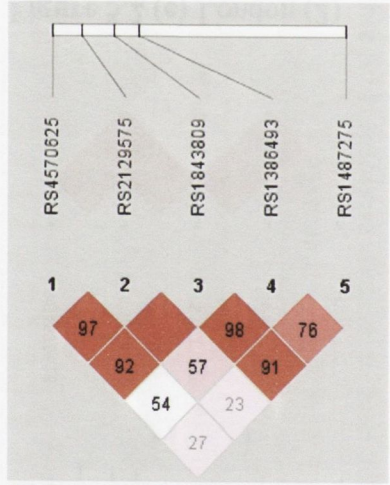


Figure 5.2 (h) Rotterdam

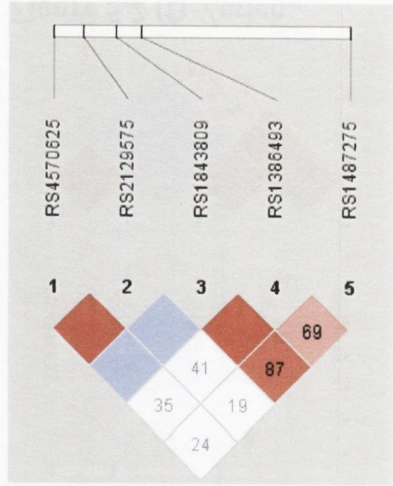


Figure 5.2 (i) Spain

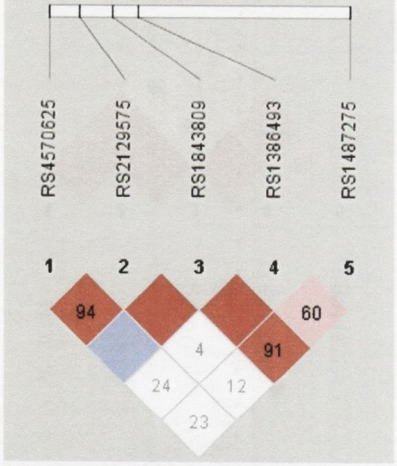


Figure 5.2 (j) Essen

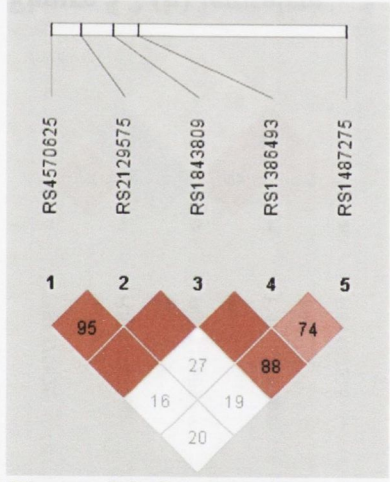


Figure 5.2 (k) Goettingen

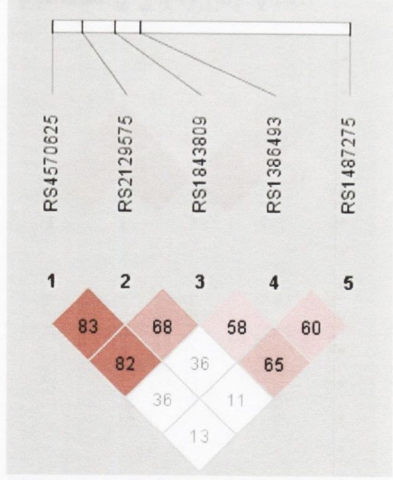
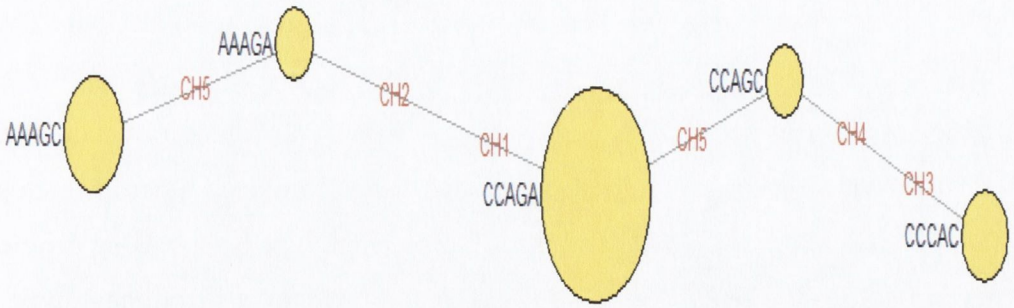
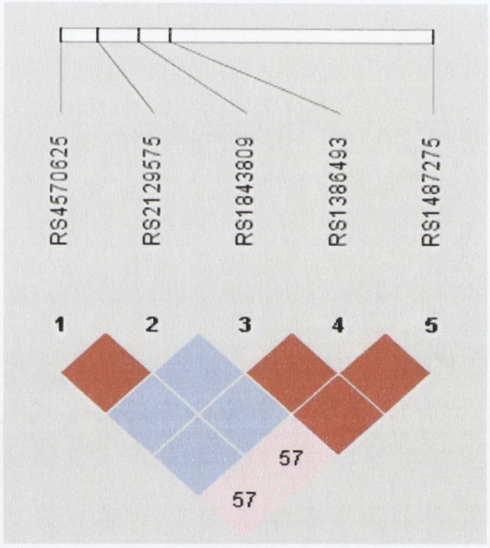


Figure 5.2 (l) Ireland

For all figures: LD is measured using the D' statistic and calculated using the Haploview programme



(a)



(b)

Figure 5.3 **(a)** Haplotype network and **(b)** LD plot for Zurich. LD shows high pairwise D' values which is reflected in the uncomplicated haplotype tree.

The theoretical number of possible haplotypes from five SNPs should be 32 in a large population. However, only nineteen haplotypes were observed (Table 5.2, defined as h1 to h19 based on their frequency from high to low) and only five of these make up the majority of each population (Table 5.1). Together the most common haplotypes account for approximately 94% of all haplotypes encountered. The two most frequent haplotypes, h1 and h2 differ from each other at three sites (markers rs1843809, rs1386493 and rs1487275). Haplotype 3 shows one difference from haplotype 4 at the marker rs1487275. Haplotype 5 differs from haplotype 1 at marker rs1487275 also. Haplotype 6 differs from haplotype 5 at marker rs1386493.

Not all haplotypes were present in every population and those of less than 1% were excluded from analyses. The haplotype "C-C-A-G-A" was the most prevalent (mean haplotype frequency = 55.1%) and appeared in every population with the highest frequency in Spain (68%) and the lowest in Dublin IMAGE (49.8%). The haplotype "C-C-C-A-C" was the second most common (mean = 11.9%). The haplotype "A-A-A-G-C" was the third most common (mean frequency = 8.6%) and was quite frequent in Zurich (mean frequency = 16.2%) while not as common in Belgium (frequency = 5.9%) and Spain (frequency = 6.5%). Haplotype blocks were found to be consistent across all populations (Figures 5.2 (a) – 5.13 (l)).

As mentioned above (section 5.2.3) haplotype data can be used to construct a tree (cladogram) or a haplotype network. The eHap program requires that all loops in a network must be broken before association analysis can proceed. The program incorporates an algorithm that will break loops according to Crandall and Templeton (1993) but it only offers one possible cladogram for analysis. While this algorithm has been proven robust (Seltman et al, 2003) it is possible to confirm the cladogram output by deducing the ancestral haplotype for the set of SNPs under investigation. The ancestral allele for each SNP in this study was determined by analyzing the sequence data obtained from the chimpanzee sequence available from Ensembl (www.ensembl.org). Iyengar et al (1998) state that to obtain an ancestral sequence, data should be obtained from at least three of the following: chimpanzees, gorillas, bonobos and orang-utans. However, the consensus sequence data for the TPH2 gene from gorillas, bonobos and orang-utans are not yet publicly available. Therefore the chimpanzee sequence was only used to determine the ancestral haplotype which was found to be "A-C-C-G-A". Haplotype "C-C-A-G-

A", with the highest frequency in all populations, is a 2-step mutation from the ancestral form. The ancestral haplotype was incorporated into simulated data and run through the eHap program. The results confirmed that all eHap cladogram output and analysis were correct for each population.

Table 5.2 Estimated five-locus haplotype frequencies in parents amongst twelve separate population samples

		Frequency (%) of each haplotype in parents per population											
Order	**Haplotype**	**Tel Aviv**	**Jerusalem**	**Spain**	**Belgium**	**Rotterdam**	**Essen**	**Goettingen**	**Zurich**	**London (1)**	**London (2)**	**Dublin Image**	**Ireland**
h1	C-C-A-G-A	53.7	61.6	68	64.7	56.6	53.6	54.7	61.3	55.8	56.7	49.8	53
h2	C-C-C-A-C	10.5	12.1	8	9.4	4	16.5	15.3	10.8	13.1	12	15	10
h3	A-A-A-G-C	13.1	12.1	6.5	5.9	10.5	11.3	8.5	16.2	11.8	9.4	12	8
h4	A-A-A-G-A	8.7	9.1	9	12.9	9.3	7.2	8	6.3	11.1	7.8	13	9
h5	C-C-A-G-C	5.2	4	5.5	2.4	3	2.1	5.5	5.4	2.1	6	4	4
h6	C-C-A-A-C	0	0	0	0	0	0	0	0	0	0	0	4
h7	C-C-C-G-A	0	0	0	0	0	0	0	0	0	0	0	3
h8	C-A-A-G-A	1.5	0	0	2.4	2.8	1	2	0	1.4	2.8	2	3
h9	A-A-A-A-A	2.9	2	1.5	1.2	2.1	3.1	2	0	1.7	2.2	2.5	2
h10	C-C-C-A-A	2	0	1.5	1.2	1	1	1.6	0	2.1	1.6	2	0
h11	C-A-A-G-C	1	0	0	0	1	1	1	1.8	1	0	0	0
h12	A-C-A-G-A	1	0	0	0	0	1	1	0	0	0	0	0
h13	A-A-A-A-C	0	0	0	0	0	1	1	0	0	0	0	0
h14	A-C-A-A-A	0	0	0	0	0	1	0	0	0	0	0	0
h15	A-C-C-A-C	0	0	0	0	0	0	0	0	0	1	0	0
h16	C-A-A-A-A	1	0	0	0	0	0	0	0	0	0	0	0
h17	C-C-A-A-A	0	0	0	0	0	0	0	0	0	0	0	1
h18	C-C-C-G-C	0	0	0	0	0	0	0	0	0	0	0	1

5.4.2. Results for Cladistic-based association analysis using TPH2 haplotypes

Across all populations, six of the 32 possible haplotypes reached a frequency of at least 1% in at least one population sample (Table 5.2). Discarding rare haplotypes (i.e. <1% in any population) reduces the number of haplotypes in the analyses and therefore decreases the degrees of freedom required. Haplotypes were used to construct an initial network which was then subdivided into clades using the results from the eHap program which incorporates the algorithm of Templeton et al. (1987). A full cladistic analysis was completed on all populations despite the similarity of haplotype frequency distributions as, in some cases, it can provide greater power to detect association and associated alleles differ between the populations. Five marker haplotype analyses using eHap showed an association between haplotype C-C-C-G-A and ADHD in the Irish population sample only. No other five marker haplotype was shown to be associated with ADHD in any of the other population samples, including the Dublin IMAGE sample. Therefore all results below reflect analysis of the Irish sample only.

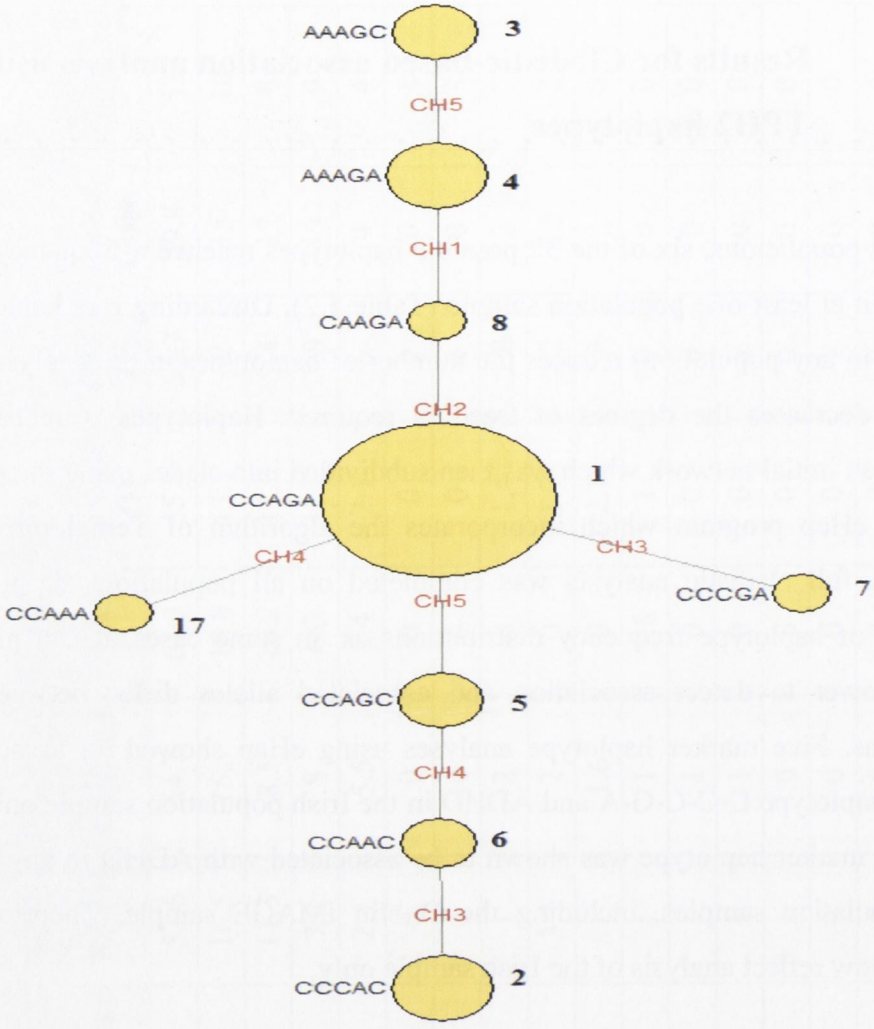


Figure 5.4 A Zero-step clade constructed using the haplotype frequencies from the Irish sample used throughout this thesis. The circles (nodes) represent the 10 most common haplotypes (frequency >1%) found in the Irish population sample and are numbered according to table 5.2. The circle size reflects the frequency of that haplotype in the population. The characters Ch and a number represent a single-step mutation at a specific SNP e.g. Ch1 means a change at position 1 of the haplotype etc. The individual haplotypes (1-8 and 17) occurring as nodes on the tree are referred to as zero-step clades. Zero-step refers to the fact that no analysis has yet been performed therefore there has been no grouping of similar haplotypes into larger clades. Most haplotypes in the cladogram are separated by a single mutational step, and with the exception of haplotype C-A-A-G-A, the most common haplotypes appear at the central nodes.

The initial step in the analysis is to compare all haplotypes on the tree to one another to test if any one individual haplotype has a relative risk equal to the nearest internal node. Each test has 1 df and is performed independently. If it is found that no single haplotype has more of an effect on the disorder than the other haplotypes then the cladogram-collapsing algorithm implemented in the eHap program should state that all haplotypes have the same relative risk (measured as score-test p-value). If the score test p-value is not significant the haplotype is collapsed into the nearest cluster in the following one-step clade.

Table 5.3 **Output from eHap for comparison of zero-step clades.**
T stands for terminal node. Numbers correspond to nodes in figure 5.4.

Cladogram:
8: (8) <-> [1, 4] (Comparing RR of haplotype 8 to that of haplotype 1 and 4) T17: (17) <-> [1] 6: (6) <-> [2, 5] 1: (1) <-> [7, 5, 17, 8] 5: (5) <-> [1, 6] 4: (4) <-> [8, 3] T2: (2) <-> [6] T7: (7) <-> [1] T3: (3) <-> [4]
Check 17 => 1 | 8 | 6 | 5 | 4 | 2 | 7 | 3 Score test p-value=0.1827: collapse
Check 2 => 6 | 8 | 17 | 1 | 5 | 4 | 7 | 3 Score test p-value=0.8975: collapse
Check 7 => 1 | 8 | 17 | 6 | 5 | 4 | 2 | 3 **Score test p-value=0.0024: reject**
Check 3 => 4 | 8 | 17 | 6 | 1 | 5 | 2 | 7 Score test p-value=0.0114: collapse

From the results above (highlighted in orange and bold), haplotype 7 (C-C-C-G-A) was found to have a significantly different relative risk to the other haplotype and the test is rejected. The haplotype is therefore not collapsed, i.e. it will not be grouped together with haplotypes with a similar relative risk into a clade and future analysis will test the relative risk of this haplotype against the relative risk of a clade of similar haplotypes (see one-step clade, Figure 5.5). Haplotype A-A-A-G-C also has a significant score test p-value of 0.0114 but is not rejected as the program concludes that any deleterious allele is likely to be associated with the clade exhibiting higher estimated levels of risk i.e. haplotype C-C-C-G-A.

The next step has conditions based on the previous results. Clades A, B and C represent one step clades which were produced by moving backward one mutational step from the zero-step clades towards the internal nodes to produce haplotype clades (Figure 5.5). Haplotypes 5 and 8 are both internal and as such can belong to two different one-step clades i.e. haplotype 5 is both one-step away from haplotype 1 and haplotype 6 and haplotype 8 is one-step away from haplotypes 1 and 4. Though they do not have significantly different relative risk to the surrounding haplotypes they are not included in the one-step clades, instead they are incorporated into the two-step clades in the next analysis. Haplotype 7, which was found to have significantly different relative risk to the remaining haplotypes, is left alone.

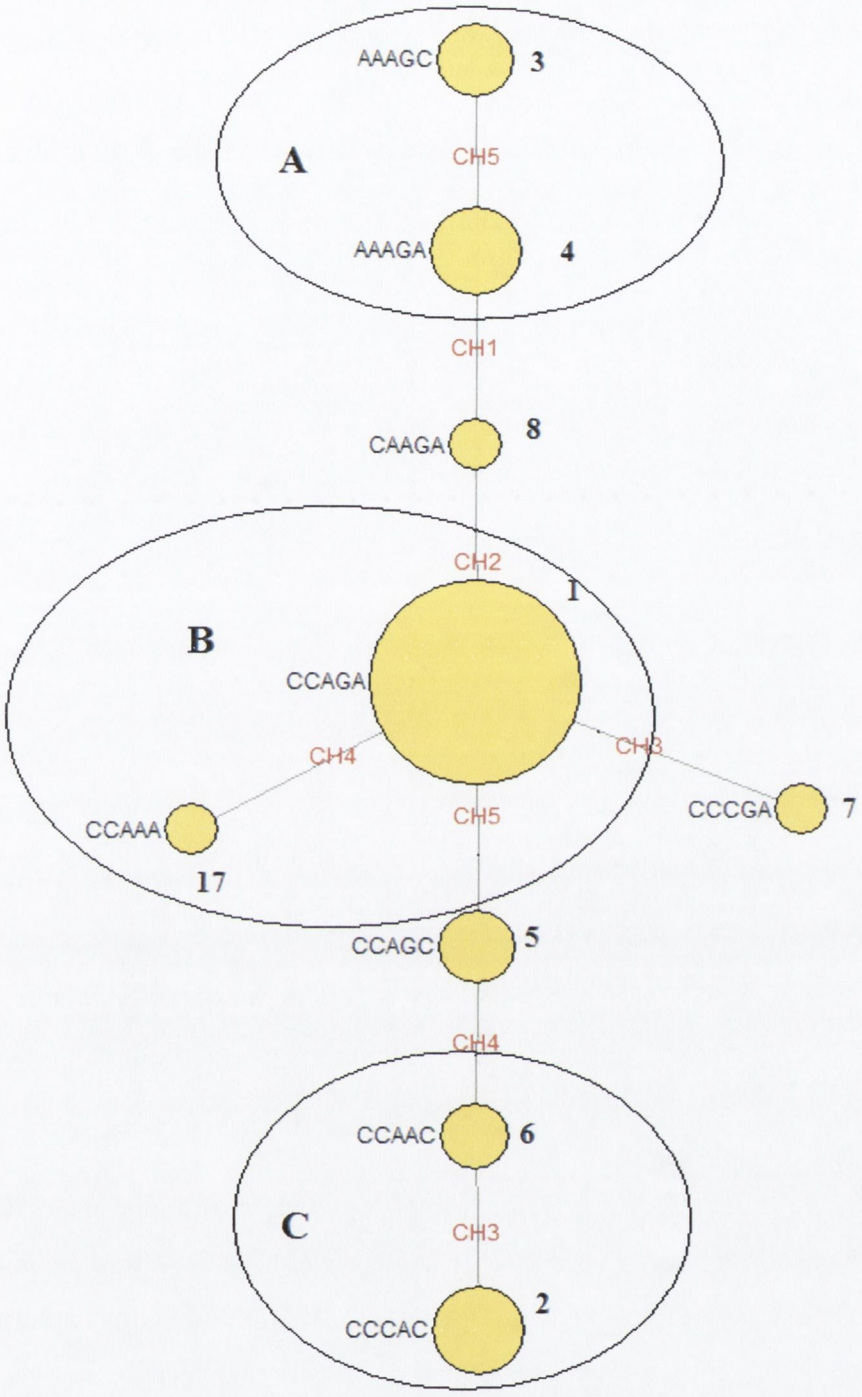


Figure 5.5 One-step cladogram.

The relative risk of haplotype clades A, B and C were compared to each other and to the single haplotypes 5, 7 and 8 to test for significant association with ADHD. All relative risks were declared to be equal therefore the one-step clades were further collapsed into two-step clades.

Table 5.4Output from eHap for one-step clade test.

Cladogram:
8: (8) <-> [1, 4] T6: (6, 2) <-> [5] 1: (1, 17) <-> [7, 5, 8] 5: (5) <-> [1, 6] T4: (4, 3) <-> [8] 7: (7) <-> [1]
Check 6, 2 => 5 | 1, 17 | 4, 3 | 8 | 7 Score test p-value=0.0927: collapse
Check 4, 3 => 8 | 6, 2 | 1, 17 | 5 | 7 Score test p-value=0.1440: collapse

The procedure is repeated to produce a two-step clade containing clades I and II. The clade containing haplotypes C-C-A-A-A and C-C-A-G-A was not collapsed into a two-step clade (Figure 5.6) with haplotype C-C-C-G-A as this haplotype was found to be significantly different to the rest in the zero-step analyses.

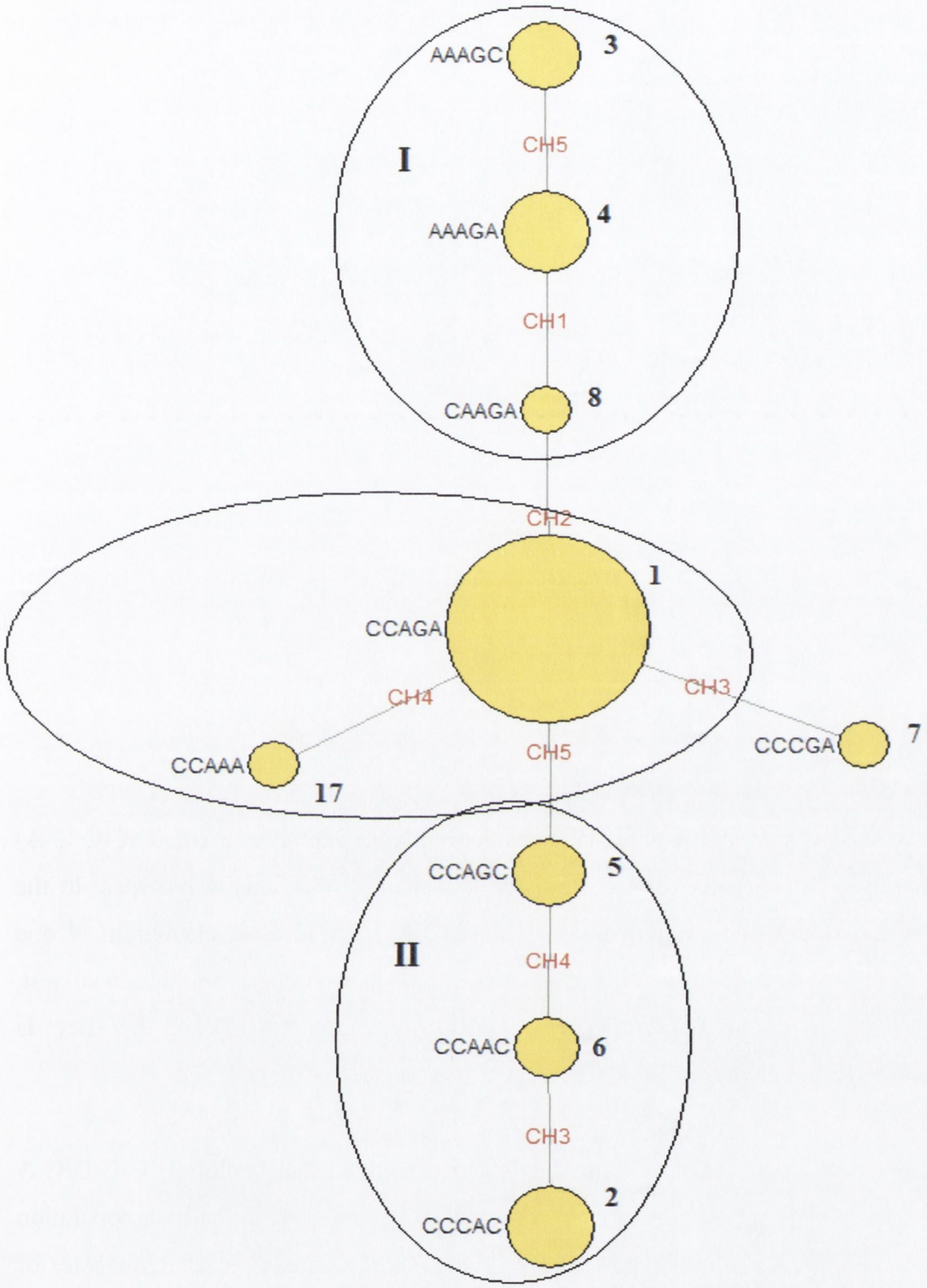


Figure 5.6 Two-step clade

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The results showed no association with either of the two-step clades (Table 5.5).

Table 5.5eHap output for analysis of two-step clades.

Cladogram:
T8: (8, 4, 3) <-> [1] 1: (1, 17) <-> [7, 5, 8] T5: (5, 6, 2) <-> [1] 7: (7) <-> [1]
Check 8, 4, 3 => 1, 17 | 5, 6, 2 | 7 Score test p-value=0.0823: collapse
Check 5, 6, 2 => 1, 17 | 8, 4, 3 | 7 Score test p-value=0.0507: collapse

The final output shows a three step cladogram with haplotype 7 (i.e. C-C-C-G-A) having a significantly different relative risk than all the other haplotypes. In the process of moving through the tests defined by this 10-node cladogram, 9 one degree of freedom tests were conducted, rather than a single omnibus 9 df test. Overall there is an association with ADHD (p-value = 0.0019) and the effect is attributed to haplotype C-C-C-G-A.

The overall analysis of twelve ADHD samples found that haplotype C-C-C-G-A was the only haplotype associated with ADHD and this was in the Irish population sample only. This haplotype was not found to be present, even at frequencies of <1%, in any other population sample from the IMAGE study therefore the results could not be replicated using the five-site analysis. However, LD values decrease between markers 1, 2 (rs4570625, rs2129575) and 4, 5 (rs1386493, rs1487275) in most populations (Figures 5.2 (a)-5.2 (l)). When recombination is present the LD values will be small and the evolution of haplotypes may not be reflected correctly in the cladogram. As recombination can affect construction of haplotype trees the markers were subdivided (as suggested by Seltman et al, 2001) and the analysis was rerun using markers 1-3 and 3-5 separately for all populations. The sample

from Ireland again highlighted haplotype C-C-C for markers 1-3 and C-G-A for markers 3-5. These two combined give the haplotype C-C-C-G-A, found to have a significantly different relative risk compared to all other haplotypes in the 5 marker analysis. Interestingly, the Dublin IMAGE sample also flagged the haplotype C-C-C as having a significantly different relative risk than the others. No other haplotypes or clades were separated out for any of the remaining populations.

5.5. Discussion

This study was undertaken to explain the conflicting findings between the IMAGE study and the findings reported in this thesis and narrow down the region of association between the TPH2 gene and ADHD. This was achieved through the use of an evolutionary-based association analysis using haplotype data from the two samples.

Conflicting association findings are not unique in the field of psychiatric genetics. Mutsuddi et al (2006) for example observed that risk alleles and haplotypes appear to differ between studies reporting associations with the DTNBP1 (Dysbindin) gene in schizophrenia. In studies where the same SNP was used there were instances in which the associated allele differed among samples. The differences between samples were attributed to differences in the genetic architecture of the sampled populations. A difference in LD between populations is a possible cause for conflicting patterns of association with a marker, especially when a non-causal marker is examined. However, LD analysis of all populations in this study found that LD structure was relatively similar across all populations in Europe and Israel, with the exception of Zurich (Figure 5.3). This could be attributed to the restrictive immigration policy imposed by Switzerland during World Wars 1 and 2 (1914-1945). In addition, during the 16th and 17th centuries Zurich adopted an aristocratic and isolationist attitude and built a second ring of city ramparts which were not pulled down until 1839. In contrast to this, Essen has a less uniform LD structure which is reflected in the haplotype frequencies of the parents from this sample. The population of Essen rose rapidly from 1818 to 1900 when the number of coal and ore mines in the area increased. This led to a population explosion from 4,496 inhabitants in 1818 to approximately 119,000 occupants by 1900 with people migrating to the area for work from Eastern Europe. A single haplotype tree can represent small portions of past historical events and gene-flow patterns though it is subject to error due to recombination events (Templeton, 2005). In addition, haplotypes provide information on evolutionary histories, beyond what can be learned from individual markers.

The haplotype frequencies of all twelve population samples are similar suggesting that each European and Israel-derived sample is genetically similar and that population differences cannot explain differences in results of single SNP association studies. Cladistic analysis groups haplotypes according to the closeness of their evolutionary relationships to test for association with a disorder at several levels of hierarchy. In this study it was possible to construct simple phylogenetic trees that explain the evolution of haplotypes occurring at a frequency of >1% in each population.

Given the results of this study it is feasible that the 5' region of the TPH2 gene, covered by the first three markers analysed (rs4570625, rs2129575 and rs1843809), contains a functional polymorphism that contributes risk to the development of ADHD in the Irish population. In support of this a recent study (Scheuch et al, 2007) reported that a polymorphism (rs11178996) in the putative promoter region results in a 22% decrease in TPH2 transcriptional activity in primary serotonergic neurons. This SNP is in high LD with four of the SNPs examined here (rs4570625, rs2129575, rs1843809 and rs1386493). It is possible that this variant may play a role in the aetiology of ADHD. A 22% decrease in TPH2 activity could lead to a decrease in serotonin biosynthesis which would lead to hyposerotonemia, a condition which is believed to, at least partly, play a role in the pathophysiology of ADHD. Lin et al (2007) reported that flip-flop associations can occur when an associated marker is linked with a functional variant at another locus. This may explain why allele C of marker rs1843809 exhibited a trend towards association with the IMAGE Dublin sample while allele A of the marker was observed to be highly significantly associated with the Irish sample.

The cause of the discrepancies between the findings of the IMAGE and this study has not been completely resolved despite the efforts to elucidate the underlying cause. It does however appear to confirm the association of TPH2 and ADHD in two separate samples from Ireland. Differences in environmental factors, ascertainment, and diagnosis could have lead to the original contradictory findings between the Irish sample and the IMAGE sample populations. In addition, all samples were of varying size. As some haplotypes are only carried by a few individuals, the small sample size of some of the populations complicates the assessment of their possible effect on susceptibility to the disorder. Because all the

samples have a similar genetic structure, the conflicting results between the IMAGE study and the results of this thesis cannot simply be attributed to population differences. It is also possible that the original association findings were false positive results. It is important to unambiguously confirm the association of TPH2 with ADHD and identify the risk alleles for the disorder. The IMAGE phase II sample might shed some light on the area. In addition future analyses employing this method in more diverse populations may help. The advantage to this study is that the first four markers are closely linked but to complete analysis of the entire gene may involve including additional markers between markers rs1386493 and rs1487275 which are 55kb apart.

The use of haplotypes (composed of multiple markers) distributed through and around the gene is a powerful tool for resolving the controversial issues of association studies based on individual polymorphisms. There is a growing amount of evidence that several polymorphisms in a gene can have an effect on phenotype which is a natural biological consequence of the induced change in the protein product. The total effect cannot be distinguished by sequential tests for effects in a series of polymorphisms. The exclusion of haplotypes of less than 1% frequency in any population should not affect the outcome of results as these haplotypes are younger than more frequent and centrally located haplotypes and may therefore have no major role in the transmission of ADHD.

Single locus associations may be confounded by other, functional, loci when numerous loci act simultaneously to cause a disease. In the Dublin IMAGE sample a trend toward association with the C allele of marker rs1843809 was observed while in the Irish sample a highly significant association was observed with the A allele of this marker. This marker is intronic so unlikely to have a direct effect on the TPH2 gene function. However, evolutionary-based haplotype analysis pointed to a region of the TPH2 in both samples which may harbour a functional allele. If multi-locus effect is in effect here then this may explain why both samples from an Irish population were found to have association with different alleles of the same marker.

Cladistic based analyses are pioneering techniques to detect an association between a candidate gene and a disorder and to localise susceptibility loci for the disorder. The number of conflicting reports of association of single markers at numerous loci with specific disorders suggests that attempts to find additional approaches to association analyses would be time well spent.

Chapter 6

General Discussion

6.1. Summary of major findings

This thesis describes an investigation of the role of serotonergic candidate genes in the aetiology of ADHD in an Irish sample using a range of molecular genetic and statistical methodologies. The focus was primarily on genes shown to be associated with ADHD in published studies, or those with strong *a priori* support to suggest a role in the aetiology of the disorder. TPH1, TPH2, 5-HTT, VMAT2, 5-HT1B, 5-HT2A, MAO-A and MAO-B were therefore identified as prospective serotonergic candidate genes for ADHD. These genes were investigated using a range of approaches, including genetic association studies, mutation screening, sub group analysis, and additional use was made of the multi-centre IMAGE sample.

Associations were identified with variants in several genes, most notably with TPH2 and MAO-A. TPH2 was considered to be a strong candidate gene from evidence from mouse knockout studies. A highly significant association was observed with marker rs1843809 in intron 5 of this gene and with several multi-marker haplotypes encompassing this marker, although there is no evidence to suggest that marker rs1843809 is functional given that it is an intronic marker. If the association is a true finding then it may be in LD with one or more functional variants in the gene.

Marker rs1843809 was subsequently found to be associated with ADHD in the IMAGE study. However, this association was with the opposite allele to the findings presented in this thesis. Further analyses were performed to try to explain this "flip-flop" association. This was achieved using a relatively unexplored technique of haplotype grouping that focuses on particular haplotypes or groups of haplotypes (also known as cladograms) and uses the evolutionary relationships between the haplotypes to assist in the discovery of risk variants for a disorder. This method highlighted the region surrounding marker rs1843809 as potentially harbouring a functional variant not yet identified. This finding emphasises the importance of this region of the TPH2 gene, at least in the Irish population. A recent study by Scheuch et al (2007) reported that a polymorphism in the putative promoter region of the gene results in a 22% decrease in TPH2 transcriptional activity in primary serotonergic neurons. This marker is in strong LD with marker rs1843809 which strengthens the reported association between ADHD and TPH2.

In addition, this finding adds evidence to support the hyposerotonergic theory of ADHD as a reduction in TPH2 transcriptional activity could lead to a decrease in serotonin levels in the brain.

Sequence analysis of exons 4 and 5 that surround marker rs1843809 revealed no novel DNA variants that could account for the association with ADHD. Recently, a complete sequence analysis of the 11 TPH2 exons revealed a novel, rare C-T substitution in exon 6 of the gene that results in a pro-ser change at position 206 (Cichon et al, 2007). The group reported the mutation to be significantly associated with bipolar disorder in samples from Russia and Germany. Although this marker was not examined in this thesis it is possible there is LD between this marker and marker rs1843809. This very recent development will need to be further explored. This finding indicates the possible importance of rare variants in the predisposition to psychiatric disorders, including ADHD. In support of this, recent analysis of the IMAGE sample showed a highly significant association of a rare variant (rs11568324) of the NET gene with ADHD ($p = 0.003$; Asherson et al, 2007). This finding has recently been confirmed in two additional samples in Ireland (Dr. Ziarah Hawi, personal communication) and in the USA (Kim et al, 2007). Future analysis of the TPH2 gene should consider sequence analysis of the coding as well as control regions to identify rare variants of the gene that potentially contribute to the development of ADHD.

Pharmacological and mice knockout studies indicated the importance of the MAO-A gene as a candidate for ADHD. A significant association was observed with the marker 941G/T in this study. Further support for this finding has recently been reported in a Taiwanese ADHD sample (Xu et al, 2007). This marker is a silent variant in exon 8 of the gene and has been reported to be associated with low (941T) and high (941G) MAO-A activity. The 941G allele was found to be associated with ADHD in this study which fits with the hyposerotonergic hypothesis of ADHD as high MAO-A activity should result in low serotonin levels in the brain.

Stratifying the genotype data from all genes by subtypes has shown that a refined phenotype may reveal association not apparent in the total sample. For example markers from VMAT2 and MAO-A were observed to be associated with the

inattentive subtype only. These findings are important considering the theory that the inattentive subtype should indeed be classified under ADHD or in a category of its own. Several markers that were not observed to be associated with the entire ADHD sample also exhibited an association/trend toward association when the sample was stratified based on the presence of co-morbid ODD (ADHD-ODD). This is particularly true in the case of 5HT1B (Chapter 3, section 3.5.5). Clearly, however, these findings are preliminary, are not corrected for multiple tests, and need to be replicated in other samples before being taken further.

Quantitative trait analysis of parent ratings of impulsivity for the TPH2 and 5-HTT genes showed that three markers of the TPH2 gene, not found to be associated with the categorical classification of ADHD, were associated with parent reported impulsivity scores. Possession of a C allele of any of these markers resulted in higher impulsivity ratings than possession of the T allele. These findings provide evidence for a trait specific relationship with these markers and ADHD.

6.2 Strengths and limitations of this study

There are strengths and limitations to all scientific research. These may depend on a number of factors including cost, time, availability and accessibility of necessary resources.

The strengths of this thesis are:

1. The use of a relatively homogenous sample
2. The stringent diagnostic methods applied to the sample
3. The reasonable coverage of the genes
4. The moderately large sample size compared to others in the field
5. The availability of the IMAGE data

The limitations of this study are:

1. The size of the sample given the small affect sizes being revealed
2. The exclusion of variants with frequencies less than 10%
3. The broad spectrum ADHD diagnosis
4. The issue of multiple testing

6.2.1 The Irish Sample

The clinical sample is the most important resource for any genetic association study. Ideally the sample should be homogenous and individuals with the particular phenotype under study should be drawn at random from the entire population (to exclude bias).

The sample used in this thesis was drawn from the Irish population. This should decrease genetic heterogeneity as Ireland is regarded as having a genetically relatively homogenous population due to its location on the periphery of Europe and the social history of limited migration into the country. The Great Famine in Ireland (1845-1849) resulted in large migration leading to genetic drift and gene flow out of the country. However, since then Ireland has experienced relatively little immigration of people of other ethnicities, until the last few years. An

advantage of a homogenous sample is that it may contain fewer susceptibility loci, with possible higher odd ratios than more genetically heterogeneous populations. In theory this should help in the search for causative variants of ADHD, in the Irish population at the very least.

For any complex disorder the method of diagnosis is extremely important. For the sample in this thesis, all ADHD individuals have been diagnosed according to DSM-IV criteria using the CAPA diagnostic instrument. The result is a reliable diagnosis of ADHD, leading to a more clinically homogenous sample than those using a less rigorous method of diagnosis. However, although this diagnosis is reliable it is still quite broad. The sample contains children diagnosed with all three categories of ADHD (hyperactive-impulsive, inattentive and combined types), as well as co-morbid disorders (ODD and CD). As seen in sections 3.5.5 and 3.5.6 in the case of the 5-HT1B markers rs1228814 and rs1157585 and the 5-HT2A marker rs927544, there is a significant over transmission of alleles to ADHD-ODD probands that was not observed in the overall sample. In addition, the analysis of the three subtypes of ADHD suggests that markers from the TPH2, VMAT2, MAO-A and MAO-B genes are only associated with the inattentive subtype of ADHD. These results suggest that a narrower, more refined phenotype may be more valuable to the search for ADHD risk variants than the broad DSM-IV diagnosis.

It is likely that there are a number of genes of small effect involved in the aetiology of ADHD. The addition of these effects and/or interaction between functional variants in these genes potentially leads to the development of ADHD. Detection of these variants requires large sample sizes. The collection and diagnosis of samples for neuropsychiatric disorders can be quite costly and time consuming and requires individuals specifically trained to diagnose the phenotype in question. In addition, there are a number of required ethical restraints involved when dealing with a childhood onset disorder such as ADHD. The Irish sample used in this study is relatively large and homogenous and thus is a valuable resource for the purpose of genetic association investigations. However, the complex nature of ADHD and the likely small effect size of susceptibility genes mean that definitive results are not easy to achieve. Therefore it is unsurprising

6.2.2. Gene Coverage

To ensure complete coverage of a gene in association studies requires genotyping a number of variants in the gene, including those in the promoter region and long-range enhancer regions. However, due to the difficulties in identifying these regions, few genes have been covered completely in any association study. Therefore, methods of selecting and prioritising variants are vital for association studies. Within this thesis, preference was given to genotyping any putative functional polymorphisms identified and to ensuring LD coverage.

The markers examined in this thesis were selected based upon their heterozygosity (those with the highest heterozygosity being the most informative), inter-marker distance and those that map to coding regions or that are close to intron-exon boundaries. In addition, markers examined by other investigators prior to this thesis were also selected for examination to confirm or exclude a reported association. It is therefore important to state that as no gene was completely covered, a lack of association found here does not eliminate the possibility that the genes tested contain other variants associated with ADHD.

The potential effect of long-range control of gene expression is also important to mention as it may mean that variants or haplotypes within or near the gene may not show association. It is not yet known how far upstream of a gene, markers should be placed to capture association by such enhancer sequences. These questions are inherent in candidate gene studies but are likely to be addressed in whole genome association studies currently underway.

6.2.3. Multiple testing

The issue of multiple testing is a much debated topic in psychiatric genetics. The theory behind multiple testing is that with every additional test performed on data the chances of a false positive result increases. To correct for this the p-value of a positive association should be corrected for the number of tests performed on the data. In addition there is an argument that the results should be corrected for the number of tests that *could* be performed as well. If the p-value is still significant (i.e. <0.05) after correction then the corresponding odds ratios and other statistical measures are regarded as reliable findings.

In this thesis, raw p-value results are presented for the initial data analysis and for the results of the additional tests performed. It is important that all studies not corrected for multiple testing are clearly reported as such so as to ensure that other researchers are not misguided. Replication of findings by other groups is an important procedure that can help identify false positive results. If a finding is replicated by an independent sample this increases the likelihood of it being a true association. Furthermore, a replication study of a very specific *a priori* hypothesis consists of only one test. This process is one way of dealing with the problem of multiple testing. However, the problem with replication studies is that to replicate a reported finding it is estimated that the second sample should be at least twice the size of the sample with the first reported association. As a result literature searches reveal a combination of significant and non significant reports of association between the variant in question and the disorder with no definitive answer. Possibly the best approach for this situation is a joint/meta analysis which will yield a sufficient sample size to definitively confirm or disprove a reported association.

6.3 General Conclusions and future directions

6.3.1 General conclusions

The results of this study add to the large body of work being carried out on the disorder at present. TPH2 is in particular an interesting candidate given its crucial role in serotonin biosynthesis.

The increasing efficiency and decreasing costs of genotyping means that the number of genes that can be analysed for any one disorder is rapidly growing. This in turn will produce results faster than before, which is an essential part of candidate gene and whole genome studies. Consequently, this could mean an increase in false positive and negative results. False positive results are an unavoidable issue in the field of complex genetic disorders. Replication studies and meta-analyses are techniques used to confirm initial associations or reveal false-positive results. Therefore inter group collaborations are extremely important in testing candidate genes. Large sample sizes are required to find associations with genes of small effect. In the case of ADHD there are presumed to be many genes involved in the aetiology of the disorder, each of moderate to small effect, and hence large sample sizes are crucial for genetic association tests.

ADHD phenotype is an important aspect that should be addressed in future studies. Using diagnostic criteria, according to DSM IV, an individual's diagnosis is dichotomised as being either affected or unaffected for ADHD. This qualitative approach may mask the quantitative nature of some of the component sub-phenotypes of the disorder. Refining the phenotype in secondary analyses of molecular genetic findings may help identify susceptibility genes that alter the phenotypic expression of ADHD. A section of chapter 4 (section 4.2) of this thesis focused on a quantitative trait approach to analyse parent ratings of impulsivity for two serotonergic candidate genes. ADHD is thought to reflect the extreme end of a set of behaviours that are distributed quantitatively in the general population. Therefore utilizing an approach that makes use of continuously distributed quantitative traits, such as impulsive behaviour, can provide the power to help

detect a relationship between specific characteristics of ADHD behaviour and variation in the genetic risk for the disorder.

6.3.2. Future Directions

A complex phenotype such as ADHD with multiple genetic and non-genetic factors acting together presents challenges to understanding the genetic structure of the disorder. This is why replication of association studies has been inconsistent and linkage scans have identified largely non-overlapping chromosomal regions as potentially harbouring susceptibility genes. These issues stand in the way of gaining true insight into the molecular genetics of ADHD. These problems may be due to the heterogeneity of methods used to analyse serotonergic genes in relation to ADHD and low statistical power to detect genes of small effect, common setbacks to complex, polygenic disorders such as ADHD. Utilizing endophenotypes or latent class analyses, to define subtypes of ADHD, is a potential solution to address these problems.

6.3.2.1. Endophenotypes and Latent Class Analysis

Endophenotypes offer the potential approach to generating more homogenous subtypes of the current ADHD diagnosis. An endophenotype may be described as "a phenotype that is more proximal to the biological etiology of a clinical disorder that its signs or symptoms and is influenced by one or more of the same susceptibility genes" (Doyle et al, 2005). It is thought that an endophenotype that underlies a disorder will be less genetically complex than the disorder itself. Because they are thought to be influenced by fewer genes than ADHD as a whole, this should, in theory, result in a greater statistical power to detect the effects of a specific gene. This could lead to a greater understanding of the disorder at the molecular genetic level and detection of the genetic variants that contribute to the aetiology of the disorder.

Latent class analysis (LCA) has also been suggested as a means to refine the ADHD phenotype through the use of symptoms to separate individuals into subtypes. The ensuing subtypes contain individuals that are phenotypically

homogenous that may share a common genetic risk factor for ADHD symptom expression. LCAs include the three DSM-IV subtypes but also reportedly include six to eight clusters underlying the overall ADHD phenotype. Hudziak et al (1998) defined eight latent classes through the analysis of female twins and these results have been replicated in other samples (Neuman et al, 2001; Rasmussen et al, 2002). In addition they have proven to be independently transmitted, familial, and heritable (Todd et al, 2001; Rasmussen et al, 2004). Though these classes have not been applied to clinical samples and could possibly be biased by population types, latent class analysis could provide more homogenous ADHD subtypes for candidate gene studies.

6.3.2.2. Future association and functional studies

The completion of the human genome project has lead to an effort to record variation across the genome primarily focusing on SNPs. An increase in available markers should benefit the search for susceptibility genes for polygenic disorders such as ADHD. Freely available and easily accessible marker databases online makes the investigation of specific genes both quicker and easier. However, many of the polymorphisms in these databases are unconfirmed, numerous SNPs occur at low frequencies, others are only present in specific populations, and others don't exist at all and are merely a result of sequencing errors. In addition, it is probable that there are still many polymorphisms yet to be discovered. It is also reasonable to assume that disease causing variants are present at much higher frequencies in or are specific to affected individuals and therefore may not be detected in scans of unaffected individuals. Therefore mutation screening remains a vital component of candidate gene studies. Efficient novel SNP genotyping methodologies and high-throughput capillary-based fluorescent genotyping machines mean that the major limits on association studies are now sample collection, data analysis, and financial. Many of the genes that have been reported to lack association with ADHD may have been overlooked too early on the basis of a lack of association with a single SNP. More thorough studies of these genes may be required. This may be addressed in whole genome studies but the coverage of candidate genes may not be complete. In addition, high throughput sequencing is likely to be required to properly assess the multiple rare variant hypothesis.

Expression work is an essential follow on step for association testing. Once a gene has been found to be associated with the disorder the focus should shift to clarify how the associated variants affect gene function. The variant may not affect function itself but may be in LD with a functional variant that increases risk for the disorder. Chapter 5 of this thesis examined a method that uses the evolutionary relationships between haplotypes to aid the discovery of risk variants for a disorder. This method assumes that haplotypes that are associated with a disorder will contain many of the same variants and therefore might induce similar effects on disease predisposition. This should help direct the search for functional variants by pinpointing a specific region of a gene as harbouring a potential functional variant and therefore reduce the amount of investigation for functional variants in a gene found to be associated with ADHD.

6.3.2.3. Serotonin and Brain Development

Chapter 3 of this thesis reports on several serotonergic candidate genes that have been observed to be associated with ADHD. Research has shown that serotonin levels are lower in ADHD individuals, which is thought to be a result of genetic risk variants. However, future research into serotonergic functioning in ADHD probands could involve examination of the role of serotonin during brain development. Serotonin regulates both the development of serotonergic neurons and the development of serotonergic target tissue and is thought to help guide the development of the other monoamines (e.g. dopamine and noradrenalin). A disruption in this process can result in lasting changes in the development and function of the brain and of behaviour (Whitaker-Azmitia, 2001). The right prefrontal cortex has been shown to be smaller in children with ADHD (Castellanos et al, 1996) possibly indicative of disruption in brain development. The removal of serotonin during early foetal development has been shown to result in a permanent reduction in the quantity of neurons in the hippocampus and cortex of the adult brain of rats (Lauder and Krebs, 1976). This group also observed that drugs and stress can interfere with the development of serotonergic circuitry by serotonin during the gestational stage when monoamines are required as humoral signals during pre- and potentially postnatal development in rats and chicks (Lauder et al, 1981). In addition, nicotine and alcohol have also been reported to alter the balance of expression of 5-HT receptors in rats and primates (Slotkin et

al, 2007; Macri et al, 2007). These findings are also important in relation to gene-environment interactions in ADHD.

6.3.2.4. Gene-Environment and Gene-Gene Interactions

As discussed in chapter 1 of this thesis (section 1.2.2), environment plays a role in the aetiology of ADHD and the casual process that leads to the development of ADHD is likely to be influenced by gene-environment interaction. Variation in exposure to maternal alcohol consumption and smoking during pregnancy are environmental factors of interest. The detection of specific genetic risks for gene-environment interactions should be helped by focussing on groups at high risk of environmental exposure. For example, a study by Young et al (2006) reported that low MAO-A activity is associated with an increased risk for antisocial behaviour resulting from maltreatment in the first 10 years of the patients life.

Gene-gene interaction studies may also be important in untangling and helping resolve the complex nature of ADHD. ADHD is a complex disorder and is most likely that a number of genes are involved in the aetiology of the disorder with each of the genes having a small effect on the phenotype. It is therefore necessary to consider the simultaneous effect of several SNPs at different loci on the ADHD phenotype. There are two aims to gene-gene interaction: (1) to identify SNPs that are associated with the phenotype and (2) to classify affected individuals based on genotype in a manner that captures information about risk of disease. It would be interesting to explore potential gene-gene interactions across the serotonergic neurotransmitter system and examine the interaction of TPH2 in relation to some of the dopamine candidate genes (e.g. DAT1, DRD4 and DRD5) as both serotonin and dopamine are known to interact with each other. It is hypothesised that serotonin has a regulatory role over dopamine neurotransmission. Therefore any disruption of serotonin function will affect the dopamine system which will in turn affect dopamine mediated behaviours (Quist and Kennedy, 2001). Serotonin neurons have been shown to project to dopamine cell bodies that are located in midbrain regions and to dopamine terminals in the prefrontal cortex. These serotonergic innervations allow serotonin to functionally regulate dopamine release and neuronal firing. Increasing serotonin levels in mice that have the dopamine transporter knocked out (DAT-KO) have suggested that the increase balances the

high dopamine levels that result from absence of the transporter. This is an important finding given that Methylphenidate (MPH, Ritalin®) has been shown to block DAT1 and suggests the potential additional use of SSRIs in ADHD treatment considering that MPH is not efficacious in approximately 25-35% of ADHD probands (Quist and Kennedy, 2001)

6.3.2.5. Future Pharmacogenetic studies

The eventual aim of molecular genetic studies of complex disorders like ADHD is the complete understanding of biological basis of the disorder with a view, *inter alia*, to developing pharmacologic therapies that are beneficial to the majority of patients. Methylphenidate is the most common medication prescribed for ADHD symptoms but it is not efficacious in all cases and therefore possibly does not deal with the basic underlying aetiology of the disorder. The area of pharmacogenetics (the study of heritable differences in response to medication) requires more research as a study has shown that ADHD probands with the DAT1 10-repeat allele may respond better to stimulant drugs than probands with the 9-repeat allele (Kirley et al, 2003).

6.3.3 Final Word

The search for genetic risk factors that are involved in the aetiology of ADHD has seen considerable advances over the past number of years. This thesis underlines some of the current methods being used to reveal risk genes for ADHD and other complex psychiatric disorders. In conclusion future studies should look to increasing sample sizes through collaborative work between labs and consider focusing on refinement of phenotypes. An increase in understanding of the loci associated with ADHD will lead the field towards functional studies. Research into gene function and how they interact with other genes and the environment should increase the understanding of the biological underpinnings of this complex disorder.

The work presented would not be possible without the participation of the families who have donated their DNA and time to make these studies possible. The way forward for investigations into the aetiology of ADHD will, hopefully, through intelligent experimental design and some measure of insight, bear fruit. The hope and expectation of researchers in this field is that the advances can one day be of direct benefit to the patients and their families.

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Appendix 1

Reagents and Solutions

1.1. DNA Extraction Buffers

Lysis Buffer (10X)

- Sucrose 547.7g
- 2M Tris (pH 7.5) 25ml
- 1M $MgCl_2$ 25ml
- Triton X100 50ml
- H_2O up to 1L

Suspension Buffer

- 2M Tris (pH 7.5) 5ml
- 0.5M EDTA (pH 8.0) 20ml
- 5M NaCl 2ml
- H_2O up to 100ml

TE Buffer

- 2M Tris (pH 7.5) 1ml
- 0.5M EDTA (pH 8.0) 40µl
- H_2O up to 200ml

1.2. PCR Reagents

MgCl2 Buffer

- 2M KCl 2.5ml
- 1M Tris (pH 8.9) 1ml
- 1M $MgCl_2$ 150µl
- Triton X100 100µl
- H_2O 3.75ml

1.3. Gel electrophoresis and visualisation

TAE Buffer

- Trizma Base 141.1g
- 0.5M EDTA (pH8.0) 50ml
- Glacial acetic acid 60ml
- H_2O up to 2L

SNaPshot Buffer

- 1M Tris (pH9.0) 8ml
- 2mM $MgCl_2$ 8ml
- H_2O 34ml

Appendix II

Amplification and SNaPshot™ conditions

This Appendix lists the primers used for each variant genotyped in this thesis.

PCR primers are labelled 'F' for forward primers and 'R' for reverse primers. They are orientated 5' to 3'. All primers were designed using the Primer3 programme (www.es.embet.org/cgi-bin/primer3_ww.cgi). SNaPshot primers were designed by hand using the surrounding sequence of the SNP. These primers are labelled 'e' and listed in the same fashion as the PCR primers. All primers were ordered from invitrogen (www.invitrogen.com).

All variants (excluding the SERT SNPs) were amplified using the following conditions: an initial denaturing step at 95°C for 3mins 30secs followed by approximately 30 cycles at 95°C for 30secs, annealing temperature for 30secs and 72°C for 30secs. A final extension step at 72°C for 5min ended the programme.

Table I **Primer sequences and conditions for TPH1**

Marker	Forward Primer	Reverse Primer	Annealing Temperature	Genotyping method
rs4537731	TGGCATTGAAGTAAGAGCAC	GTTTCATGCAGGTATTAGTG	54°C	RFLP

Table II **RFLP conditions for TPH1**

Marker	Restriction Enzyme	Enzyme Incubation Conditions	Gel Type	Concentration of Gel	Fragment Sizes (bp)
rs4537731	*Sau3A1*	37°C overnight in a water bath	Agarose	2.5%	280 (G) 194 + 86 (A)

Table III **Primer Sequences and conditions for TPH2 variants. All variants were analyzed using the SNaPshot technique**

Marker	Forward Primer	Reverse Primer	Extension Primer
rs2129575	ACTGGAAAGCATTTGGCAAG	GGGAGCATTTGGAAATAGGA	CAATGCCTGGACACTAAA
rs1386488	TTGACTGCAGGCCATGTTTA	CCATGTGGTCCTGGTTTTTA	GTGTGGGCTTTGTTTATAAAAAGGGATTTGAC
rs10879345	TGCTTCATCTGGGAAGTGTG	TGGGGAGGAATATGACTGCT	TAATACGCTTGTTTATATGCCATTTACAAATAATGTAGCT
rs1843809	AAAGAGGCCCTGAGCTCCTA	CATGAGGTTCATGGGCTACC	TTAAGCAAACAAGAGTGG
rs1386496	GTGGTAGAGCGGGAATTCAA	AAGCAATCTGTCTGCCTTGG	CTGCCACATCTTCTGCTA

rs1386494	CAAATTGAATGTGCCTGCTG	ATCAATTGCCAGGGATGTGT	GATAAGCTAGCAACTAGTAGTAAAAACC
rs1386493	GAAATTCCCAGTCGCCAATA	AGTGAAATGGGTGGGTGAAT	TGATTATCCTACAGCTCATGGTC
rs1023990	ACCATCCTCTTGGTGCTGTC	ATCTGTTTCTGGGGGACCTC	TCCCTTCGCCATGTGAAGTGTTC
rs1386497	CAAGAGCCACATACCATCCA	CATGCATGGTATCCAACAGG	GATAGTCTTGAAAAAAAA
rs1487278	GGCCCCTGGAGAGTTTCTTA	TCCAAGGAGAAGCTCGACAT	TCTAACTTTCAACAACTCACGTT
rs1487275	GGGCCCTTTTAAGATGGATT	TGGGCTGCTTTTTGGACATA	AACATAACAAAAAGTTATAGGCTAAATT
rs1487279	TTGCTGTGGGGGAAAAAGTA	GGGGCTAAATTTTCCTGAGA	CATATTTATTTTGTTTCTATCTTCAAAAAGAAT

Table IV Primer sequences and conditions for 5-HTT variants

Name	Forward Primer	Reverse Primer	Annealing Temperature	Genotyping method
5-HTTLPR	GGCGTTGCCGCTCTGAATGC	GAGGGACTGAGCTGGACAACCAC	62°C for 2 cycles then 61°C for 34 cycles	Size discrimination
Intron 2 VNTR	GTCAGTATCACAGGCTGCGAG	TGTTCCTAGTCTTACGCCAGTG	62°C	Size discrimination
3'UTR SNP	CCGCTTGAATGCTGTGTAACACAC	GTACCCTTCCAATAATAACCTCC	56°C	RFLP

Table V Genotyping conditions for 5-HTT markers

Marker	Restriction Enzyme	Enzyme Incubation Conditions	Gel Type	Concentration of Gel	Fragment Sizes (bp)
5-HTTLPR	*N/A*	N/A	Agarose	3%	484 – Short allele 528 – Long allele
Intron 2 VNTR	*N/A*	N/A	Agarose	3%	250 – 9 Repeat 267 – 10 Repeat 301 – 12 Repeat
3'UTR SNP	*Mse I*	37°C overnight in a water bath	Agarose	3%	741 (G) 689 + 52 (T)

Table VI Primer Sequences and conditions for 5-HT1B variants

Name	Forward Primer	Reverse Primer	Extension Primer	Annealing Temperature
T-261G	*TCAAAGCCTTCTCCTCAAGC	*CAGGTTTGTCCCCAGTTGAT	GCTCTTAGCAACCCAGG	56°C
-182Ins/ -181Del	*TCAAAGCCTTCTCCTCAAGC	*CAGGTTTGTCCCCAGTTGAT	GGGTGCGGCAGCCAAGGCAG	56°C
A-161T	*TCAAAGCCTTCTCCTCAAGC	*CAGGTTTGTCCCCAGTTGAT	TGCCTTGGCTGCCGCACCC	56°C
rs1228814	GGACCGGACCTGGACTCTAT	CTCTGCCCTCCCTCTCTTTT	CGAGCAGCCGCAACTCCAGC	59°C
G861C	AAAGATGCCTGCTGGTTCC	CTGGCTTCTCAGGATCCATT	N/A	55°C
rs6297	AAAGATGCCTGCTGGTTCC	CTGGCTTCTCAGGATCCATT	AGGCGACCCCACTGCAAA	55°C

*PCR amplification of the promoter region encompassing all three SNPs

Table VII RFLP methods for 5-HT1B variants

Marker	Restriction Enzyme	Enzyme Incubation Conditions	Gel Type	Concentration of Gel	Fragment Sizes (bp)
G861C	*Hin*CII	60°C for 35 rounds	Agarose	2.5%	452 + 96 (C) 310 + 142 + 96 (G)
-182Ins/-181Del	BsaXI	37°C for 4 hours	Agarose	2.5%	Cuts at positions 164, 194 if deletion present

Table VIII Primer sequences and conditions for MAO-A variants

Name	Forward Primer	Reverse Primer	Annealing Temperature	Genotyping Method	Restriction Enzyme
30bp VNTR	CCCAGGCTGCTCCAGAAAC	GGACCTGGGCAGTTGTGC	56°C		-
CA(n) Repeat	AGAGACTAGACAAGTTGCAC	CACTATCTTGTTAGCTCACT	56°C	semi automated florescent method	-
941G/T	GACCTTGACTGCCAAGAT	CTTCTCCTTCCAGAAGGCC	54°C	RFLP	*Fnu4HI*
rs979605	GCTGCTACACGGCCTACTTC	AGAAATGGGGATTTTGACAAC	64°C	RFLP	*TspRI*

Table IX Primer sequences and conditions for MAO-B variants

Name	Forward Primer	Reverse Primer	Annealing Temperature	Genotyping method
rs1799836	(T):CACTGGCAAATAGCAAAAGT (C):CACTGGCAAATAGCAAAAGC	GGATTTACTTTGCAGGCACC	64°C	Allele specific PCR
CA(n) Repeat	GTGTTGGTGTGAAGGAAGCA	GATTGAGTAAGAGGGAAATGG	64°C	Semi automated florescent method ABI 3100

Appendix III

Analysis software – input files and operating commands

1.1 Haploview File Structure

This software is available for downloading at http://www.broad.mit.edu/mpg/Haploview/index.php. Files are created using the "phased structure".

1.1.1 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. Input file is the same as that used for TRANSMIT (see section 1.2 below)

1.1.2 Additional Haploview Files

An additional file known, as a map file can also be included, however it is not necessary. This file is created in excel and saved as a text tab delimited file:

- Column 1: Marker Name (in rs format or other as necessary).
- Column 2: Position (provides the physical position of the variant).

An example of an input file with three variants:

```
rs1111    1000
rs2222    2000
rs3333    3000
```

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

1.2 TRANSMIT

TRANSMIT was run on the HGMP website (http://www.hgmp.mrc.ac.uk/) using the GLUE (a graphical interface) application. Files were set up as follows in a ped file format in Excel and saved as a text (tab delimited) file:

- Column 1: family id (same for all members of the family).
- Column 2: individual id within the family (i.e. 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 = unknown, 1 = unaffected, 2 = affected).
- Column 7: variant 1, allele 1 (alleles must has individual numbers, 1 = A; 2 = C; 3 = G; 4 = T; 0 = no data).
- Column 8: variant 1, allele 2.

Subsequent columns may contain additional variants if available.

An example of an input file with two variants:

94	1	0	0	1	0	0	0	2	2
94	2	0	0	2	0	2	2	2	2
94	3	1	2	1	2	2	2	2	2
95	1	0	0	1	0	1	2	1	1
95	2	0	0	2	0	1	2	2	2
95	3	1	2	2	2	1	2	1	2

Using the GLUE interface, the number of markers to be included in each analysis, markers to be excluded, sex of transmitting parent etc, can be selected before analysis.

1.3. ehap (evolutionary-based haplotype analysis) programme

The software is available for downloading at http://wpicr.wpic.pitt.edu/wpiccomp.gen/. There are two distinct programmes:

1. eHap - determines the initial cladogram and haplotypes.
2. HapLinMod – statistical program that determines the final cladogram and the estimated regression coefficients.

The interface between the two is seamless and most of the interaction with the programs is through eHap.

There are three input files required

1. Pedigree file in pre-linkage format (see Transmit input files above, see section 1.2).
2. Locus file: specifies the number of marker loci genotyped (as opposed to the usual pre-linkage file, which also counts a disease locus), the number of alleles per locus and their names. For example if there are three markers typed, with alleles 1/2, 1/2 and 1/2. Then the file would be organized as follows:

   3
   2
   1 2
   2
   1 2
   2
   1 2

3. Control file: eHap implements a large number of analyses by using directions in the control file and the contents of several data files.

Contents of the control file:

 # Files

pedfile = [pedigree.txt]	pedigree file.
lfile = [Locus.txt]	locus file.
cladefile = [clade.txt]	cladogram output file.
out = [rslt.txt]	file name for HapLinMod standard output.
err = [err.txt]	HapLinMod error file.
eerror = [error.txt]	file containing non-Mendelian configurations of haplotypes.
ehap=[eHap.txt]	collapsed cladogram file passed from HapLinMod to eHap.

#Settings

#input data

transmit = [1]	for TDT tests use 1, otherwise use 0.
generation = [1]	to analyze the phenotypes of the parental generation use 0, otherwise use 1. If transmit = 1, generation must = 1.
ocv = [affection]	outcome variable to use for this analysis.

#eHap

zerocut = [0.01]	cutoff for eliminating haplotypes by frequency (haplotypes with estimated frequency less than zerocut are treated as if they had frequency 0).
mdist = [1]	minimum distance for connection in terms of mutation.

#HapLinMod

verbose = [1]	verbosity of HapLinMod (0 = show only final cladogram / 1 = also show tests and p-values / 2 = additional detail including option and column id settings / -1 = quiet mode (p-values only)).
family = [tdt]	GLM family type (normal/poisson/binomial/ tdt).

method = [sc]	analysis method (score/LR). Specify type of hypothesis test.