THE ROLE OF DOPAMINE-RELATED GENES IN AUTISM SPECTRUM DISORDERS: EVIDENCE FOR SPECIFIC GENES AND RISK FOR ASD IN FAMILIES WITH AFFECTED MALES

by

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Abstract

Individuals with autism spectrum disorders (ASDs) are impaired in cognitive processes and emotional regulation, and exhibit stereotyped behaviours. Dopamine (DA) modulates executive functions, learning, memory, emotional processing and social cognition; all of which are impaired in individuals with ASDs. Because DA modulates a number of processes that are impaired in individuals with ASDs, genes in the dopaminergic pathway are good candidates for genes influencing autistic behaviours. As our previous findings suggested a role for a dopamine-related gene in families with only affected males, this thesis describes a comprehensive study of five genes affecting DA synthesis, levels and function in mothers and affected males with ASDs in an initial TEST cohort of 112 male-only affected sib-pair families as well as a replication study in three additional male-only family cohorts. I genotyped three to five polymorphisms in the *TH*, *SLC6A3*, *DRD1*, *DRD2* and *PPP1R1B* genes and performed population-based single marker case-control comparisons, family-based association tests, quantitative transmission disequilibrium tests as well as haplotype-based analyses and tests for gene-gene interactions. I found evidence for association of the *DRD1* (P=0.0027-0.040), *DRD2* (P=0.0002-0.007) and *PPP1R1B* (P=0.00042-0.001) genes with autism in affected males from the TEST cohort. Evidence for DA-related gene interactions were found between polymorphisms in *DRD1*, *DRD2* and *PPP1R1B* (P=0.0094-0.012) in affected males relative to a comparison group. Furthermore, I found that polymorphisms in the *TH* and *DRD1* genes were associated with the risk for mothers having sons with ASD in the TEST families (P=0.007-0.025) and putative risk alleles in *DRD1* and *DRD2* were preferentially transmitted from

mothers (P=0.016) and fathers (P=0.023) respectively, to affected children. All findings remained significant following corrections for multiple testing. The TEST cohort findings were not replicated in other family cohorts. However, an examination of dysmorphology data for the different family sets revealed phenotypic differences and thus, genetic differences are to be expected. In summary, I found evidence for a contribution of DA-related genes in a specific family cohort with ASDs. Additional functional and phenotypic studies will enable a better understanding of the contributions and implications of these findings to our understanding of autism.

Co-Authorship

I am responsible for the majority of the experimental design, data collection, and analysis in this thesis. I produced the first draft of this thesis with subsequent drafts incorporating input from my supervisor, Dr. Jeanette J.A. Holden. As my supervisor, Dr. Holden had an essential and invaluable role during my studies. Cuiling Zhang aided in the data collection for one of the twenty-one polymorphisms examined in my study; she genotyped the HUMTH01 polymorphism in individuals from 74 families from the TEST cohort.

In loving memory of my grandparents, Joseph and Mary Hettinger,

Who shaped my past,

And

To my girlies, Dina, Stephany and Melanie,

Who are my present and my future.

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5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine (serotonin)
6-OHDA.....	6-hydroxydopamine
10i	imperfect 10-repeat allele
10p.....	perfect 10-repeat allele
A.....	adenine
ABC	Autism Behavior Checklist
ACC	anterior cingulate cortex
ADDM	Autism and Developmental Disabilities Monitoring Network
ADHD	attention-deficit hyperactivity disorder
ADI	Autism Diagnostic Interview
ADI-R	Autism Diagnostic Interview-Revised
ADOS.....	Autism Diagnostic Observation Schedule
ADOS-G	Autism Diagnostic Observation Schedule -Generic
AGRE.....	Autism Genetic Resource Exchange
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ARPP-21	human cAMP-regulated phosphoprotein (M_r 32kDa) gene
Arpp-21	mouse cAMP-regulated phosphoprotein (M_r 32kDa) gene
ASD.....	autism spectrum disorder
ASD-CARC	Autism Spectrum Disorders-Canadian American Research Consortium
BH	Benjamini and Hochberg method
bp.....	base pair
ºC.....	degrees Celsius
C.....	cytosine
cAMP	3'-5'-cyclic adenosine monophosphate

CARS	Childhood Autism Rating Scale
CDD	childhood disintegrative disorder
cDNA	complementary deoxyribonucleic acid
CGH	comparative genomic hybridization
CGI.....	Clinical Global Impression Scale
CI.....	confidence interval
CNV	copy number variation
CSF	cerebrospinal fluid
DA.....	dopamine
DAceptive	dopaminoceptive
DAergic.....	dopaminergic
DARPP-32	dopamine- and cAMP-regulated phosphoprotein of molecular weight 32kDa
DBH	dopamine-β-hydroxylase
dbSNP	database of single nucleotide polymorphisms
dCTP	deoxycytidine triphosphate
DNA.....	deoxyribonucleic acid
DRD1	human dopamine D1 receptor gene
Drd1a	mouse dopamine D1 receptor gene
DRD2	human dopamine D2 receptor gene
Drd2	mouse dopamine D2 receptor gene
Drd5	mouse dopamine D5 receptor gene
DSM-III.....	Diagnostic and Statistical Manual, Third Edition
DSM-III-R.....	Diagnostic and Statistical Manual, Third Edition - Revised
DSM-IV	Diagnostic and Statistical Manual, Fourth Edition
DSM-IV-TR.....	Diagnostic and Statistical Manual, Fourth Edition – Text Revision

DZ ..... dizygotic

EPI..... epinephrine

ETDT ..... extended-transmission disequilibrium test

F ..... forward primer

FBAT ..... family-based association test

FDR..... false discovery rate

fMRI..... functional magnetic resonance imaging

G..... guanine

GABA ..... γ-aminobutyric acid

GI ..... gastrointestinal

GP ..... globus pallidus

htSNP ..... haplotype-tagged SNP

HVA..... homovanillic acid

HWE ..... Hardy-Weinberg equilibrium

ICD-10 ..... International Classification of Diseases, Tenth Revision

ID/ED..... Intradimensional/Extradimensional

IMGSAC..... International Molecular Genetic Study of Autism Consortium

Ins/Del..... insertion/deletion

kb..... kilobase

kDa..... kiloDaltons

L ..... long (insertion) allele

L-AADC..... human L-aromatic amino-acid decarboxylase gene

L-DOPA..... L-dihydroxyphenylalanine

LD ..... linkage disequilibrium

LTD..... long-term depression

LTP ..... long-term potentiation

MAF..... minor allele frequency

$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
min	minute
MLS	maximum lod score
mM	millimolar
MPX	multiplex (multiple-incidence)
M_r	molecular weight
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MSH	α-melanocyte stimulating hormone
MSN	medium spiny neuron
MZ	monozygotic
NAc	nucleus accumbens
NCBI	National Center for Biotechnology Information
NE	norepinephrine
ng	nanogram
NMDA	N-methyl-D-aspartate
OCD	obsessive compulsive disorder
OFC	orbitofrontal cortex
OR	odds ratio
PCR	polymerase chain reaction
pDARPP-32	phosphorylated dopamine- and cAMP-regulated phosphoprotein of molecular weight 32kDa
PDD	pervasive developmental disorder
PDD-NOS	pervasive developmental disorder-not otherwise specified
PDDBI	Pervasive Developmental Disorder Behavior Inventory
PET	positron emission tomography

PFC ..... prefrontal cortex

PKA..... protein kinase A

PKU..... phenylketonuria

PNMT ..... phenylethanolamine N-methyltransferase

PPP1R1B ..... human protein phosphatase 1, regulatory subunit 1B gene

Ppp1r1b..... mouse protein phosphatase 1, regulatory subunit 1B gene

QTDT..... quantitative transmission disequilibrium test

R..... reverse primer

rCBF..... regional cerebral blood flow

RFLP ..... restriction fragment-length polymorphism

RRA ..... retrorubral area

S ..... short (deleted) allele

sec ..... second

SLC6A3 ..... human dopamine transporter gene

Slc6a3..... mouse dopamine transporter gene

SNc..... substantia nigra pars compacta

SNP ..... single nucleotide polymorphism

SPX ..... simplex (single-incidence)

SSRI ..... selective serotonin reuptake inhibitor

STh..... subthalamic nucleus

T ..... thymine

TDT..... transmission disequilibrium test

TH ..... human tyrosine hydroxylase gene

Th ..... mouse tyrosine hydroxylase gene

TMS ..... transcranial magnetic stimulation

ToL..... Tower of London

ToM..... Theory of Mind

U.....	unit of restriction enzyme
μl	microlitre
UTR.....	untranslated region
UV.....	ultraviolet
VNTR.....	variable number tandem repeat
VTA	ventral tegmental area
WHO.....	World Health Organization
Y-BOCS.....	Yale-Brown Obsessive Compulsive Scale

Chapter 1

Introduction

A PubMed search for research articles containing the word ‘autism’ finds a large body of work with almost 12,000 peer-reviewed articles. However, although autism has been known for over sixty years, approximately 75% of research in the field has been performed since 1990 with over 50% of published research performed since 2000. There are three reasons why there has been such a concerted effort towards understanding autism: 1) autism is a severely debilitating condition; 2) it is highly prevalent in the general population; and 3) considerable quality of life issues and financial considerations exist regarding affected individuals, their family members and society.

1.1 Identification and Characterization of Autism Spectrum Disorders

1.1.1 Clinical Features

In 1943, Leo Kanner used the term ‘infantile autism’ to describe eleven children who were socially isolated, demonstrated behavioural inflexibility and had impairments in communication. His use of the word ‘autism’ from the Greek ‘auto’ meaning ‘self’, was also coined the following year by Hans Asperger who used the term ‘autistic psychopathy’ to describe children who exhibited similar features to the children described by Kanner but did not have the severe impairments in communication. Neither work initially received much attention. At the time, the term ‘autism’ had already been used to describe schizophrenia and children exhibiting these impairments were diagnosed with childhood schizophrenia (Bender & Grugett, 1956). The original study by Asperger

was published in German and went largely unnoticed until it was translated into English by Lorna Wing in 1981.

Although autism became a diagnostic entity in 1980 following the release of the Diagnostic and Statistical Manual, Third Edition (DSM-III) and new diagnostic criteria were added in DSM-III-Revised (reviewed by Tidmarsh & Volkmar, 2003), the definition of autism was considered too broad and there was no information regarding the diagnosis of other Pervasive Developmental Disorders (PDDs) such as Asperger syndrome (Volkmar *et al.*, 1994). The release of DSM-IV in 1994 by the American Psychiatric Association provided the diagnostic refinement needed for an autism diagnosis and included other PDD classifications that remain in use in clinical and research settings. The formal DSM-IV classifications are: autistic disorder (autism), Asperger's disorder (Asperger syndrome), Rett's disorder (Rett syndrome), childhood disintegrative disorder (CDD) and pervasive developmental disorder-not otherwise specified (PDD-NOS). These improvements in DSM-IV aligned PDD classifications and criteria between the DSM-IV from the American Psychiatric Association and the International Classification of Diseases, Tenth Revision (ICD-10) from the World Health Organization (1992). In 2000, the American Psychiatric Association released DSM-IV-Text Revision (TR) to maintain the currency of DSM-IV however, DSM-IV-TR is considered a minor revision to DSM-IV and does not have any major changes to PDD classifications or criteria.

PDDs, which are also known as autism spectrum disorders (ASDs), are characterized by severe impairments in reciprocal social interaction and communication, and the presence of repetitive behaviours and stereotypies (American Psychiatric

Association, 2000). Examples of impairments in social interaction include eye gaze avoidance and the lack of facial expressions or the absence of emotional reciprocity. Impairments in verbal and nonverbal communication are manifested as the inability to participate in or start a conversation or delays in or the lack of language skills. Restricted repetitive behaviours include the presence of inflexible routines or mannerisms or an excessive preoccupation with movements or objects. In addition to this triad of features, individuals with ASD often have intellectual impairment.

Although ASDs are commonly defined by these three core features, significant differences exist among these conditions (American Psychiatric Association, 2000). The manifestation of autism usually occurs within the first three years of life and persists into adulthood, although the extent of impairments varies greatly among individuals. Intellectual impairments are present in many cases of autism, as are abnormalities in eating, sleeping and self-injurious behaviours, such as head-banging or finger-biting. In contrast, Asperger syndrome usually has a later age of onset and individuals have no significant delay in either language or cognitive development, although impairments in reciprocal social interaction and repetitive behaviours or interests are present. Rett syndrome and CDD are characterized by normal development followed by regression, the onset of which partially defines the disorder. Rett syndrome is characterized by regression occurring between 5 and 48 months of age while the age of regression in CDD is typically after 2 years. PDD-NOS is used when there are impairments in social interaction and communication, and the presence of stereotyped behaviours but the criteria for a specific PDD are not met. This classification includes ‘atypical autism’ (i.e. core features are present for autism diagnosis but a later age of onset).

1.1.2 Diagnostic Tools

Although DSM-IV defines the criteria for an ASD, diagnostic tools are required for the diagnosis of the condition. Two of the earliest rating scales developed for ASDs for use by researchers are the Autism Behavior Checklist (ABC)(Krug *et al.*, 1980) and the Childhood Autism Rating Scale (CARS)(Schopler *et al.*, 1980). The ABC contains 57 items which are rated as either present or absent. Examples of items includes 'Does not attend to social stimuli', 'Does not imitate other children at play', 'Flaps hands' and 'Actively avoids eye-contact'. The CARS consists of 15 items which are scored on a scale from one to four with higher scores indicating greater severity. Items from the CARS includes 'Impairments in human relationships', 'Inappropriate affect', 'Bizarre use of body movement and persistence of stereotypies' and 'Nonverbal communication'. However, because of limitations in identifying those individuals with autism with less severe impairments, the need to assess individuals across as wide a chronological or mental age as possible, and improved refinements to DSM criteria, newer tools were developed.

The Autism Diagnostic Interview (ADI) was developed to address these needs (Le Couteur *et al.*, 1989). The ADI had good inter-rater reliability and it could be applied to a broader age range of individuals. A revised version, the ADI-R was later developed which improved upon the original interview and is now considered a gold standard by clinicians and researchers for autism diagnosis (Lord *et al.*, 1994). The ADI-R was shortened, reorganized and modified to be appropriate for anyone over the mental age of two years. It is a parent-based interview and consists of questions relating to four domains which are: 'Qualitative abnormalities in reciprocal social interaction',

‘Qualitative abnormalities in communication’, ‘Restricted, repetitive, and stereotyped patterns of behaviour’ and ‘Abnormality of development evident at or before 36 months’. Items within these domains are scored and an algorithm based on DSM-IV and ICD-10 criteria is applied with cutoffs to establish an autism diagnosis. The second gold standard used by clinicians and researchers for the diagnosis of autism is the Autism Diagnostic Observation Schedule (ADOS)(Lord *et al.,* 1989). The ADOS was developed as an interactive schedule and consists of eight tasks that can be administered in any order. These tasks are developed such that a given task targets a specific behaviour. For example, a construction activity such as doing a puzzle tests whether the subject asks the examiner for help to obtain more puzzle pieces. Ratings are made on a Likert scale and an algorithm is applied to determine an autism diagnosis. Similar to the ADI-R, the ADOS has good inter-rater reliability (Lord *et al.*, 1989), however clinicians administering either test require significant training and reliability assessment. A limitation of both the ADI-R and ADOS is the inability to diagnose ASDs other than autism. Thus there is the continued need for the development of additional diagnostic instruments such as the ADOS-Generic (ADOS-G) and PDD Behavior Inventory (PDDBI). The ADOS-G consists of four 30 minute modules and was developed to diagnose children and adults with autism or PDD-NOS (Lord *et al.*, 2000) while the PDDBI was designed to assess children with autism, Asperger syndrome, CDD and PDD-NOS (Cohen *et al.*, 2003). The PDDBI has been shown to correlate well with the ADI-R and, unlike the ADI-R and ADOS, may be better suited for evaluating treatment outcomes of individuals with ASDs and can be administered by parents or teachers (Cohen, 2003).

1.2 Epidemiology of ASDs

A review of epidemiological studies over the past forty years shows an increased prevalence (the proportion of affected individuals in a population at a specific time) of autism and ASDs over time, raising the public concern of whether there is an 'autism epidemic'. For example, Fombonne (2003) performed a meta-analysis of thirty-two autism surveys published between 1966 and 2001 and found that the median prevalence rates for autism for sixteen surveys performed from 1966 to 1991 and for sixteen surveys performed from 1992 to 2001 was 4.4/10000 and 12.7/10000, respectively. Lotter (1966), Wing *et al.* (1976) and McCarthy *et al.* (1984) estimated the prevalence of autistic disorder in children to be 4.1/10000, 4.8/10000 and 4.3/10000 in surveys performed in 1964, 1970 and 1978, respectively while Matsuishi *et al.* (1987), Honda *et al.* (1996) and Baird *et al*. (2006) estimated the prevalence of autism in children to be 15.5/10000, 21.1/10000 and 38.9/10000 in surveys performed in 1984, 1994 and 2000, respectively.

Similar to the increase seen in prevalence rates of autism, prevalence estimates for all ASDs have also shown increases over time. Prevalence estimates of ASDs in the United States in a cohort of 289456 children 3 to 10 years old in 1996 were 34.0/10000 (Yeargin-Allsopp *et al.*, 2003) and 66.0/10000 in a cohort of 407578 eight year old children in 2002 (Autism and Developmental Disabilities Monitoring Network Surveillance Year 2002 Principal Investigators & Centers for Disease Control and Prevention, 2007) while a Canadian study by Ouellette-Kuntz *et al.* (2006) determined the administrative prevalence of ASDs in children 1 to 14 years of age in 2002 in two provinces, Manitoba and Prince Edward Island, to be 28.4/10000 and 35.2/10000,

respectively. In the United Kingdom, the estimated prevalence of all ASDs in cohorts of school age children in 2000 was 116.1/10000, or approximately 1% of the general population (Baird *et al.*, 2006).

Based on studies of prevalence estimates since the 1960s, it is apparent that the prevalence of autism and ASDs is much higher than previously anticipated. This increase may be attributed to methodological factors such as improved inclusion criteria and standardized diagnosis as well as under-identification and diagnostic substitution. If changes in the prevalence of autism are because of improved diagnostic capability, then the rate can be expected to plateau. Newschaffer *et al.* (2005) examined birth cohort curves of children 6 to 17 years of age with autism in the United States between 1992 and 2001 and found that autism prevalence increased among younger cohorts over time (i.e. 13.3/10000 for 6 year olds compared to 9.3/10000 for 10 year olds) but the rate of increase was less in younger cohorts suggesting a possible levelling of prevalence rates.

Other methodological explanations for this increase in prevalence may be under-identification and diagnostic substitution. A Canadian study by Coo *et al.* (2008) examined the assignment of special education codes to school children in the province of British Columbia between 1996 and 2004 and found that ~46% of the increase in autism prevalence from 12.3/10000 to 43.1/10000 was because not all children with autism were diagnosed upon entering school and ~33% of the increase in autism prevalence was contributed by children with a special education classification other than autism being later assigned an autism designation.

Whether these factors account for the change in prevalence or the rate of autism and ASDs are truly increasing is not known. Further epidemiological research and public

health monitoring programs such as the Autism and Developmental Disabilities Monitoring (ADDM) Network (Rice *et al.,* 2007), which is a multi-site surveillance program in the United States designed to study autism prevalence over time in 600,000 children, would provide the long-term longitudinal data required to understand the factors contributing to ASD prevalence.

1.3 The Impact and Costs of ASDs

There is a significant personal impact in terms of a reduced quality of life for individuals with ASDs. Although ASDs have a highly variable course, the severity of impairments in social interaction and communication and the extremely disruptive effects of stereotypies have a major effect on affected individuals. Although some individuals with ASDs have favourable outcomes, Seltzer *et al.* (2004) found 15% to 25% of adults with autism live and work independently and six studies (sample sizes ranging from 9 to 43) which examined the outcome of adults with high-functioning autism or Asperger syndrome, found 5% to 44% of individuals were employed and 16% to 50% lived independently (reviewed by Howlin, 2000), the majority of individuals with ASDs require constant assistance or care either from family members or professional service providers.

Reduced quality of life is also found in family members of individuals with ASDs. Mothers of children with autism reported greater stress than mothers of typically developing children as measured using Exner scoring of the Rorschach test (Duarte *et al.*, 2005). Blacher and McIntyre (2006) found decreased well-being was reported by mothers of young adults with autism compared to mothers of young adults with Down

syndrome, intellectual impairments or cerebral palsy while Allik *et al.* (2006) examined quality of life measures using the Short Form Health Survey in parents (31 mothers and 30 fathers) of children with Asperger syndrome or high-functioning autism and found that mothers had decreased physical well-being which was directly related to the social abilities of the child.

There is also a considerable economic cost to ASDs which includes medical expenses such as physician and clinic services, prescription medications and behavioural therapies, nonmedical costs such as respite and family care, and indirect costs such as lost productivity of individuals with autism and their parents. A 2003 estimate of the economic cost of caring for an individual with ASD over a lifetime is $3.2 million USD with a total estimate of $35 billion USD for all individuals with ASDs in the United States (reviewed by Ganz, 2006). Because of differences in the severity of ASDs, the total cost may range from $13 billion USD to $76 billion USD and the true cost may be in the upper range since a prevalence of 27.5/10000 was used in these calculations and more recent prevalence estimates for ASDs are as high as 116/10000 (~1% of the population)(Baird *et al.*, 2006).

1.4 Genetics of ASDs

Because of the impact of ASDs on the quality of life of affected individuals and their families as well as the significant economic costs associated with these conditions, research towards understanding the cause of ASDs has become a priority over the past several years with a considerable focus on identifying genes contributing to autism.

1.4.1 Family and Twin Studies

For decades, the etiology of ASDs was unclear. Hypotheses ranged from biological to psychological origins of the condition. However, family and twin studies have demonstrated a major etiological role for genetic factors in autism. A family study on 99 autistic individuals found a 2.9% recurrence of autism among siblings of an affected individual (Bolton *et al.*, 1994) while a review of ten studies found the recurrence risk of autism to be in the range of 2% to 6% (Bailey *et al.*, 1998) with more recent estimates of between 6% and 7% (Ouellette-Kuntz *et al.*, 2006).

Studies examining co-occurrence in monozygotic (MZ) and dizygotic (DZ) twins have also supported a strong genetic component for autism. Bailey *et al.* (1995) found 60% of MZ twins (N=25) were concordant for autism while no concordance was found in 20 DZ twins. In two additional studies, Ritvo *et al.* (1985) and Steffenburg *et al.* (1989) examined 40 and 21 twin pairs, respectively, using DSM-III criteria for autism and found 91%-96% concordance in MZ pairs and 0%-24% concordance in DZ pairs. The findings of high concordance in MZ twins (who share 100% of their genome) and a recurrence risk in siblings of affected individuals higher than current prevalence estimates of ~1% in the general population, strongly supports an underlying genetic basis for autism.

1.4.2 Mode of Inheritance

Although a genetic basis for autism is well-established, determining its mode of inheritance has proven difficult. A straight-forward single gene model with Mendelian inheritance fails to adequately explain several aspects of ASD, such as the biased sex ratio (~4:1 males to females)(Jones *et al.*, 1996; Fombonne, 1999), low recurrence rate

and the large differences in concordance rates seen between MZ and DZ twin pairs. Instead, it is more likely that a multifactorial mode of inheritance is involved, whereby the interaction of several genes with environmental factors leads to manifestation of the disorder (Jorde *et al.*, 1991; Bailey *et al.*, 1995). Furthermore, because autism is associated with other disorders such as Fragile X syndrome (Feinstein & Reiss, 1998) and tuberous sclerosis (Smalley, 1998), it appears that ASDs are genetically varied or heterogeneous. Such genetic heterogeneity complicates determining the mode of inheritance of ASDs.

Several studies have attempted to determine the number of genes involved in ASD and results are variable. Several linkage studies all concluded that a relatively small number of loci are involved in the etiology of autism (International Molecular Genetic Study of Autism Consortium (IMGSAC), 1998, 2001; Ashley-Koch *et al.*, 1999; Barrett *et al.*, 1999; Philippe *et al.*, 1999). While Pickles *et al.* (1995) determined from latent class modeling of family and twin data that three loci are involved, a linkage study by Risch *et al.* (1999) concluded that over fifteen major loci are needed to confer susceptibility to autism. Therefore, although ASDs are polygenic, there is no agreement on how many are involved.

1.4.3 Threshold Model

In spite of the fact that the extent of genetic heterogeneity in ASD is not known, autism can be considered a quantitative trait (i.e. various alleles which may be found in the general population may interact and lead to an ASD phenotype). Furthermore, the proportion and combination of these alleles likely determines the severity or type of ASD

which develops. This threshold model accounts for clinical observations made by various groups. Piven *et al*. (1994) found that fathers of children with autism had greater social deficits and stereotyped behaviours than fathers with a Down syndrome child and mothers of children with autism had greater impairments in communication while Bolton *et al.* (1994) reported that approximately 20% of unaffected siblings with an autistic proband had some form of social or communicative impairment. Thus different types of ASDs can be seen within families with mild manifestations, known as the broader autism phenotype, falling below a threshold for diagnosis of an ASD.

1.4.4 Identification of Susceptibility Genes in Autism

Three traditional approaches have been used to identify susceptibility genes in autism: reports of chromosomal abnormalities and cytogenetic studies, genome scans, and examination of candidate genes.

1.4.4.1 Cytogenetic Analyses and Chromosomal Abnormalities

The study of locations of chromosomal abnormalities or breakpoints can be very informative for mapping and localizing susceptibility genes; however, a broad range of abnormalities for almost all chromosomes has been described including deletions, translocations, and inversions in individuals with ASDs (Gillberg, 1998). Most commonly, several groups have significantly associated abnormalities of chromosome 15 with autism in approximately 1% of cases. These structural abnormalities are usually either supernumerary isodicentric chromosome 15 (Flejter *et al.,* 1996) or 15q11-q13 duplications (Bundey *et al.,* 1994) of maternal origin. Interestingly, deletion of the

15q11-q13 region gives rise to Angelman or Prader/Willi – syndromes with autistic-like aspects (Steffenburg *et al.,* 1996; Jiang *et al.,* 1999).

More recently, copy number variations (CNVs), or submicroscopic deletions or duplications of chromosomal regions that may lead to changes in gene expression (reviewed by Freeman *et al.,* 2006), have been associated with autism. For example, Weiss *et al.* (2008) used comparative genomic hybridization (CGH) to identify CNVs in 751 multiple-incidence (multiplex – MPX) families and found a *de novo* microdeletion on chromosome 16p11.2 in affected children from four MPX families while Christian *et al.* (2008) used CGH in a cohort of 397 unrelated individuals with ASDs and found 12% (51/397) of affected individuals had CNVs (deletions and duplication) in several regions of chromosomes 2, 7 and 15, most of which (86%) were inherited from their parents.

1.4.4.2 Genome Scans

Because multiple genes are implicated in ASDs, linkage analysis using large-scale genome scans is also a valid strategy for identifying specific chromosomal regions or locations associated with ASD. Literally thousands of genetic markers are available which can be used to examine the entire genome at various resolutions. The first such whole-genome scan for autism was performed by IMGSAC in 1998. A total of 39 MPX families were initially screened with 354 markers to identify chromosomal regions of interest followed by a second screening using 99 MPX families and an additional 175 markers. Segments of chromosomes 4, 7, 10, 16, 19, and 22 were identified as potential sites for autism susceptibility genes, with a region of chromosome 7q having a maximum lod score (MLS) of 3.6 (IMGSAC, 1998). Further evidence for linkage was reported for

chromosome 7q as well as 16p (IMGSAC, 2001) while Barrett *et al.* (1999) and Philippe *et al.* (1999) reported linkage to chromosome 7 as well as to 12 additional regions including 15q11-q13.

The highly varied findings across genome scans is largely attributed to the heterogeneity associated with autism, although the possibility exists that different susceptibility loci are relevant within specific cohorts of families with ASDs. However, there are a few chromosome regions that have shown evidence of association to autism in more than one study. Initially identified by IMGSAC in 1998, chromosome 7q was associated with autism in several studies including Barrett *et al.* (1999), Philippe *et al.* (1999), IMGSAC (2001) and Ylisaukko-oja *et al.* (2006) but the identification of different regions of chromosome 7q suggests that more than one susceptibility locus is present on this chromosome.

1.4.4.3 Candidate Gene Approach

Another common molecular strategy is the assessment of candidate genes for the identification of susceptibility loci in ASDs. Both single-incidence (simplex – SPX) and MPX families are screened using polymorphisms (usually single nucleotide polymorphisms - SNPs) at the locus of interest and statistical analyses (including case-control and family-based comparisons as well as genotype-phenotype associations) determine whether an association exists between the locus of interest and the condition. There has been an enormous number of candidate gene studies performed over the past several years using various rationales.

Candidate genes have been selected on the basis of chromosomal localization in relation to cytogenetic abnormalities or chromosome regions with positive lod scores from genome scans. For example, the maternal copy of the *UBE3A* gene, localized at chromosome 15q11-q13, is preferentially expressed in the brain and is associated with Angelman syndrome in offspring (Vu & Hoffman, 1997). Evidence of linkage and linkage disequilibrium has been reported with the *UBE3A* gene in 94 families with at least two autistic individuals (Nurmi *et al.*, 2001). The *EN2* gene, which is localized to chromosome 7q36, has been studied as a candidate gene of autism based on evidence of linkage to chromosome 7q. For example, Benayed *et al.* (2005) genotyped two SNPs in the *EN2* gene and found evidence for association with autism in their cohort of 532 families. A recent study by Brune *et al.* (2008) failed to find evidence for association in their cohort, illustrating one of the greatest difficulties facing candidate gene studies, the inconsistent replication of findings.

Another approach to selecting candidate genes is based on their role or importance in certain biochemical pathways or physiological systems that are altered in autistic individuals. For example, *MECP2* and *FMR1*, X-linked genes associated with Rett syndrome and Fragile X syndrome, respectively, are involved in regulating gene transcription. *MECP2* encodes a methyl CpG-binding protein that regulates gene expression and chromatin remodelling (Amir *et al.*, 1999) while *FMR1* encodes the FMRP protein which is involved in mRNA transport and translation at dendritic spines (Bagni & Greenough, 2005). The *RELN* gene, which is located at chromosome 7q22, encodes the reelin protein which has a critical role in prenatal cortical development and neuronal migration as shown in mice lacking the *Reln* gene (D'Arcangelo *et al.*, 1995).

Based on neuroanatomical evidence for altered brain development in individuals with autism (Kemper & Bauman, 2002) and positional evidence from genome scans (i.e. Philippe *et al.*, 1999), Zhang *et al.* (2002) examined the *RELN* gene as a candidate gene for ASDs in 126 MPX families and found evidence of association with autism using a family-based association approach. As described in the following section, genes involved in the synthesis and function of neurotransmitters are of particular interest in autism.

1.5 Genetic Studies of Neurotransmitter Systems and Autism

The deficits seen in persons with ASDs can be accounted for by abnormalities in different neuronal and neurochemical pathways including the glutamate, γ-aminobutyric acid (GABA) and serotonin (5-hydroxytryptamine, 5-HT) pathways.

1.5.1 Glutamate and GABA Pathways

Glutamate and GABA are two very common neurotransmitters in the brain. Glutamate acts as the major excitatory neurotransmitter while GABA is the major inhibitory neurotransmitter; both are integral to brain activity and function and are involved in brain maturation and development (reviewed by Manent & Represa, 2007; Pardo & Eberhart, 2007). There are two classes of glutamate receptors, metabotropic and ionotropic. Ionotropic receptors are divided into N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate families. GABA is synthesized from glutamate by glutamic acid decarboxylase (GAD1 and GAD2) and has both ionotropic and metabotropic receptor families. $GABA_A$ and $GABA_C$ receptors are

ionotropic while $GABA_B$ receptors are metabotropic (reviewed by McDougle *et al.*, 2005). Both neurotransmitters are implicated in autism.

Shinohe *et al.* (2006) found increased serum glutamate in 18 adults with autism compared to 19 matched controls ($P<0.001$) while Dhossche *et al*. (2002) found increased plasma GABA levels in nine children with autism compared to age-matched children with Attention-Deficit Hyperactivity Disorder (ADHD)($P=0.01$). Purcell *et al.* (2001) used cDNA microarrays to identify changes in gene expression in postmortem brains of ten individuals with autism compared to twenty-three matched controls and found increased expression of *GluR*1 and *GABRA5*, genes which encode ionotropic AMPA and $GABA_A$ receptors respectively, and *SLC1A3* which encodes a glutamate transporter. Yip *et al.* (2007) and Fatemi *et al.* (2002) found decreased GAD1 messenger ribonucleic acid (mRNA) and protein levels in cerebella of post-mortem brains of individuals with autism compared to matched controls.

A number of studies have examined genes in the glutamatergic and GABAergic pathway for evidence of association with autism. Genetic studies of the glutamatergic pathway have focused on genes which encode both ionotropic and metabotropic receptors. For example, an early study by Jamain *et al.* (2002) found strong evidence of linkage ($P=0.0005$-0.0009) of *GluR6* to autism and identified a 2-marker SNP haplotype which was over-transmitted from mothers to affected males ($P=0.002$) while Serajee *et al.* (2003) examined *GRM8*, which is located on chromosome 7q, in a cohort of 196 MPX families and found maternal transmission of a 3-marker SNP haplotype to affected children.

Genetic studies of the GABAergic pathway have predominantly looked at genes that encode receptors. A region of particular interest has been a $GABA_A$ subunit gene cluster (*GABRB3*, *GABRA5* and *GABRG3*) located at chromosome 15q11-q13, a region associated with chromosomal abnormalities and autism (Bundey *et al.*, 1994; Flejter *et al.*, 1996). Cook *et al.* (1998), Buxbaum *et al.* (2002) and Kim *et al.* (2006) used family-based tests of association and found over-transmission of alleles or haplotypes at the *GABRB3* locus to affected individuals while Kim *et al.* (2008) examined 93 SNPs spanning the *GABRB3*, *GABRA5* and *GABRG3* gene cluster and found over-transmission of alleles in intragenic SNPs of all three genes (P=0.017-0.030). However, their findings were not significant following Bonferroni corrections, demonstrating the statistical difficulties in reliably identifying candidate genes in autism.

1.5.2 Serotonin Pathway

Serotonin has been the most extensively studied neurochemical in autism. It is synthesized from tryptophan in two reactions with the initial hydroxylation by tryptophan hydroxylase (encoded by *TPH1* and *TPH2*) being rate-limiting. Depletion of dietary tryptophan and its association with a worsening of stereotypies in a double-blind placebo-controlled randomized crossover study of twenty adults with autism (McDougle *et al.*, 1996a) was an early indicator of a role of the pathway in autism pathophysiology. The serotoninergic pathway regulates behaviours including mood and arousal, and 5-HT has a role in brain development and neuronal differentiation (reviewed by Lam *et al.*, 2006). A positron emission tomography (PET) study by Chugani *et al.* (1997) found increased levels of 5-HT synthesis in frontal cortex and thalamus as well as decreased synthesis in

cerebella of seven boys with autism compared to five siblings. These findings suggest that these children with autism have either 1) abnormal serotoninergic innervation or 2) altered 5-HT synthesis in anatomically normal serotoninergic neurons in these brain regions.

Measurements of 5-HT or its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), have been extensively reported in autism. In 1961, Schain and Freedman measured whole blood 5-HT in twenty-three children with autism and found increased 5-HT compared to children with mild intellectual impairments. The authors commented that some affected children were on medication (i.e. phenobarbital or chlorpromazine) but these drugs were not associated with changes in 5-HT levels. Hranilovic *et al.* (2007) found increased platelet 5-HT levels in adults with autism (N=53) compared to controls (N=45) while Cohen *et al.* (1977) and Narayan *et al.* (1993) did not find differences in cerebrospinal fluid (CSF) measurements of 5-HIAA between autistic children (N=10 and N=17, respectively) and comparison cohorts (N=9 and N=15, respectively).

Further support for a role of 5-HT in the pathophysiology of autism stems from pharmacological studies (reviewed by Buitelaar & Willemsen-Swinkels, 2000). Several studies have administered selective serotonin reuptake inhibitors (SSRIs), which are 5-HT transporter antagonists, to affected individuals in controlled trials. For example, a double-blind, placebo-controlled study of fluvoxamine in thirty adults with autism found 53% (8/15) of individuals who received fluvoxamine responded to treatment and showed improvements in measures of social relatedness, repetitive behaviours and aggression compared to placebo (McDougle *et al.*, 1996b) while an open-label study of escitalopram administered to twenty-eight children with ASDs (6 to 17 years old) found 61% of

participants showed improvements in stereotypies, hyperactivity, inappropriate speech, and irritability (all *P*=0.001) as measured using the ABC-community version, and showed an overall reduction (*P*=0.001) in the severity of the condition as measured using the Clinical Global Impression scale (CGI)(Owley *et al.*, 2005).

The considerable interest in the 5-HT pathway and autism is seen in the number of 5-HT-related genes that have been tested for association with autism. Although some studies have examined genes which encode enzymes involved in 5-HT synthesis (i.e. Coon *et al.*, 2005; Ramoz *et al.*, 2006) or receptors (i.e. Lassig *et al.*, 1999; Veenstra-VanderWeele *et al.*, 2002), the majority of genetic studies have examined the *SLC6A4* gene (also known as *SERT* and *5-HTT*) which encodes the 5-HT transporter. Studies of two functional variants, a 44 base pair (bp) insertion/deletion (5-HTTLPR) found ~1 kilobase (kb) upstream of the *SLC6A4* gene (Lesch *et al.*, 1996) and a variable number tandem repeat (VNTR) polymorphism located in intron 2 (MacKenzie & Quinn, 1999), have given conflicting results. Cook *et al.* (1997), Klauck *et al.* (1997) and Devlin *et al.* (2005) found evidence of over-transmission of 5-HTTLPR alleles to individuals with autism but Cook *et al.* (1997) and Devlin *et al.* (2005) reported the deleted (short - S) allele while Klauck *et al.* (1997) found the insertion (long - L) allele was associated with autism. Both Cook *et al.* (1997) and Klauck *et al.* (1997) found over-transmission of different 5-HTTLPR-Intron 2 VNTR haplotypes to affected individuals (*P*=0.018 and *P*=0.049, respectively) while a lack of association of either marker or other variants at the *SLC6A4* locus has been found in several different family cohorts (i.e. Betancur *et al.*, 2002; Guerini *et al.*, 2006; Koishi *et al.*, 2006).

1.5.3 Other Neurochemical Pathways and Autism

Considerable attention has been directed to the study of the neurotransmitters glutamate, GABA and 5-HT and a number of studies have examined genes involved in the synthesis and function of glutamate , GABA and 5-HT for a role in autism. In contrast, study of the dopamine pathway and dopamine-related genes in ASDs have received little attention.

1.6 The Dopamine Pathway

Dopamine (DA) modulates processes and behaviours that are abnormal in individuals with ASDs including motor functions (Vilensky *et al.*, 1981), cognitive processes (Hughes *et al.*, 1994; Mostofsky *et al.*, 2000), emotional regulation (Gaigg & Bowler, 2007) and homeostatic processes such as sleeping and waking (Gail *et al.*, 2004) and blood pressure (Ming *et al.*, 2005).

1.6.1 Dopamine as a Catecholamine

Dopamine belongs to the catecholamine family of neurotransmitters that also includes norepinephrine (NE) and epinephrine (EPI). They are called catecholamines because their core molecular structure consists of a catechol group (1,2-dihydroxybenzene) and a primary or secondary amine (Figure 1.1). The initial substrate for catecholamine biosynthesis is the amino acid L-tyrosine. It is hydroxylated to L-dihydroxyphenylalanine (L-DOPA) by the rate-limiting enzyme, tyrosine hydroxylase (TH), in a reaction that requires tetrahydrobiopterin, molecular oxygen and ferrous iron (Fe^{2+})(Nagatsu *et al.*, 1964)(Figure 1.1). L-DOPA is decarboxylated to DA by L-

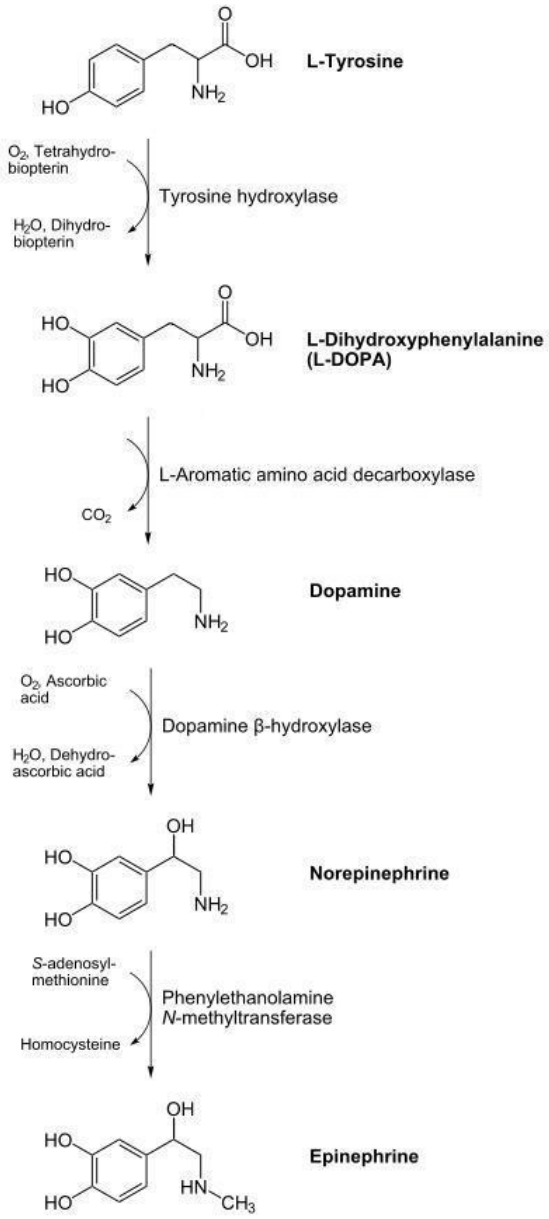

Figure 1.1: Biosynthesis of catecholamine neurotransmitters. Dopamine (DA), norepinephrine (NE), and epinephrine (EPI) are sequentially synthesized from tyrosine. Tyrosine is converted by the rate-limiting tyrosine hydroxylase (TH) to L-dihydroxyphenylalanine (L-DOPA) which is converted to DA by L-aromatic amino acid decarboxylase (L-AADC) in dopaminergic neurons. Dopamine β-hydroxylase (DBH) converts DA to NE while phenylethanolamine N-methyl transferase (PNMT) converts NE to EPI in noradrenergic and adrenergic neurons, respectively (adapted from http://en.wikipedia.org/wiki/dopamine).

aromatic amino-acid decarboxylase (L-AADC) using vitamin B6 as a cofactor (Holtz, 1959). As shown in Figure 1.1, catecholamine synthesis continues in a sequential manner (Udenfriend & Wyngaarden, 1956). NE is synthesized from DA by the hydroxylation of the side chain of DA by dopamine-β-hydroxylase (DBH)(Levin *et al.*, 1960) and EPI is derived by the addition of a methyl group to the amine of NE by phenylethanolamine N-methyltransferase (PNMT)(Axelrod, 1962).

The characterization and biological study of catecholamines from the 1930s to 1960s was focused primarily on NE and EPI. Although researchers during this period (i.e. Udenfriend & Wyngaarden, 1956) found that catecholamines were sequentially synthesized in the order of DA to NE to EPI, DA was considered nothing more than the immediate substrate for NE. However, Arvid Carlsson in the late 1950s provided evidence that DA, like NE and EPI, was a neurotransmitter.

1.6.2 Dopamine as a Neurotransmitter

In 1957, Carlsson *et al.* injected L-DOPA into reserpine-treated mice and rabbits and found that administration of L-DOPA ameliorated the akinetic effects of reserpine (which causes depletion of catecholamine stores). Although these findings alone did not provide direct evidence for DA as a neurotransmitter, Carlsson *et al.* (1958) found that increased levels of DA, but not NE, in brain following L-DOPA injections in reserpine-treated rabbits coincided with resumption of normal motor function and behaviours. This work showed that DA was more than just a substrate for NE but a neurotransmitter in its own right. These findings, that DA has a role in motor functions and that depletion of DA could be restored with administration of L-DOPA, led to the development of L-DOPA

therapy for the treatment of Parkinson disease (reviewed by Carlsson, 2001), and earned Arvid Carlsson a Nobel Prize in 2000.

1.6.3 Dopamine and Movement

Following the insights that 1) the experimental akinesia found in reserpine-treated animals was similar to the bradykinesia associated with Parkinson disease and 2) DA was required for motor functions, subsequent research led to a greater understanding of the role of DA as a neurotransmitter. In 1964, Dahlström and Fuxe used formaldehyde histofluorescence to identify catecholaminergic neurons in rat midbrain and lower brain stem including twelve cell groups (named A1-A12). Of these, dopaminergic (DAergic) neurons were found in midbrain groups A8-A10, which are distributed in a continuum with no discernable boundaries and anatomically span the retrorubral area (RRA), substantia nigra pars compacta (SNc) and ventral tegmental area (VTA), respectively (reviewed by Bentivoglio & Morelli, 2005). Anden *et al.* (1964) identified a major DAergic pathway called the nigrostriatal pathway which originates from the SNc and projects to the caudate nucleus and putamen of the striatum. The observations that high levels of DA were found in the caudate of dog (Carlsson, 1959) and human post-mortem brain (Ehringer & Hornykiewicz, 1998; originally published in German in 1960 but republished in English in 1998), but not in the caudate and putamen of individuals with Parkinson disease (Ehringer & Hornykiewicz, 1998), led to the realization that it was the loss of nigrostriatal DAergic neurons which results in the bradykinesia seen in Parkinson disease (reviewed by Carlsson, 2001).

The striatum is the major input structure of the basal ganglia which are composed of several nuclei including the striatum, globus pallidus (GP) and subthalamic nucleus (STh)(reviewed by Bentivoglio & Morelli, 2005). The basal ganglia process cortical information through two pathways called the direct and indirect pathways. Nigrostriatal DAergic neurons modulate striatal activity by different dopamine receptors expressed by GABAergic medium spiny neurons (MSNs) from these pathways. There are two classes of dopamine receptors defined by their effect on adenylate cyclase activity (Kebabian & Calne, 1979). The D1 class of DA receptors is composed of D1 and D5 receptors which are coupled to stimulatory G-proteins that increase the activity of adenylate cyclase while the D2 class of receptors is composed of D2, D3 and D4 receptors which are coupled to G-proteins that decrease adenylate cyclase activity (reviewed by Svenningsson *et al.*, 2004). The opposing downstream effects of D1 and D2 receptor activation are mediated by the dopamine- and cAMP-regulated phosphoprotein of molecular weight 32kDa (DARPP-32), which is a critical modulator of DA signal transmission and controls a variety of downstream physiological effects (reviewed by Svenningsson *et al.*, 2004). Immunohistochemical methods have shown high densities of dopamine D1 and D2 receptors and DARPP-32 protein in MSNs in striatum of rat, non-human primate and human brain (Ouimet *et al.*, 1984; Ouimet *et al.*, 1992; Levey *et al.*, 1993; Bergson *et al.*, 1995) with dopamine D1 receptors found in striatonigral neurons of the direct pathway and D2 receptors found in striatopallidal neurons of the indirect pathway (Gerfen *et al.*, 1990); DARPP-32 is found throughout the striatum (Ouimet *et al.*, 1992). Nigrostriatal DAergic neurons differentially modulate the output of the basal ganglia using both pathways and disruption of either pathway has an effect on motor functions. For

example, striatal administration of haloperidol (a D2/D3/D4 receptor antagonist partially selective for D2 receptors) induces catalepsy in rats (Meyer *et al.*, 1993); an effect which is attenuated in mice lacking the *Ppp1r1b* gene which encodes DARPP-32 (Fienberg *et al.*, 1998).

Further support for the function of DA in the control of movement is found using mutant mouse lines. Nishii *et al.* (1998) generated transgenic mice which were deficient in DA but not NE and EPI synthesis and found these *Th*-/- 'DA-deficient' mice were hypokinetic. Similarly, Baik *et al.* (1995) and Jung *et al.* (1999) generated two mouse lines lacking the gene which encodes the dopamine D2 receptor (*Drd2*) and found that animals from both strains displayed abnormalities in gait and locomotion with bradykinesia-like movements similar to that seen in Parkinson disease. In contrast, mice lacking the dopamine D1 receptor gene, *Drd1a*, or the gene which encodes the dopamine transporter, *Slc6a3*, are hyperactive (Xu *et al.*, 1994; Giros *et al.*, 1996).

1.6.4 Dopamine and Cognition

Since Arvid Carlsson's finding of dopamine's role in movement, additional roles for DA have been identified in a broad variety of cognitive processes including working memory, attentional set-shifting, planning, learning and memory. Processes such as working memory, set-shifting and planning are known as executive functions, an umbrella term used to describe the processes involved in complex, goal-directed, problem-solving tasks (reviewed by Royall *et al.*, 2002). The identification of a role for DA in these processes was possible following the mapping of the mesocortical and mesolimbic DAergic pathways (commonly known as the mesocorticolimbic pathway).

Cortical regions receive DAergic innervation from the mesocortical pathway in a rostral-caudal gradient in which dense DAergic innervation is found in the prefrontal cortex (PFC), the anterior cingulate cortex (ACC), the orbitofrontal cortex (OFC) and the motor cortex with less DAergic innervation in the temporal cortex and the parietal cortex in rats, non-human primates and humans (Fallon *et al.*, 1978; Berger *et al.*, 1988; Lewis *et al.*, 1988; Gaspar *et al.*, 1989). Williams and Goldman-Rakic (1998) used TH-immunostaining and retrograde transport to show that DAergic neurons, predominantly from the A9 and A10 cell groups, contributed to the mesocortical pathway in non-human primates. Early evidence for a role of the mesocortical DA pathway in cognitive functions was found by Brozoski *et al.* (1979) and Simon *et al.* (1980). Brozoski *et al.* (1979) used intracortical injections of 6-hydroxydopamine (6-OHDA), a DAergic toxin, to selectively deplete DA in the PFC of non-human primates and found that the degree of impairment in working memory performance was comparable to the deficit found following surgical removal of the cortical region (the dorsolateral PFC). Simon *et al.* (1980) used 6-OHDA to selectively lesion DAergic projections from the A10 cell group in rats and found that lesioned animals were severely depleted in PFC DA and were impaired in working memory as measured using the T-maze test. Similar deficits in working memory were found in rats and non-human primates following administration of FG7142, an anxiogenic β-carboline which mimics a mild stress response and results in increased DA release in the PFC (Murphy *et al.*, 1996). The seminal work by Patricia Goldman-Rakic showed an important role for the D1 class of dopamine receptors in working memory function and the PFC. The administration of high doses of SCH23390 (a D1/D5 receptor antagonist) in the PFC disrupts working memory performance

(Sawaguchi & Goldman-Rakic, 1991) while low doses enhance performance in non-human primates (Williams & Goldman-Rakic, 1995). In contrast, the administration of raclopride (a D2/D3 receptor antagonist) in the PFC had no effect on working memory (Sawaguchi & Goldman-Rakic, 1991). These findings led to the concept of an 'inverted U-shaped' function to describe the relationship between dopamine function in the PFC and working memory performance in which there is an optimal level of performance and either hypoactivation or hyperactivation of D1/D5 receptors impairs performance (Williams & Goldman-Rakic, 1995).

The role of dopamine and working memory is not exclusive to the cortical function of D1 receptors. The findings that systemic administration of low doses of quinpirole (a D2/D3 receptor agonist) causes impairments in working memory while higher doses improve performance in non-human primates (Arnsten *et al.*, 1995) and that transgenic mice which overexpress striatal dopamine D2 receptors are impaired in working memory (Kellendonk *et al.*, 2006), indicate that other brain regions are involved. Alexander *et al.* (1986) proposed that information is processed by several different looped circuits in the brain. These circuits are composed of cortical projections to the basal ganglia, the major output of which projects to the thalamus, which projects back to cortical regions. These corticostriatal circuits underlie information processing and different circuits are involved in different functions such as movement and cognitive processes. Thus, the frontostriatal circuitry which underlies working memory function is modulated by DA from the mesocortical pathway and dopamine D1 receptors in the PFC as well as the nigrostriatal pathway and dopamine D2 receptors in the striatum.

In addition to working memory, DA is integral to other executive functions such as planning and attention. Owen *et al.* (1995) found that individuals with Parkinson disease are impaired in planning as measured using the Tower of London (ToL) task. Mehta *et al.* (1999) administered sulpiride (a D2/D3/D4 receptor antagonist) to adult male volunteers and also found impairments in planning using the ToL task. Furthermore, Mehta *et al.* (2003) used PET to measure regional cerebral blood flow (rCBF) and found that sulpiride-impaired planning in humans was associated with decreased rCBF in caudate nuclei. Crofts *et al.* (2001) used 6-OHDA to lesion PFC DAergic neurons in non-human primates and found lesioned animals were impaired in attentional set-shifting as measured using the Intradimensional/Extradimensional (ID/ED) task and were easily distracted. Simon *et al.* (1980) described similar behavioural changes in PFC DA-depleted rats. Monchi *et al.* (2006) used PET and raclopride as a radiotracer to measure DA release in humans and found increased extracellular DA in caudate during tests of cognitive flexibility. Ragozzino (2002) and Floresco *et al.* (2006) found that rats that received PFC infusions of SCH23390 or eticlopride (a D2/D3 receptor antagonist) respectively, were incapable of switching attention in a response and visual cue discrimination task while Mehta *et al.* (1999) found that systemic administration of sulpiride impaired set-shifting performance on the ID/ED task in humans. These findings support roles for the mesocortical and nigrostriatal DA pathways in cognitive flexibility.

The mesolimbic pathway originates from the VTA (A10 cell group) and, to a lesser extent the SNc (A9 cell group), with DAergic projections to the amygdala, the hippocampus and the ventral striatum which includes the nucleus accumbens

(NAc)(Fallon *et al.*, 1978; Swanson, 1982; reviewed by Bentivoglio & Morelli, 2005). Goto and Grace (2005) recorded electrophysiological responses in the NAc during localized administration of quinpirole to the NAc of rats performing a response and visual cue discrimination task and found that dopamine D2 receptor stimulation in the NAc attenuated PFC-evoked activity and animals were unable to shift their attention during task switching. The mesolimbic DA pathway modulates behaviours such as motivation, goal-directed behaviours and behaviour reinforcement (i.e. reward) through the NAc, behaviours which underlie executive functions and other cognitive processes including memory and learning.

Several forms of memory and learning are subserved by neuroanatomical circuits modulated by different overlapping contributions from the mesocortical, mesolimbic and nigrostriatal DAergic pathways. The frontostriatal circuitry, which underlies working memory, is modulated not only by mesocortical and nigrostriatal DA but also by mesolimbic DA. Taghzouti *et al.* (1985) used localized injections of 6-OHDA to deplete DA in the NAc of rats and found that lesioned rats were impaired in working memory using a T-maze test. DA is required for episodic memory, which is the recollection of personal experiences such as events, places and knowledge. Knecht *et al.* (2004) performed a randomized double-blind study with forty healthy volunteers and found that individuals who received L-DOPA remembered new words longer than individuals taking placebo while individuals who received low doses of apomorphine (an indirect DA agonist that at low doses acts on DA autoreceptors and results in decreased DA levels) had impaired episodic memory (Montoya *et al.*, 2008). Cervenka *et al.* (2008) used PET and raclopride as a radiotracer to measure DA release in humans during tests of episodic

memory and found increased extracellular DA in caudate and NAc while Lepage *et al.* (1998) performed a meta-analysis of PET studies of memory and found that the hippocampus was involved in episodic memory acquisition and retrieval. These findings led to the proposal that episodic memory is controlled by a functional circuit which includes parts of the dorsal and ventral striatum as well as the hippocampus, and that this circuit is modulated by the mesolimbic and nigrostriatal DA systems.

Procedural memory is associated with the long-term retention of skills that are developed by repetition or practice. It is usually associated with motor skills such as riding a bike but can include non-motor skills as well such as learning to read inverted words. Although the role of DA in procedural memory is not fully understood, there is evidence for a role of DA. Cohen and Pourcher (2007) found that procedural memory is impaired in individuals with Parkinson disease while Floel *et al.* (2005) performed a randomized, double-blind study with twenty healthy volunteers and found that older individuals who received L-DOPA showed improved procedural memory comparable to that of younger volunteers.

Memory and learning are inter-related cognitive processes. Learning is required for the initial encoding of memory and if learning is impaired then memory consolidation cannot occur. Thus, the role of DA in the modulation of one often, but not always, affects the other. For example, El-Ghundi *et al.* (1999) used the Morris water maze test to examine spatial learning and memory and found that *Drd1a*-/- mice initially had difficulty locating a hidden platform and were impaired in remembering its location in later trials. In contrast, Cohen and Pourcher (2007) used a non-motor procedural

learning/memory paradigm and found that individuals with Parkinson disease were not impaired in learning how to read inverted words but were impaired in remembering them.

Dopamine has a profound role in procedural, spatial, associative and reversal learning. Although the neuroanatomical circuitry subserving these forms of learning overlap, there are differences and thus are modulated by different midbrain DAergic pathways. Meintzschel and Ziemann (2006) measured changes in motor cortex plasticity using transcranial magnetic stimulation (TMS) in humans during a procedural learning task and found that individuals who received an indirect DA agonist (methylphenidate) or a D2 receptor antagonist (haloperidol) showed improved or impaired practice-dependent learning respectively, while Willuhn and Steiner (2008) administered SCH23390 to the striatum of rats and found that blockade of striatal D1 receptors impaired procedural learning in a wheel-skill test. These findings showed that procedural memory and learning are modulated by mesocortical and nigrostriatal DA on corticomotostriatal circuits. In contrast, the mesolimbic and mesocortical pathways modulate spatial learning in rats. El-Ghundi *et al.* (1999) found that *Drd1a*-/- mice are impaired in the Morris water maze (a test of spatial learning and hippocampal function) while Taghzouti *et al.* (1985) used localized injections of 6-OHDA to deplete DA in the NAc of rats and found that lesioned rats had difficulties in learning a spatial discrimination task. Rinaldi *et al.* (2007) found that antagonism of either dopamine D1 or D2 receptors in the PFC caused spatial learning impairments in mice. Kellendonk *et al.* (2006), examining transgenic mice over-expressing striatal D2 receptors, and Kruzich *et al.* (2006), testing *Drd2*-/- mice lacking dopamine D2 receptors, found that mice from both strains were impaired in associative learning (the ability to form a relationship between a stimulus and

an outcome), suggesting that an optimal level of striatal D2 receptors is required for associative learning and thus there is a role for the nigrostriatal DA pathway in this process. Kruzich *et al.* (2006) used a two-odour discrimination task while Heyser *et al.* (2000) used a discriminated operant task and found that, respectively, *Drd2*-/- mice and *Ppp1r1b-/-* mice were impaired in reversal learning. Calaminus and Hauber (2008) administered dopamine D1 (SCH23390) or D2 (eticlopride) receptor antagonists to the OFC of rats and found administration of either antagonist impaired rats in reversal learning as measured using the reaction time task and Taghzouti *et al.* (1985) used localized injections of 6-OHDA to deplete DA in the NAc of rats and found that lesioned rats had difficulties in reversing previously learned rules. Thus, both mesocortical and mesolimbic DAergic pathways modulate reversal learning using dopamine D1 receptors, dopamine D2 receptors and DARPP-32, and demonstrates a specialized role of the OFC.

1.6.5 Dopamine, Emotional Processing and Social Interaction

Similar to its importance in motor functions and cognitive processes, dopamine has an important role in emotional and social processes such as fear conditioning and Theory of Mind (ToM), and social interactions including pair bonding (partner formation) and mother-infant interactions, all of which are abnormal in autism.

Nishii *et al.* (1998) tested *Th*-/- DA-deficient mice using an emotional processing paradigm (i.e. fear conditioning - the association of a negative stimulus such as foot shock with a neutral context or stimulus such as a room or auditory tone) and found transgenic mice had delays in emotional learning while Benke *et al.* (1998) and Breitenstein *et al.* (1998) assessed emotional processing using a language task and found

that individuals with Parkinson disease were impaired in emotional prosody (i.e. the ability to express or understand feelings in verbal communication). While the findings of Benke *et al.* (1998) and Breitenstein *et al.* (1998) provide indirect evidence for a role of nigrostriatal DA in emotional processing, Morrow *et al.* (1999) and Fernandez (2003) used localized 6-OHDA administration to the medial PFC in rats and found that lesioned animals had longer extinction rates than sham-lesioned controls, which supports a role of mesocortical DA and fear conditioning. Although there is evidence for the nigrostriatal and mesocortical DA pathways, the mesolimbic DA pathway has the major role in emotional regulation (reviewed by Pezze & Feldon, 2004). McCullough *et al.* (1993) used an avoidance task in rats and found that increased DA levels in the NAc of rats correlated with successful avoidance behaviour (i.e. pressing a lever to avoid a shock) while rats which had 6-OHDA depletion of DA in the NAc did not engage in avoidance behaviour. Furthermore, Fenu *et al.* (2001) found that NAc DA in emotional learning during a conditioned taste aversion task in rats was mediated by dopamine D1 receptors. Emotional processing is also affected by mesolimbic DAergic projections to the amygdala and is modulated by dopamine D2 receptors. Guarraci *et al.* (2000) and Greba *et al.* (2001) found that administration of D2 antagonists to the amygdala of rats impaired both emotional learning and memory during a fear conditioning task with similar impairments found following administration of D2 agonists, which act on presynaptic autoreceptors and suppress DA release, to mesoamygdalic DAergic neurons in the VTA (Nader & LeDoux, 1999).

ToM, or mentalizing, is defined as the awareness or understanding of the mental or emotional state of other individuals (Baron-Cohen *et al.*, 1985). Although ToM has

been extensively studied, few studies have examined the role of DA in ToM and available evidence for a role is indirect. Saltzman *et al.* (2000) and Mengelberg and Siegert (2003) found that individuals with Parkinsons disease were impaired in several ToM tasks compared to age-matched controls. Furthermore, the OFC, medial PFC and amygdala receive considerable DAergic innervation (Fallon *et al.*, 1978; Berger *et al.*, 1988) and have roles in processes (i.e. reversal learning and emotional regulation) that are modulated by DA. Thus, because these brain regions are also required for ToM tasks (i.e. Stone *et al.*, 1998, 2003; reviewed by Gallagher & Frith, 2003), DA may modulate their role in ToM.

Social interactions are complex, dynamic actions between individuals that are modified or adapted depending upon the responses or actions of the other individual. Two examples are partner-pair formation and mother-infant interactions, both of which are modulated by DA from midbrain DAergic cell groups as well as DAergic cell groups from the diencephalon. Wang *et al.* (1999a) found that systemic administration of high doses of apomorphine (an indirect DA agonist that at high doses results in increased extracellular DA levels) or the D2 receptor agonist, quinpirole, induced partner preference formation in female prairie voles while administration of the D2 receptor antagonist, eticlopride, blocked pair bonding (prairie voles are a monogamous rodent species commonly used as a non-primate animal model of social attachment and biparental behaviour). Aragona *et al.* (2006) used male voles and found that administration of dopamine D2 receptor agonists (quinpirole) maintained pair bonding while administration of SKF38393 (a dopamine D1/D5 receptor agonist) prevented pair bonding. Furthermore, DA has a role in partner pair formation by regulating hormone

release. The secretion of oxytocin and vasopressin from the pituitary is modulated by two diencephalic DAergic pathways, the tuberoinfundibular DA pathway (A12) and the periventricular-hypophysial DA pathway (A14), which originate from the hypothalamus and innervate the anterior and posterior pituitary, respectively (reviewed by Lookingland & Moore, 2005). Williams *et al.* (1994) found that intraventricular administration of oxytocin induced partner formation in female voles while Winslow *et al.* (1993) found that pair bonding in male voles was facilitated following intraventricular administration of vasopressin. Thus, the role of DA in pair bond formation is both as a neurotransmitter and as a neurohormone.

A dual role of DA is seen in mother-infant interactions, which is also described as maternal responsiveness or behaviour toward infants. Strathearn *et al.* (2008) used functional magnetic resonance imaging (fMRI) and found several DA-innervated regions including the caudate, NAc, medial PFC, OFC, and dorsolateral PFC as well as regions which have DAergic cell groups such as the SNc, VTA and hypothalamus, were activated in first-time mothers viewing images of their infant compared to an unknown infant. Lorberbaum *et al.* (2002) used fMRI and found similar regional activations in first-time mothers listening to recordings of infant cries compared to white noise. Direct evidence for a role of midbrain DAergic pathways in maternal behaviours are found in rat studies. Hansen *et al.* (1991) lesioned DAergic neurons from the VTA in female rats using 6-OHDA and found that lesioned animals were impaired in pup retrieval while Byrnes *et al.* (2002) found that systemic administration of D1 antagonists or D2 antagonists disrupted maternal behaviours (pup retrieval and grooming). Champagne *et al.* (2004) measured DA levels in NAc of lactating rats with pups and found NAc DA increased

during periods of pup grooming with decreased NAc DA levels found in mothers which infrequently engaged in pup grooming behaviours while Silva *et al.* (2003) administered pimozide (a D2/D3/D4 receptor antagonist) to the NAc of female rats and found that dams took longer to retrieve pups, initiate nursing and build nests.

Diencephalic DAergic neurons have a neurohormonal role in maternal behaviours by regulating the release of oxytocin and prolactin. Fahrbach *et al.* (1984) and van Leengoed *et al.* (1987) found that intraventricular administration of oxytocin or an oxytocin receptor antagonist facilitated or impaired, respectively, the onset of maternal behaviours in rats while Pedersen *et al.* (1994) found that oxytocin-induced maternal behaviours were attenuated following administration of localized oxytocin receptor antagonists in the VTA of rats, suggesting an interaction between diencephalic DAergic neurons which mediate oxytocin release and midbrain DAergic neurons in maternal behaviours. Prolactin also has a role in maternal behaviours. This neurohormone is secreted from the anterior pituitary and its release is modulated by DA from the tuberoinfundibular and the periventricular-hypophysial DAergic pathways (reviewed by Lookingland & Moore, 2005). Basal DAergic activity on prolactin release is inhibitory and mediated by dopamine D2 receptors. Kelly *et al.* (1997) used *Drd2*-/- mice and found mutant mice had upregulated prolactin secretion (i.e. hyperprolactinemia) and anterior pituitary hyperplasia. Although Kelly *et al.* (1997) did not examine the effects of increased levels of prolactin on maternal behaviours in *Drd2*-/- mice, Bridges and Mann (1994) administered prolactin in the anterior hypothalamus of female rats and found that injected animals had a faster rate of onset of maternal behaviours while Bridges and Ronsheim (1990) administered bromocriptine (a D2/D3/D4 receptor agonist) in rats at a

dosage which acts on postsynaptic receptors, and found that animals which received bromocriptine had decreased prolactin levels and took longer to develop maternal behaviours. While these studies support a role for prolactin in the development of maternal behaviours in rats, the findings of Sarkar (1989), who transplanted anterior pituitaries to generate hyperprolactinemic rats and found increased levels of oxytocin in the hypothalamus, suggests that the effect of prolactin on maternal behaviours may be because of the action of oxytocin.

1.6.6 Other Roles of Dopamine

In addition to dopamine's role in movement, cognition, emotion and social behaviours, DA has a role in regulating several homeostatic processes including sleep and waking, appetite and body weight (energy homeostasis), temperature, blood pressure and sodium transport as well as gastrointestinal (GI) function.

Wisor *et al.* (2001) administered amphetamine (an indirect DA agonist) to the caudate of dogs and found increased striatal DA levels were associated with increased wakefulness. They also found an association between increased DA levels and wakefulness in mice. *Slc6a3*-/- mice are hyperdopaminergic and are awake longer than wild-type mice with a similar disruption in sleep-wake patterns found in wild-type mice following administration of GBR12909 (a dopamine transporter antagonist). Monti *et al.* (1988) administered apomorphine and bromocriptine in rats at a dosage which acts on DAergic D2 autoreceptors and results in decreased DA release, and found that treated animals had decreased periods of wakefulness while Trampus *et al.* (1993) administered A68930 (a D1/D5 receptor agonist) in rats and found that animals had increased waking

time which was attenuated following administration of dopamine D1/D5 receptor antagonists.

Dopamine modulates other homeostatic functions such as body weight, temperature and blood pressure. Szczypka *et al.* (1999) administered L-DOPA to *Th*-/- DA-deficient mice and found that food intake in mutant mice was proportional to the dose of L-DOPA while Sotak *et al.* (2005) used a different strain of *Th*-/- DA-deficient mice and found that hypophagic mutant mice resumed normal eating behaviours following viral-mediated rescue of *Th* gene expression in nigrostriatal DAergic neurons. These findings as well as those of Gerardo-Gettens *et al.* (1989) and Poggioli *et al.* (1986) who found that increased levels of prolactin or α-melanocyte stimulating hormone (MSH), both of which are under diencephalic DAergic regulation and released from the pituitary (reviewed by Lookingland & Moore, 2005), stimulated or inhibited food intake in rats, provide evidence for a role of DA in appetite and energy homeostasis. DA also has a role in body temperature regulation. Ito *et al.* (2008) used methamphetamine (an indirect DA agonist) to induce hyperthermia and found that fewer *Drd1a*-/- mice (7%) and *Drd2*-/- mice (4%) than wt mice (27%) died, suggesting a role for both the dopamine D1 and D2 receptor in body temperature regulation. Furthermore, Drago and Amir (1984) used anterior pituitary transplants to generate hyperprolactinemic rats and found that animals with increased prolactin levels had decreased core body temperatures (i.e. were hypothermic) while Opp *et al.* (1988) administered MSH to rabbits and found a dose-dependent decrease in brain temperature.

DA has an important role in the regulation of blood pressure and sodium balance (reviewed by Zeng *et al.*, 2007). In the periphery, DA is synthesized in non-neuronal

cells of the kidney and dopamine receptors from both classes are found in blood vessels and kidneys (reviewed by Missale *et al.*, 1998). Although DA's involvement in blood pressure and fluid balance is not fully understood, there is evidence of differing contributions of all dopamine receptor subtypes to blood pressure by different mechanisms (reviewed by Zeng *et al.*, 2007). For example, Zeng *et al.* (2004) used mesenteric arteries from rats and found that administration of either dopamine D1 or D3 receptor agonists caused vasodilation of blood vessels while Hollon *et al.* (2002) found that the increased blood pressure seen in *Drd5*-/- mice was associated with dysregulation of the sympathetic nervous system. Mouse and rat studies have provided evidence for a role of DA in sodium transport, which is a contributing factor to blood pressure but also important in the regulation of fluid balance. Wang *et al.* (1997) used rats on high-salt diets and found that increased renal DA levels were associated with increased urinary sodium excretion (natriuresis) and urine output (diuresis). Although the findings of Wang *et al.* (1999b), who administered D1 receptor antisense oligodeoxynucleotides to the renal cortex of the kidney in rats and found that injected animals had decreased sodium excretion and urine output, supports a significant role for the dopamine D1 receptor in DA's effect on kidney function, findings in mice lacking either the *Drd2* or the *Drd3* gene suggests that the natriuretic effects of DA requires both classes of dopamine receptors (Asico *et al.*, 1998; Ueda *et al.*, 2003).

In addition to the kidney, DA is synthesized in the enteric nervous system of the GI tract. Li *et al.* (2004) used immunohistochemical methods and found the greatest density of DAergic neurons in the GI tract of mice and guinea pigs was in the submucosal and myenteric plexuses of the small intestine with less DAergic innervation found in the

stomach and colon. Although the role of DA in GI function is not fully understood, there is evidence for a role of DA. Singaram *et al.* (1995) used immunohistochemical methods and found fewer myenteric DAergic neurons in individuals with Parkinson disease who had chronic constipation, which is one of the most common GI complaints in individuals with Parkinson disease (reviewed by Pfeiffer, 2003). Furthermore, Hardoff *et al.* (2001) found that individuals with Parkinson disease had delayed gastric emptying and complained of abdominal bloating; GI symptoms were significantly improved in affected individuals who received domperidone (a D2/D3 receptor antagonist which does not cross the blood-brain barrier)(Soykan *et al.*, 1997).

1.7 Purpose of Study

ASDs are generally considered to be genetically complex, characterized by multifactorial inheritance involving several genes of small or moderate effect (reviewed by Bacchelli & Maestrini, 2006). No culprit genes have been conclusively identified that account for idiopathic forms of ASDs, undoubtedly due in large part to genetic heterogeneity and the considerable phenotypic variation among affected individuals. One approach to identifying genes contributing to ASDs is through the selection and study of candidate genes involved in systems or pathways of which there is evidence of an impairment in individuals with autism. The purpose of this thesis was to test genes involved in the DA pathway for evidence of association with specific ASD endophenotypes in an effort to identify a subgroup within the ASD population whose members share an underlying pathophysiology.

1.7.1 Evidence for a Role of Dopamine in Autism

As mentioned in section 1.6, dopamine modulates a broad variety of processes, functions and behaviours that are abnormal in individuals with ASDs including motor functions, cognitive processes, emotional regulation, social interaction and homeostatic processes such as blood pressure and sleep patterns as well as GI function. The following sections review the evidence supporting abnormalities in DA in autism as related to each of these processes.

Vilensky *et al.* (1981) performed a kinesiologic analysis of gait patterns in 21 children with autism and 15 typically developing children and found gait differences in affected children compared to controls which were similar to that found in individuals with Parkinson disease (i.e. decreased stride length). Individuals with autism are impaired in cognitive processes including executive functions, memory and learning. Hughes *et al.* (1994) and Tsuchiya *et al.* (2005) found decreased performance in measures of set-shifting and planning in children with autism compared to matched controls. Both individuals with autism (Reed, 2002; Steele *et al.*, 2006) and those with Asperger syndrome (Morris *et al.*, 1999) have been found to perform poorly in several tasks of working memory while Crane and Goddard (2008) found that individuals with autism were impaired in episodic memory compared to matched controls. Mostofsky *et al.* (2000) used a serial response time task to measure procedural learning and found that individuals with autism (N=11) took significantly longer to learn compared to matched controls (N=17) while Coldren and Halloran (2003) found that children with autism were impaired in reversal learning as measured using a spatial-reversal task. Children with autism have difficulty in understanding and expressing emotional prosody (Peppe *et al.*,

2007) while adults with Asperger syndrome are impaired in emotional processing compared to matched controls as measured using a fear conditioning task (Gaigg & Bowler, 2007). As core features of ASDs, individuals with autism are impaired in aspects of social interaction including ToM tasks (Baron-Cohen *et al.*, 1985; Bauminger & Kasari, 1999; Kaland *et al.*, 2008) and have repetitive behaviours and stereotypies, of which there is evidence for a role of DA in the pathophysiology (Karler *et al.*, 1994; Karler *et al.*, 1998). Furthermore, Gail *et al.* (2004), Ming *et al.* (2005) and Nikolov *et al.* (2008) found that children with autism had altered sleep-wake cycles (i.e. increased night-time waking), elevated blood pressure, and GI problems (i.e. constipation or diarrhea) respectively, compared to matched controls.

Direct evidence in support of a role for DA in autism comes from measurements of DA or its metabolites. Ernst *et al.* (1997) found decreased DAergic activity in the medial PFC in children with autism, and proposed that these deficits contribute to the cognitive impairment characteristic of many of these children. In other studies, children with autism were found to have higher levels of the major metabolite of DA, homovanillic acid (HVA), in CSF (Gillberg *et al.*, 1983; Gillberg & Svennerholm, 1987) and urine (Martineau *et al.*, 1994) compared to matched controls.

1.7.2 A Model for Autism Susceptibility

In a previous study, our group found reduced DBH activity in mothers having two or more sons with autism (Robinson *et al.*, 2001). Reduced DBH activity was associated with a 19-bp deletion, which was later found to be in high LD with a putative functional polymorphism that accounted for about 45% of the variance of plasma DBH activity

(Zabetian *et al.*, 2001). Based on these findings, we proposed a model that autism susceptibility is determined by a combination of maternal effects including genetic factors (e.g. DBH), fetal susceptibility genes and fetal sex (Robinson *et al.*, 2001). Encouraged by these findings, I initiated a comprehensive study of genes affecting DA levels and function in both mothers and sons with ASDs. I anticipate that there will be a number of common variants (Lander, 1996) as well as rare variants (Pritchard, 2001; Pritchard & Cox, 2002) in different individuals. I hypothesize that different families will possess different combinations of these variants, and that the varying combinations of DAergic gene variants in different families will be one of the factors mediating the phenotypic variability that is well-documented in patients with ASDs. I will test this hypothesis in an initial family cohort (TEST) followed by a replication study using additional family cohorts (REPLICATION) to determine the extent of the contribution of the variants identifed in TEST families with different families with ASDs.

1.7.3 Rationale for Selection of Dopamine-related Candidate Genes

1.7.3.1 *TH*

The tyrosine hydroxylase (*TH*) gene consists of fourteen exons (Kobayashi *et al.*, 1988) and maps to chromosome 11p15 (Moss *et al.*, 1986). Because it encodes the enzyme responsible for the rate-limiting step of DA synthesis (Nagatsu *et al.*, 1964), changes in *TH* expression or function may influence the processes or behaviours modulated by DA. For example, *Th-/-* mice are impaired in measures of emotional processing, learning, and memory (Kobayashi, 2001; Denenberg *et al.*, 2004; Robinson *et al.*, 2006) and these impairments in learning and memory are ameliorated following administration of L-

DOPA or insertion of a functional *Th* gene in DA-deficient mice (Denenberg *et al.*, 2004; Robinson *et al.*, 2006). Brozoski *et al.* (1979) found that nonhuman primates with DAergic depletion of the PFC were impaired in tests of working memory while Creese and Iversen (1974), and Canales and Graybiel (2000) found that the administration of DA agonists (amphetamine and apomorphine) in striatum resulted in hyperactivity and increased stereotypies in rodents.

To date, five association studies have examined the role of *TH* as a candidate gene in autism. Three studies used restriction fragment-length polymorphisms (RFLPs) to genotype markers at the *TH* locus and found no association to individuals with autism (N≤50) compared to controls (N≤50)(Herault *et al.*, 1993, 1994; Martineau *et al.*, 1994). Two studies used the HUMTH01 polymorphism, which is a functional variant associated with changes in serum HVA levels (Wei *et al.*, 1997), and found no evidence of association to autism in either thirty-nine affected sib-pair families (Philippe *et al.*, 2002) or individuals with autism (N=66) compared to controls (N=89)(Comings *et al.*, 1995). These studies examined relatively small numbers of individuals with autism and used either a single polymorphism or polymorphisms which are difficult to identify (i.e. no primer sequences). I examined three markers which span the *TH* locus, including two SNPs with no known functional effects and the functional variant, HUMTH01. HUMTH01 has seven alleles with 5 to 10 repeats of the TCAT motif (Polymeropoulos *et al.*, 1991). There are two 10-repeat alleles; one consists of ten perfect repeats (10p) and the other has ten repeats with a single base pair deletion in the fifth core repeat (10i). Both 10-repeat alleles (10i and 10p) are associated with increased HVA levels in healthy volunteers (Wei *et al.*, 1997). Because the 10-repeat allele of HUMTH01 is associated

with increased HVA levels in healthy volunteers (Wei *et al.*, 1997), and increased HVA levels are found in individuals with autism (i.e. Gillberg *et al.*, 1983), I hypothesize that the 10-repeat allele or haplotypes containing this allele will be increased in families with ASDs relative to a comparison group.

1.7.3.2 *SLC6A3*

The dopamine transporter is encoded by the *SLC6A3* gene which is located at chromosome 5p15.3 and consists of 15 exons (Vandenbergh *et al.*, 1992). The dopamine transporter takes up extracellular DA into presynaptic terminals and thus plays a major role in DAergic neurotransmission (Giros *et al.*, 1996; Jones *et al.*, 1999). *Slc6a3-/-* mice show impairments in spatial learning and memory (Gainetdinov *et al.*, 1999) and social interaction (Rodriguiz *et al.*, 2004) as well as disrupted sleep-wake patterns (Wisor *et al.*, 2001). Furthermore, mice either lacking the *Slc6a3* gene (Gainetdinov *et al.*, 1999) or expressing 10% of normal gene function (Berridge *et al.*, 2005) were hyperdopaminergic and had increased stereotypies. In humans, individuals with different genotypes of functional variants at the *SLC6A3* locus have shown differences in DAergic activity affecting neuronal networks involved in working memory (Bertolino *et al.*, 2006) and episodic memory (Schott *et al.*, 2006).

No association studies have examined the role of *SLC6A3* as a candidate gene in autism. I examined five functional polymorphisms which spanned the *SLC6A3* gene. Because these polymorphisms are associated with changes in gene expression that may affect extracellular DA levels (Fuke *et al.*, 2001; Greenwood & Kelsoe, 2003; Guindalini *et al.*, 2006)(summarized in Table 1.1) and there is evidence that decreased dopamine

Table 1.1: Summary of *SLC6A3* expression and dopamine transporter (DAT) binding studies of markers at the *SLC6A3* locus

Polymorphism	Allele[1]	Functional effect	Reference
rs2975226	T	Increased expression	Greenwood and Kelsoe (2003)[2]
	A	Decreased expression	
I8 VNTR	6-repeat	Increased expression	Guindalini *et al.* (2006)[2]
	5-repeat	Decreased expression	
rs2550936	C	Increased expression	Greenwood and Kelsoe (2003)[2]
	A	Decreased expression	
rs28363149	Del	Increased expression	Greenwood and Kelsoe (2003)[2]
	Ins	Decreased expression	
EX15 VNTR	10-repeat	Increased expression	Fuke *et al.* (2001)[3]
	9-repeat	Decreased expression	
Polymorphism	**Genotype**	**Functional effect**	**Reference**
I8 VNTR	6/6	Increased mRNA	Brookes *et al.* (2007)[4]
	5/6	Decreased mRNA	
EX15 VNTR	10/10	Increased mRNA	Brookes *et al.* (2007)[4]
	9/9 & 9/10	Decreased mRNA	
EX15 VNTR	10/10	Increased DAT binding	Heinz *et al.* (2000)[5]
	9/10	Decreased DAT binding	

[1]Alleles associated with decreased expression were tested for evidence of association with autism susceptibility in this study
[2]Luciferase construct in SN4741 dopaminergic cell line
[3]Luciferase construct in COS-7 cell line
[4]Measured from post-mortem midbrain tissue
[5]SPECT measurements of DAT availability in striatum

transporter activity results in impairments in episodic memory and stereotypic behaviours in mice that resemble those in individuals with autism, I hypothesize that alleles associated with decreased *SLC6A3* expression or dopamine transporter availability resulting from the rs2975226 A, I8 VNTR 5-repeat, rs2550936 A, rs28363149 Ins or EX15 VNTR 9-repeat alleles (or haplotypes containing these alleles) will be increased in families with ASDs.

1.7.3.3 *DRD1*

The *DRD1* gene consists of two exons separated by a single intron (Minowa *et al.*, 1992), is located at chromosome 5q35.1 (Grandy *et al.*, 1990), and encodes the dopamine D1 receptor which modulates many of the DA-related behaviors that are abnormal in individuals with autism. For example, the administration of high doses of D1 receptor antagonists to the PFC was found to disrupt performance on working memory tasks in non-human primates (Sawaguchi & Goldman-Rakic, 1991; Williams & Goldman-Rakic, 1995) and attentional set-shifting in rats (Ragozzino, 2002) while D1 receptor blockade in the OFC or striatum of rats impaired reversal learning (Calaminus & Hauber, 2008) and procedural learning (Willuhn & Steiner, 2008), respectively. Dopamine D1 receptors modulate a feed-forward inhibitory circuit involved in amygdala activation (Marowsky *et al.*, 2005), which is a key structure involved in emotional regulation and social behaviour, for which there is evidence of dysfunction in individuals with autism (reviewed by Baron-Cohen *et al.*, 2000) while the administration of D1 receptor agonists or antagonists induced or attenuated, respectively, stereotypies in a DA-deficient mouse model (Chartoff *et al.*, 2001).

To date, no association studies have examined the role of *DRD1* as a candidate gene in autism. In this study, I examined three polymorphisms which span the *DRD1* locus. As it is not known whether any of these polymorphisms are functional, I do not have a hypothesis that specific alleles will be associated with autism susceptibility.

1.7.3.4 *DRD2*

The *DRD2* gene consists of eight exons (Eubanks *et al.*, 1992) and maps to 11q22-q23 (Grandy *et al.*, 1989). In addition to its role in postsynaptic neurons, the dopamine D2 receptor acts as an autoreceptor mediating DA synthesis (Onali & Olianas, 1989) and neurotransmission (Mercuri *et al.*, 1997; Bolan *et al.*, 2007) in DAergic neurons. The dopamine D2 receptor is involved in the DAergic modulation of executive functions such as working memory, planning and attentional set-shifting (Mehta *et al.*, 1999), emotional processing (Greba *et al.*, 2001), social interactions (Wang *et al.*, 1999a; Silva *et al.*, 2003) and the regulation of homeostatic processes including blood pressure (Li *et al.*, 2001) and sleep-wake patterns (Monti *et al.*, 1988). *Drd2*-/- mice have abnormal gait patterns characteristic of individuals with Parkinson disease (Baik *et al.*, 1995; Jung *et al.*, 1999) and the administration of antipsychotic medications such as risperidone (a D2 receptor antagonist) has proven efficacious in treating symptoms associated with ASDs (McDougle *et al.*, 1998; Troost *et al.*, 2005).

Two studies have examined the *DRD2* gene as a candidate gene for autism. Comings *et al.* (1991) reported an increased frequency of the *TaqI* A1 allele in individuals with autism (N=33) compared to controls (N=314) and Philippe *et al.* (2002) found no evidence of transmission disequilibrium of an intragenic microsatellite in thirty-

nine affected sib-pair families. These studies examined small numbers of individuals with autism using a single polymorphism. I used four commonly investigated markers at the *DRD2* locus that have been used to investigate possible associations between DAergic function and behavioral abnormalities (Comings *et al.*, 1991; Noble, 2003; Dubertret *et al.*, 2004). Although rs1799732 is a functional variant with the deletion allele associated with decreased gene expression (Arinami *et al.*, 1997), no functional effects are known for rs1079597, rs1800498 and rs1800498. Since the latter three SNPs were genotyped before rs1799732, I have no hypothesis about which alleles or haplotypes will be associated with families with ASDs.

1.7.3.5 *PPP1R1B*

The protein phosphatase 1, regulatory subunit 1B (*PPP1R1B*) gene, which is located at chromosome 17q12 and consists of 7 exons (http://www.ncbi.nlm.nih.gov; GeneID 84152), encodes DARPP-32. DARPP-32 is expressed in dopaminoceptive (DAceptive) neurons (Ouimet *et al.*, 1984; Ouimet *et al.*, 1992) and mediates the effects of both dopamine D1 and D2 dopamine receptor classes (Fienberg & Greengard, 2000). For example, dopamine D2 receptor antagonism-induced catalepsy in rats is attenuated in *Ppp1r1b*-/- mice (Fienberg *et al.*, 1998) while Heyser *et al.* (2000) found that knockout mice were impaired in reversal learning. In addition, genetic (Ogden *et al.*, 2004; Meyer-Lindenberg *et al.*, 2007) and immunoblot (Albert *et al.*, 2002; Ishikawa *et al.*, 2007) studies have found evidence of association of the *PPP1R1B* locus and altered PFC DARPP-32 protein levels in schizophrenia and bipolar disorder, two conditions for which

there is evidence of DA dysfunction and which exhibit comorbidity with autism (Stahlberg *et al.*, 2004).

To date, no association studies have examined the role of *PPP1R1B* as a candidate gene in autism. In my study, I examined three polymorphisms at the *PPP1R1B* locus. As none of these polymorphisms are associated with changes in gene expression or protein function, I do not have a hypothesis that certain alleles will be associated with individuals with autism.

1.7.4 Plan of Study

Because of their roles in the synthesis or function of DA and evidence of their involvement in processes or behaviours impaired in individuals with autism, the *TH*, *SLC6A3*, *DRD1*, *DRD2* and *PPP1R1B* genes are good candidate genes and are included in my study of DA-related genes and susceptibility to autism. An additional gene, *ARPP-21* which encodes a cAMP-regulated phosphoprotein (M_r 32kDa) that is involved in postsynaptic neuronal signalling, was also examined however, it is not formally presented in this thesis and can be found in Appendix A.

Given our hypothesis that DA-related genes are important in families having only affected males and our model of autism susceptibility (Robinson *et al.*, 2001), I examined polymorphisms at the *TH*, *SLC6A3*, *DRD1*, *DRD2* and *PPP1R1B* loci in 112 male-only affected sib-pair families to determine whether there was any association between ASDs or a specific endophenotype and the mothers' or sons' genotypes.

Chapter 2

Materials and Methods

2.1 Subjects

My study was performed using a two-stage design using family cohorts in which all affected individuals were male. The initial test cohort (TEST) consisted of 112 affected sib-pair families, including 28 families from Canada (Robinson *et al.*, 2001), 5 from the South Carolina Autism Project (Schroer *et al.*, 1998) and 79 families obtained through the Autism Genetic Resource Exchange (AGRE) in the United States (Geschwind *et al.*, 2001). A listing of AGRE family identification numbers is available in Appendix B. Three additional cohorts (REPLICATION 1, REPLICATION 2 and REPLICATION 3) were available as replication cohorts for the replication study. REPLICATION 1 and REPLICATION 2 consisted of 66 affected sib-pair families (multiple incidence - MPX) and 105 single-affected families (single incidence - SPX) respectively, recruited from Canada and the United States by the Autism Spectrum Disorders-Canadian American Research Consortium (ASD-CARC; http://www.AutismResearch.com; http://www.ASDCARC.com). REPLICATION 3 consisted of 50 single-affected families recruited from New York State. This study was approved by the Research Ethics Board of Queen's University and all other participating ASD-CARC institutions. Written informed consent was obtained from parents of all participating families from Canada, the United States including New York and South Carolina, and through AGRE (Geschwind *et al.*, 2001).

All affected children (N=526) were assessed using the ADOS (Lord *et al.*, 1989) and/or the ADI-R (Lord *et al.*, 1994) with PDDBI (Cohen, 2003; Cohen *et al.*, 2003) data

available on some individuals from REPLICATION 1 and REPLICATION 2. As shown in Table 2.1, diagnoses varied in families with 414 meeting criteria for autism, 36 for "not quite autism" and 48 for "broad spectrum"; with report details for 28 individuals diagnosed with an ASD not available. The designations "not quite autism" and "broad spectrum" are not DSM-IV diagnoses but are defined by AGRE (available from http://www.agre.org/agrecatalog/algorithm.cfm). "Not quite autism" is assigned when an individual either meets the autism criteria for the "age of onset" and is within a point of meeting autism cut-off criteria in all or any of the three core domains of the ADI-R, or does not meet "age of onset" criteria but does meet autism cut-offs in all three core domains. "Broad spectrum", which includes PDD variants and Asperger syndrome, is assigned when an individual exhibits minimal deficits in all three core domains, moderate deficits in two core domains or a severe deficit in one core domain. All affected individuals were negative for Fragile X syndrome and tuberous sclerosis, and no gross chromosome abnormalities were detected in any of the affected individuals.

The comparison group for this study consisted of DNA samples from 443 individuals (233 females, 210 males), 138 of whom were individuals with no personal history of autism, 68 of whom were unaffected spouses in families with X-linked mental retardation syndromes and 55 of whom were local volunteers, or were from anonymous placentae (N=115). The remaining 190 DNA samples were from Guthrie spots from anonymous neonates obtained from the Ontario Ministry of Health as part of a phenylketonuria (PKU) screening program (Robinson *et al.*, 2001). Because there were no differences in the allele frequencies for markers at loci used in this study in males and females from the comparison cohort (P=0.14-0.95), all individuals from the comparison

Table 2.1: Diagnosis of affected individuals from affected male-only family cohorts used in this study

Family cohort[1]	Number of families	Number of affected individuals	Diagnosis			
			Autism	Not quite autism[2]	Broad spectrum[2]	ASD[3]
TEST (MPX)	112	235	195 (83.0%)	15 (6.4%)	25 (10.6%)	0
REPLICATION 1 (MPX)	66	136	86 (63.2%)	16 (11.8%)	17 (12.5%)	17 (12.5%)
REPLICATION 2 (SPX)	105	105	83 (79.0%)	5 (4.8%)	6 (5.7%)	11 (10.5%)
REPLICATION 3 (SPX)	50	50	50 (100%)	0	0	0
Total:	333	526	414 (78.7%)	36 (6.8%)	48 (9.1%)	28 (5.3%)

[1]MPX – multiplex (multiple-incidence); SPX – simplex (single-incidence)
[2]As defined by the Autism Genetics Resource Exchange (available from http://www.agre.org/agrecatalog/algorithm.cfm)
[3]Confirmed diagnosis of an ASD (personal communication with Dr. Suzanne Lewis)

group were included for case-control comparisons. Although comprehensive information regarding psychiatric and behavioural disorders is not available for the comparison group, it is unlikely that the prevalence of ASDs among this cohort is greater than that in the general population, or about 1/150 (reviewed by Newschaffer *et al.*, 2007).

2.2 Marker Selection, Amplification and Genotyping

A total of 18 polymorphisms were genotyped in this study of DA-related genes and autism and were chosen based on the following criteria: 1) evidence of a functional effect on gene expression or protein function (i.e. functional variant), 2) a high (~20%) common minor allele frequency (MAF) in the general population, 3) identification as a haplotype-tagged SNP (htSNP) using data from the International HapMap Project (http://www.hapmap.org), 4) evidence of association with other DA-releated conditions and 5) positional information from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov) such that marker coverage spans a given locus.

2.2.1 *TH*

Three polymorphisms, HUMTH01, rs6356 G/A and rs2070762 T/C, were genotyped at the *TH* locus (Figure 2.1). HUMTH01 is located in intron 1 and has seven alleles with 5 to 10 repeats of the TCAT motif (Polymeropoulos *et al.*, 1991). Rs6356 and rs2070762 are located in exon 3 and intron 12, respectively (Figure 2.1), and were identified using data from the International HapMap Project and Haploview 4.0 (Barrett *et al.*, 2005). No haplotype blocks at the *TH* locus were identified using HapMap data. The primers used

Figure 2.1: Illustration of the *TH* locus. A schematic showing gene structure, marker positions and measures of linkage disequilibrium between HUMTH01 and rs2070762 listing D' of the comparison group (N=217) followed by D' of parents from TEST families (N=209). Rs6356 was excluded from analyses. Legend: exon; intron; untranslated region.

to amplify the HUMTH01 microsatellite have been reported previously (Polymeropoulos *et al.*, 1991). The following primers were used to amplify the rs2070762 polymorphism: forward (F) 5'- CAGCCCTACCAAGACCAGAC -3' and reverse (R) 5'- GTCCTTCTCACGGATGGTGT -3'. PCR reactions for HUMTH01 and rs2070762 were carried out using 10ng of template DNA and 1mM $MgCl_2$ in either 4µl reactions for rs2070762 or in 5µl reactions for HUMTH01. HUMTH01 reactions incorporated α-P^{32} labeled dCTP. Cycling conditions for rs2070762 were: 94ºC for 5 min, 38 cycles of 94ºC for 30 sec, 54ºC for 50 sec, 72ºC for 50 sec, followed by a final extension at 72ºC for 10 minutes. Cycling conditions for HUMTH01 were: 96ºC for 5 min, 30 cycles of 92ºC for 30 sec, 56 ºC for 30 sec, 72ºC for 30 sec, followed by a final extension at 72ºC for 7 minutes. PstI (0.6U)(New England Biolabs, Pickering, ON, Canada) was used to digest the rs2070762 amplicons into cut (C allele) or uncut (T allele) digestion products which were then separated on 2% agarose gels and visualized using ethidium bromide and UV illumination. HUMTH01 amplicons were separated on 6% denaturing polyacrylamide gels and visualized following autoradiographic exposure. HUMTH01 alleles were scored against a sequencing ladder and pre-sequenced internal controls. All results from both markers were independently assessed by two persons.

Genotyping of rs6356 was performed using validated custom TaqMan SNP Genotyping Assays (http://www.appliedbiosystems.com) on an ABI Prism 7900HT (Applied Biosystems, Foster City, CA, USA). Genotypes were automatically scored with the SDS 2.2.2 software using standard parameters.

2.2.2 *SLC6A3*

Five functional variants were examined in the *SLC6A3* locus. As shown in Figure 2.2, rs2975226 A/T is located 2.3 kb 5' to the gene, I8 VNTR is located in intron 8, rs2550936 A/C is located in intron 9, rs28363149 Del/Ins is located in intron 14 and EX15 VNTR is located in exon 15 in the 3' untranslated region (UTR). One haplotype block (22kb) in the 5' region of the gene was identified using HapMap data but marker coverage of the region is unclear because the functional variants examined in this study are not included in the HapMap dataset. The following primers were used to amplify the *SLC6A3* rs2975226, I8 VNTR, rs2550936, and rs28363149 polymorphisms, respectively: rs2975226 F 5'-CTGGGCGTCCGAAGATAG-3' and R 5'-CGGGCTCTTATCCAGTAGACA-3'; I8 VNTR F 5'-GCATGTGGATGTGTTCTTGC-3' and R 5'-GCAGAAACAAGGAGGAGCAG-3'; rs2550936 F 5'-ACGCTCCCTCTGTCCTCAG-3' and R 5'-GTCAAGGACAGGAGGTCTGG-3'; and rs28363149 F 5'-CTGGCAGTGGGTACTGGTCT-3' and R 5'-GCACATGCTGGCTGAGTAAA-3'. The primers used to amplify the E15 VNTR have been reported previously (Vandenbergh *et al.*, 1992) with the exception of a nucleotide substitution from T to C in the third position of the forward primer. PCR reactions for I8 VNTR, rs2550936, rs28363149 and EX15 VNTR were carried out using 5ng of template DNA and 1mM $MgCl_2$ in either 3µl reactions for rs2550936 or in 5µl reactions for rs28363149, I8 VNTR and EX15 VNTR. Because rs2975226 is within a GC-rich (77%) region, 10ng of template DNA was amplified in 4µl reactions using Accuprime GC-rich Taq polymerase (Invitrogen Canada Inc, Burlington, Canada) and 2.75mM $MgSO_4$. Cycling conditions for I8 VNTR, rs2550936, rs28363149 and EX15 VNTR were: 94ºC

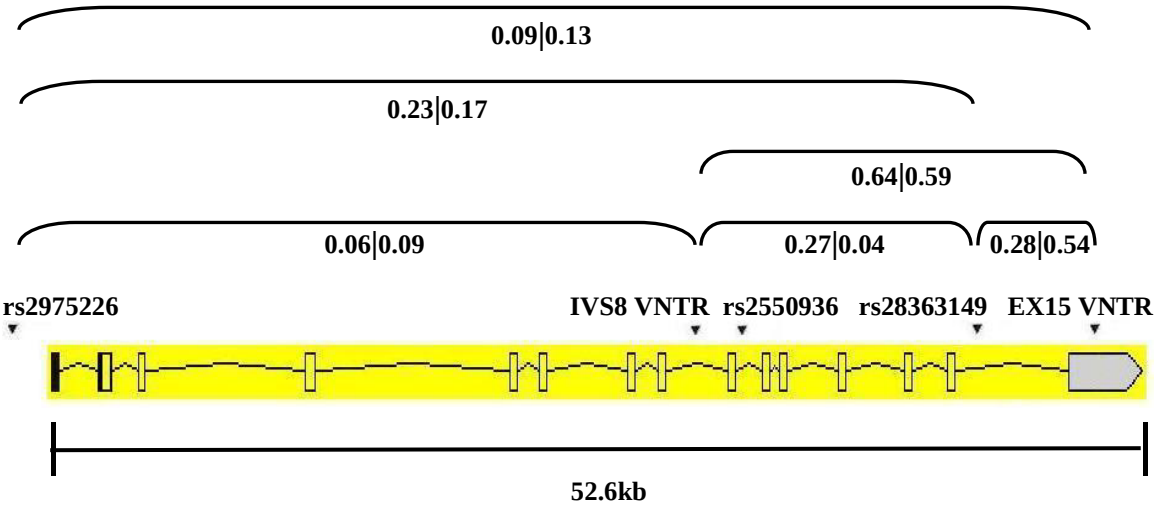

Figure 2.2: Illustration of the *SLC6A3* locus. A schematic showing gene structure, marker positions and measures of linkage disequilibrium between rs2975226, IVS8 VNTR, rs28363149 and EX15 VNTR listing D' of the comparison group (N=252) followed by D' of parents from TEST families (N=215). Rs2550936 was excluded from analyses. Legend: exon; intron; untranslated region.

for 5 min, 30 (EX15 VNTR), 34 (rs28363149) or 36 (I8 VNTR and rs2550936) cycles of 94ºC for 30 sec, 60ºC for 50 sec (45 sec for EX15 VNTR), 72ºC for 50 sec (3 min for EX15 VNTR), followed by a final extension at 72ºC for 10 minutes. Cycling conditions for rs2975226 were: 95ºC for 6 min, 37 cycles of 95ºC for 30 sec, 57 ºC for 30 sec, 72ºC for 50 sec, followed by a final extension at 72ºC for 10 minutes. Tth111I (0.3U)(New England Biolabs, Pickering, ON, Canada) was used to digest the rs2975226 amplicons into cut (T allele) or uncut (A allele) digestion products; RsaI (0.5U) (New England Biolabs, Pickering, ON, Canada) was used to digest the rs2550936 amplicons into cut (A allele) or uncut (C allele) digestion products. All digestion products were separated on 2% agarose gels and visualized using ethidium bromide and UV illumination. All results were independently assessed by two persons.

2.2.3 *DRD1*

Three polymorphisms were examined in the *DRD1* gene: rs265981, rs4532 and rs686. Rs265981 and rs4532 are 5' of the start codon and rs686 is located approximately 60bp 3' of the end of the coding region (Figure 2.3). These polymorphisms are htSNPs and define the single haplotype block which contains the *DRD1* locus. The primers used to amplify the rs265981 (-800C/T) and rs686 (+1403T/C) polymorphisms have been reported previously (Misener *et al.*, 2004). The following primers were used to examine the rs4532 (-48A/G) polymorphism: F 5'-GCAGCAAGGGAGTCAGAAGA-3' and R 5'-TCTGACACCCCTCAAGTTCC-3'. The rs265981, rs4532 and rs686 polymorphisms were previously reported as D1P.6, D1.1 and D1.7, respectively (Misener *et al.*, 2004). PCR reactions were carried out using 5ng of template DNA in 3µl reaction volumes.

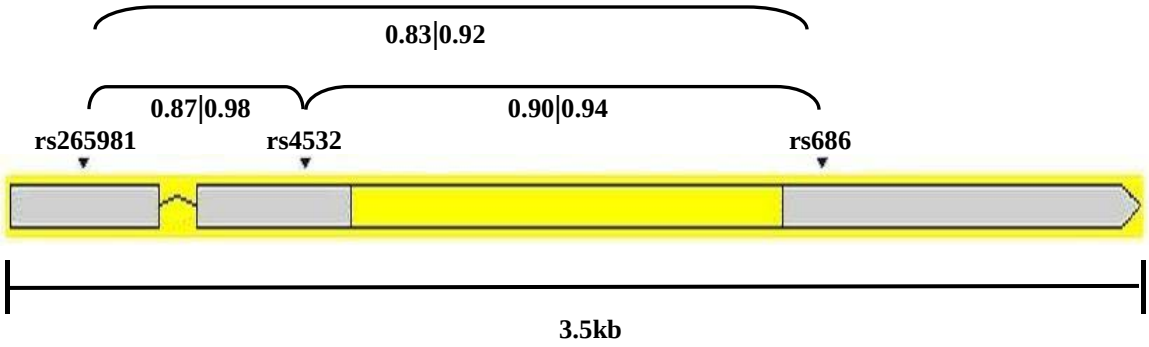

Figure 2.3: Illustration of the *DRD1* locus. A schematic showing gene structure, marker positions and measures of linkage disequilibrium between rs265981, rs4532 and rs686 listing D' of the comparison group (N=443) followed by D' of parents from TEST families (N=214). Legend: exon; intron; untranslated region.

Amplification of rs265981 and rs686 included 1.5mM $MgCl_2$, whereas rs4532 used 1mM of $MgCl_2$. Cycling conditions were: 94ºC for 5 min, cycles of 94ºC for 30 sec, 58ºC (rs265981 and rs686) or 60ºC (rs4532) for 50 sec, 72ºC for 50 sec, followed by a final extension at 72ºC for 10 min., with 35, 36, and 37 cycles for rs4532, rs686 and rs265981, respectively. The rs265981 (C allele cut; T allele uncut), rs4532 (A allele cut; G allele uncut) and rs686 (C allele cut; T allele uncut) amplicons were digested with 0.4U HaeIII, 0.3U DdeI or 0.3U Bsp1286I (New England Biolabs, Pickering, ON, Canada) respectively, and all digestion products were separated on 2% agarose gels and visualized using ethidium bromide and UV illumination. All results were independently assessed by two persons.

2.2.4 *DRD2*

Four polymorphisms were studied at the *DRD2* locus. As shown in Figure 2.4, rs1799732 Ins/Del is located 141bp upstream to the gene, rs1079597 G/A and rs1800498 T/C are located in intron 1 and intron 2 respectively, and rs1800497 C/T is located approximately 10kb 3' to the gene. The *DRD2* gene contains four haplotype blocks. While three of the blocks are small (<4kb), one block is 20kb in size. Although the polymorphisms used in this study are not part of the HapMap dataset, rs1079597 and rs1800498 are located within this block. The primers used to amplify the *DRD2* rs1079597 G/A (TaqI B), rs1800498 T/C (TaqI D) and rs1800497 C/T (TaqI A) polymorphisms have been reported previously (Dubertret *et al.*, 2004). Prior notations of these variants (B1/B2, D1/D2 and A1/A2) correspond to current allele nomenclature of A/G, T/C and T/C, respectively, with the major alleles represented as rs1079597 G, rs1800498 T and rs1800497 C. The primers used to amplify the rs1799732 Ins/Del

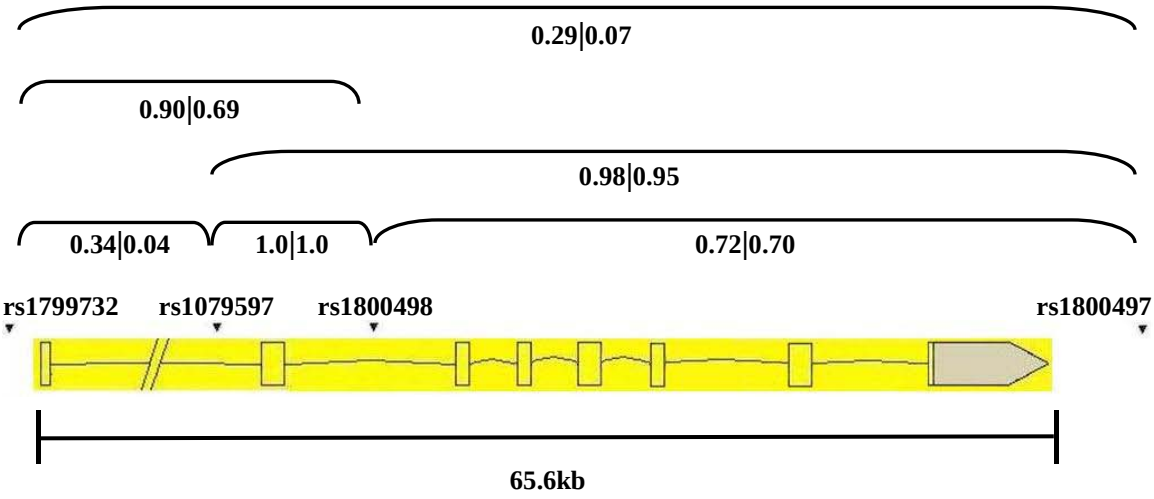

Figure 2.4: Illustration of the *DRD2* locus. A schematic showing gene structure, marker positions and measures of linkage disequilibrium between rs1799732, rs1079597, rs1800498 and rs1800497 listing D' of the comparison group (N=244) followed by D' of parents from TEST families (N=213). Legend: exon; intron; untranslated region.

(-141C Ins/Del) polymorphism were: F 5'-GAGAAGACTGGCGAGCAGAC-3' and R 5'-CCACCAAAGGAGCTGTACCT-3'. PCR reactions were carried out using 5ng of template DNA and either 1mM $MgCl_2$ in 3µl reactions for rs1079597, rs1800498 and rs1800497 or 1.5mM $MgCl_2$ in 4µl reactions for rs1799732. Cycling conditions for rs1079597, rs1800498 and rs1800497 were: 94ºC for 5 min, 34 cycles of 94ºC for 30 sec, 55ºC (rs1079597 and rs1800498) or 58ºC (rs1800497) for 50 sec, 72ºC for 50 sec, followed by a final extension at 72ºC for 10 minutes. Cycling conditions for rs1799732 were: 95ºC for 5 min, 35 cycles of 95ºC for 45 sec, 63 ºC for 30 sec, 72ºC for 30 sec, followed by a final extension at 72ºC for 6 minutes. The rs1079597 (G allele cut; A allele uncut), rs1800498 (C allele cut; T allele uncut) and rs1800497 (C allele cut; T allele uncut) amplicons were digested with 0.4U TaqI (New England Biolabs, Pickering, ON, Canada); rs1799732 (Ins allele cut; Del allele uncut) amplicons were digested with 0.05U BstNI (New England Biolabs, Pickering, ON, Canada). All digestion products were separated on either 2% (rs1079597, rs1800498 and rs1800497) or 2.5% (rs1799732) agarose gels and visualized using ethidium bromide and UV illumination. All results were independently assessed by two persons.

2.2.5 *PPP1R1B*

Three polymorphisms, rs1495099 G/C, rs907094 T/C and rs3764352 A/G, were studied at the *PPP1R1B* gene. Rs1495099 is located in intron 1; rs907094 and rs3764352 are located in intron 5 (Figure 2.5). These variants were chosen from the NCBI dbSNP Build 121 database from the Human Genome Project (available at http://www.ncbi.nlm.nih.gov/SNP/snp_summary.cgi) under the criteria that these

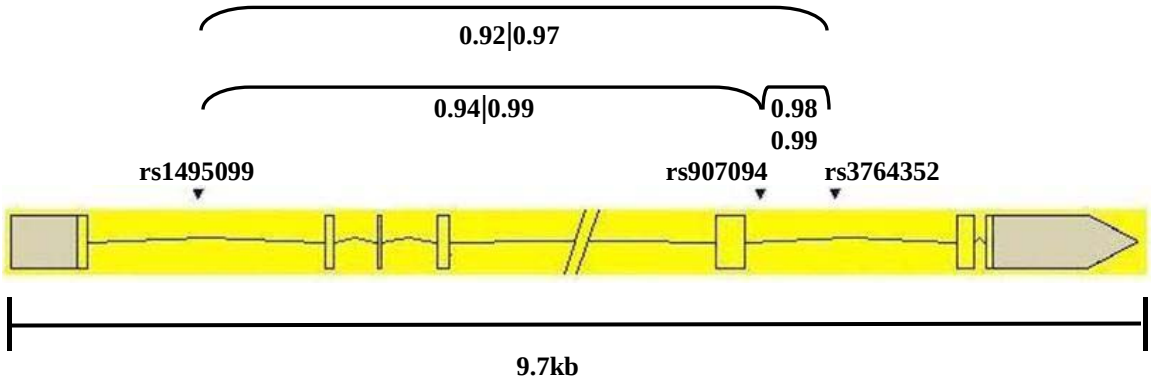

Figure 2.5: Illustration of the *PPP1R1B* locus. A schematic showing gene structure, marker positions and measures of linkage disequilibrium between rs1495099, rs907094 and rs3764352 listing D' of the comparison group (N=435) followed by D' of parents from TEST families (N=216). Legend: exon; intron; untranslated region.

markers span the *PPP1R1B* locus and have MAFs of approximately 20%. These criteria were used because of the absence of known functional SNPs in the coding region of the gene and no prior genetic studies. The *PPP1R1B* locus has a single haplotype block which includes rs907094 and rs3764352 as htSNPs. The following primers were used to amplify the *PPP1R1B* rs1495099, rs907094 and rs3764352 polymorphisms: rs1495099 F 5'-TTGTTGCTGAGCTGAGATGC-3' and R 5'-CTCCAGGGAAATGCACAAAG-3'; rs907094 F 5'-ACCTGATTGGGAGAGGGACT-3' and R 5'-GTAAGCTGAGGGGCCTGTG-3'; and rs3764352 F 5'-CTGTTTTGGAGGGGTCTCAG-3' and R 5'-TGGGAATACTGAAGAGTCAACC-3'. PCR reactions were carried out using 5ng of template DNA and 1mM $MgCl_2$ in 3μl reactions. Cycling conditions were: 94ºC for 5 min, 34 cycles of 94ºC for 30 sec, 60ºC for 50 sec, 72ºC for 50 sec, followed by a final extension at 72ºC for 10 minutes, with 34 cycles for rs907094 and 35 cycles for rs1495099 and rs3764352. PvuII (0.3U), MseI (0.3U) or BccI (0.3U)(New England Biolabs, Pickering, ON, Canada) were used to digest the rs1495099 (C allele cut; G allele uncut), rs907094 (T allele cut; C allele uncut) and rs3764352 (A allele cut; G allele uncut) amplicons, respectively, and all digestion products were separated on 2% agarose gels and visualized using ethidium bromide and UV illumination. All results were independently assessed by two persons.

2.3 Statistical Analyses

2.3.1 Test Study

2.3.1.1 Pre-analysis Quality Control

Prior to carrying out various analyses, Mendelian errors were identified in family cohorts using the FBAT program, v1.5.5 (Laird *et al.*, 2000). In families from TEST, which was the family cohort used for the first stage of this study, considerable discordance was found with *TH* rs6356 genotypes and *SLC6A3* rs2550936 genotypes which were not reconciled with repeated genotyping. Attempts to generate amplicons for rs6356 using traditional RFLP methods failed. These markers were excluded from further analyses. For the remaining polymorphisms at the *TH* and *SLC6A3* loci, a total of four and three families, respectively were excluded because of discordance while *DRD1* marker data on four families, *DRD2* marker data on five families and *PPP1R1B* marker data on two families from TEST, were excluded.

Chi-square tests for deviations from Hardy-Weinberg equilibrium (HWE) in family and comparison cohorts were performed using HWE, v1.05 as part of the linkage utility programs for human genetics package (available at http://www.genemapping.cn/util.htm). While deviations from HWE may be because of genetic drift, inbreeding and population stratification, it may also be an indicator of disease association (reviewed by Balding, 2006) or an indicator of genotyping error (Hosking *et al.*, 2004). Because of concerns of DNA quality of the 190 samples obtained from Guthrie spots for genotyping, HWE was determined and allele and genotype frequency comparisons were made between these samples and the remaining 253 DNA samples. Because deviations in HWE were found for markers from *DRD2* loci from

these 190 samples and differences in allele and genotype frequencies were found for markers from *TH* between groups, these samples were excluded from case-control comparisons in these genes. These samples were also excluded from comparisons of variants at *SLC6A3* because of difficulties in genotyping. Thus, the comparison cohort for *DRD1* and *PPP1R1B* consisted of 443 individuals while the comparison cohort for *TH*, *SLC6A3* and *DRD2* consisted of 253 individuals.

2.3.1.2 Single-gene Analyses

In the first stage of this study, allele and genotype frequency comparisons between affected individuals or their mothers from TEST, and the comparison group were made using Chi-square statistics (SPSS v14.0, SPSS, Chicago, IL, USA). Family-based association tests (FBAT), including single marker FBAT, quantitative transmission disequilibrium tests (QTDT) and haplotype TDT (HBAT), were done under an additive model using the FBAT program, v1.5.5 (Laird *et al.*, 2000) and included all affected individuals in TEST. FBAT was used for QTDT because the statistic does not require assumptions regarding the phenotype distribution (Laird *et al.*, 2000), and the ETDT program (Sham & Curtis, 1995) was used to examine the parent-of-origin of transmitted alleles. For case-control frequency comparisons and extended-transmission disequilibrium test (ETDT) analyses, one affected individual was selected at random from each family using SPSS v14.0 with the same cohort of randomly chosen individuals used for allele and genotype frequency comparisons and ETDT analyses of all polymorphisms at a given locus. Furthermore, this selected cohort from TEST was used in case-control and ETDT analyses for all genes studied.

The program, 2LD, was used to determine D' measures of linkage disequilibrium (LD)(Zhao, 2004). Haplotype frequencies in affected, mother and comparison cohorts were compared using the permutation test feature (-c flag) available with PHASE (v2.1)(Stephens *et al.*, 2001; Stephens & Scheet, 2005). PHASE compares haplotypes between cohorts under the assumption that both cohorts are part of the same population and determines whether haplotypes within a given cohort are more similar to each other than haplotypes of the other cohort, giving this permutation test greater power than traditional frequency comparisons. Although PHASE uses a default value of 100 permutations with this test, I ran 1000 permutations of my data to determine *P*-values. Because specific haplotypes could not be examined using the permutation feature, haplotype case-control comparisons between the family cohort and the comparison group were made using Chi-square statistics (SPSS v14.0, SPSS, Chicago, IL, USA) including individuals with haplotype pair calls of ≥95% estimated certainty.

2.3.1.3 Gene-gene Comparisons

Based on single gene findings, tests for gene-gene interactions between TEST and comparison cohorts were made using logistic regression from the Regression Analysis Module of HelixTree v6.3.4 (Golden Helix, Inc. Bozeman, MT, USA; available at http://www.goldenhelix.com).

2.3.1.4 Corrections for Multiple Comparisons

Because of the inconsistent and, often conflicting, findings in genetic association studies and human disease, it is recognized that statistical corrections for multiple comparisons

must be performed. However, because the contribution of a single gene to autism susceptibility is predicted to be relatively small and thus difficult to detect statistically, Bonferroni corrections for multiple testing are too conservative. Thus for my study, I applied the false discovery rate (FDR) approach using the Benjamini and Hochberg (BH) method (Benjamini & Hochberg, 1995; Benjamini *et al.*, 2001). FDR is less stringent than Bonferroni corrections, controlling the proportion of false positives within those hypothesis which are rejected (i.e. significant findings); it is a compromise between not correcting for multiple comparisons which is too lax versus Bonferroni adjustments which are too stringent. The BH method was found to be appropriate for correcting both independent and positively-dependent comparisons (Benjamini & Yekutieli, 2001), which is appropriate for genetic studies using polymorphisms. An example of FDR using the BH method is shown in Table 2.2.

In my two-stage study, FDR corrections were performed separately for single gene case-control and family-based comparisons as well as QTDT analyses and gene-gene findings using an initial FDR threshold of 0.050.

Table 2.2: Example of false discovery rate (FDR) adjustments using the Benjamini and Hochberg method and a starting alpha of 0.050

Case-Control Comparisons	Observed P-value[1]	Rank	FDR threshold[2]	Significant (FDR)	Bonferroni threshold ($\alpha/10$)	Significant (Bonferroni)
Marker 1 allele	**0.000011**	1	0.005[3]	**Yes**	0.005	**Yes**
Marker 1 genotype	**0.00049**	2	0.010	**Yes**	0.005	**Yes**
Marker 2 genotype	**0.0092**	3	0.015	**Yes**	0.005	No
Marker 3 allele	**0.010**	4	0.020	**Yes**	0.005	No
Marker 2 allele	**0.021**	5	0.025	**Yes**	0.005	No
Marker 3 genotype	**0.030**	6	0.030	**Yes**	0.005	No
Marker 4 allele	**0.042**	7	0.035	No	0.005	No
Marker 5 genotype	**0.045**	8	0.040	No	0.005	No
Marker 4 genotype	0.48	9	0.045	No	0.005	No
Marker 5 allele	0.55	10	0.050	No	0.005	No

[1]Ranked from smallest to largest. Values in bold are significant compared to a P-value of 0.050

[2]Determined by multiplying the starting threshold (i.e. 0.050) by the proportion of a given Rank divided by the total number of comparisons. Observed P-value is significant if it is less than or equal to the calculated FDR threshold

[3]Note that when using FDR the most significant finding is compared to the Bonferroni adjusted threshold

2.3.2 Replication Study

2.3.2.1 Pre-analysis Quality Control

In the second stage of this study which used family cohorts REPLICATION 1, REPLICATION 2 and REPLICATION 3 to test positive gene findings found in TEST, a total of eight families were excluded from *DRD1* data, three families were excluded from *DRD2* data and ten families were excluded from *PPP1R1B* data. The comparison cohorts for *DRD1*, *DRD2* and *PPP1R1B* which were used in the first stage of this study, were used for the replication analyses.

2.3.2.1 Genetic Analyses

The replication study approach and analyses including the random selection of affected individuals for case-control comparisons, determination of HWE in sample cohorts, allele and genotype frequency comparisons, family-based association tests and corrections for multiple comparisons were performed as described in the previous section.

Chapter 3

Results

3.1 TEST Study

I initially tested five DA-related genes for association with autism in a large cohort of male-only affected sib-pair families (TEST) using case-control comparisons, family-based tests and genotype-phenotype associations. Gene-gene interactions using logistic regression were then performed on genes of which single-gene analyses found evidence of association with autism in affected sons or their mothers in TEST families.

3.1.1 Single-gene Findings

3.1.1.1 *TH*

3.1.1.1.1 Linkage Disequilibrium of Polymorphisms at *TH* Locus

Measures of LD showed high LD between HUMTH01 and rs2070762 in both family (D'=0.81) and comparison cohorts (D'=0.80)(Figure 2.1).

3.1.1.1.2 Findings in Affected Males

3.1.1.1.2.1 Case-control Comparisons

Population based case-control analyses were performed on HUMTH01 and rs2070762. Both markers were in HWE in the comparison and family cohorts (P=0.27-0.45). As shown in Table 3.1, there were no significant differences in HUMTH01 10-repeat allele (P=0.12) or rs2070762 allele (P=0.17) frequencies in affected males (30.7% and 50.5%, respectively) compared to the comparison group (36.9% and 56.0%, respectively). No differences in HUMTH01 10-repeat containing ‘genotypes’ or rs2070762 genotype

Table 3.1: Marker allele and genotype frequencies at the *TH* locus in the comparison group and males with ASD from the TEST families

		Allele			
HUMTH01	N	10-repeat[1]	Other	χ^2 df=1	*P*
Comparison group	434	160 (36.9%)	274 (63.1%)		
Affected males[2]	218	67 (30.7%)	151 (69.3%)	2.405	0.12
rs2070762	N	T	C	χ^2 df=1	*P*
Comparison group	496	278 (56.0%)	218 (44.0%)		
Affected males[2]	220	111 (50.5%)	109 (49.5%)	1.922	0.17

		Genotype				
HUMTH01	N	10/10[1]	10[1]/other	other/other	χ^2 df=2	*P*
Comparison group	217	30 (13.8%)	100 (46.1%)	87 (40.1%)		
Affected males[2]	109	12 (11.0%)	43 (39.4%)	54 (49.5%)	2.672	0.26
rs2070762	N	T/T	T/C	C/C	χ^2 df=2	*P*
Comparison group	248	82 (33.1%)	114 (46.0%)	52 (21.0%)		
Affected males[2]	110	30 (27.3%)	51 (46.4%)	29 (26.4%)	1.800	0.41

[1]Includes perfect 10-repeat (10p) and imperfect 10-repeat (10i) alleles
[2]One affected individual was randomly chosen from each family

frequencies were found between cases and the comparison group (*P*=0.26 and *P*=0.41, respectively)(Table 3.1).

3.1.1.1.2.2 Family-based Association Tests

Because of a lack of informative families, the 10p-repeat allele was not included in family-based analyses and thus single marker FBAT analysis was performed to determine whether there was any over-transmission of HUMTH01 10i-repeat alleles or preferential allele transmission from rs2070762. As shown in Table 3.2, there was no significant over-transmission of 10i-repeat alleles (*P*=0.69) or rs2070762 alleles (*P*=0.11) in our cohort of male-only affected sib-pair families.

3.1.1.1.2.3 Genotype-Phenotype Associations

The domains, 'reciprocal social interaction', 'communication' and 'repetitive stereotyped behaviours' used for QTDT analyses were derived from total scores from the 'Qualitative Abnormalities in Reciprocal Social Interaction' (A1 to A4), 'Qualitative Abnormalities in Communication' (B1, B2(V), B3(V) and B4) and 'Restricted, Repetitive, and Stereotyped Patterns of Behaviour' (C1 to C4) subdomains in the ADI-R diagnostic algorithm (Lord *et al.*, 1994).

QTDT analysis did not find an association of either HUMTH0110i-repeat alleles or rs2070762 T alleles with ADI-R subdomain measures of reciprocal social interaction (*P*=0.94; *P*=0.28), communication (*P*=0.23; *P*=0.17) and repetitive stereotyped behaviours (*P*=0.82; *P*=0.46) in affected males (data not shown).

Table 3.2: FBAT of HUMTH01 and rs2070762 allele transmissions and HBAT of HUMTH01 10i-repeat and rs2070762 T haplotype transmissions at the *TH* locus in the TEST families[1]

Marker Locus and alleles	# Fam	Observed	Expected	Z	*P*
HUMTH01					
10i-repeat[2]	65	102.0	99.4	0.4	0.69
rs2070762					
T	82	181.0	168.8	1.6	0.11
C	82	157.0	169.2	-1.6	0.11
Haplotype					
10i-T	59	104.0	98.2	1.0	0.34

[1]All affected individuals were included in the analyses
[2]Insufficient number of families were informative to determine 10p-repeat allele transmission

3.1.1.1.2.4 Haplotype-based Findings

There was no difference in the frequency of the pooled HUMTH01 10-repeats-rs2070762 T (10-T) haplotype frequencies between affected males (28.9%) and the comparison group (35.3%)(χ^2=2.645, df=1, *P*=0.10) or evidence of over-transmission of this haplotype to affected sons (*P*=0.34)(Table 3.2). Furthermore, QTDT analysis found no association between the 10i-T haplotype and ADI-R measures of autism severity (*P*=0.31-0.87; data not shown).

3.1.1.1.3 Findings in Mothers

Because our model of autism susceptibility includes maternal effects as a contributing factor (Robinson *et al.*, 2001), I compared HUMTH01 10-repeat allele frequencies in mothers from the family cohort and the comparison group and found a decreased frequency of the 10-repeat allele (26.2% and 36.9%, respectively) and the 10-T haplotype (25.5% and 35.6%) in mothers compared to the comparison group (χ^2=7.382, df=1, *P*=0.007 and χ^2=6.349, df=1, *P*=0.012, respectively), which were significant following FDR corrections for multiple testing. The finding that the HUMTH01 10-repeat allele was more significant than that seen with the 10-T haplotype suggests that the 10-repeat allele alone is important in mothers.

3.1.1.2 *SLC6A3*

3.1.1.2.1 Linkage Disequilibrium of Polymorphisms at *SLC6A3* Locus

As shown in Figure 2.2, D' measures showed highly variable LD between markers with very low LD (D'=0.09-0.17 in parents and D'=0.06-0.23 in comparison group) between

rs2975226 and the other three polymorphisms with generally higher LD seen between EX15 VNTR and I8 VNTR and rs28363149.

3.1.1.2.2 Findings in Affected Males

3.1.1.2.2.1 Case-control Comparisons

All four markers were in HWE in both cohorts (*P*=0.11-1.0). As shown in Table 3.3, there were no significant differences in the rs2975226 A, I8 VNTR 5-repeat, rs28363149 Ins and EX15 VNTR 9-repeat allele frequencies in our cohort of affected males relative to our comparison group (*P*=0.22-0.83). Collapsed genotype comparisons of the VNTRs (6/6 *versus* pooled 5/5 and 5/6 for I8 VNTR; 10/10 *versus* pooled 9/9 and 9/10 for EX15 VNTR) were performed based on differences in mRNA levels and DA transporter binding between individuals with these genotypes (Heinz *et al.*, 2000; Brookes *et al.*, 2007). No differences between genotype frequencies of any of the markers were seen between affected and comparison cohorts (*P*=0.42-0.90)(Table 3.4).

3.1.1.2.2.2 Family-based Association Tests

Single marker FBAT analysis was performed to determine whether there was preferential allele transmission from any of the four polymorphisms and, as shown in Table 3.5, there was no significant over-transmission of any of the markers studied in the TEST family cohort (*P*=0.23-0.94).

Table 3.3: Marker allele frequencies at the *SLC6A3* locus in the comparison group and males with ASD from the TEST families

		Allele			
rs2975226	N	A	T	χ^2 df=1	*P*
Comparison group	504	293 (58.1%)	211 (41.9%)		
Affected males[1]	222	137 (61.7%)	85 (38.3%)	0.816	0.37
I8 VNTR	N	5-repeat	other	χ^2 df=1	*P*
Comparison group	496	98 (19.8%)	181 (82.3%)		
Affected males[1]	220	39 (17.7%)	398 (80.2%)	0.406	0.52
rs28363149	N	Del	Ins	χ^2 df=1	*P*
Comparison group	506	479 (94.7%)	27 (5.3%)		
Affected males[1]	220	203 (92.3%)	17 (7.7%)	1.540	0.22
EX15 VNTR	N	9-repeat	other	χ^2 df=1	*P*
Comparison group	492	124 (24.7%)	378 (75.3%)		
Affected males[1]	218	56 (25.5%)	164 (74.5%)	0.046	0.83

[1]One affected individual was randomly chosen from each family

Table 3.4: Marker genotype frequencies at the *SLC6A3* locus in the comparison group and males with ASD from the TEST families

		Genotype				
rs2975226	N	A/A	A/T	T/T	χ^2 df=2	*P*
Comparison group	252	89 (35.3%)	115 (45.6%)	48 (19.0%)		
Affected males[1]	111	44 (39.6%)	49 (44.1%)	18 (16.2%)	0.771	0.68
I8 VNTR	N	5/5 & 5/6	6/6	other/other	χ^2 df=2	*P*
Comparison group	248	81 (32.7%)	160 (64.5%)	7 (2.8%)		
Affected males[1]	110	33 (30.0%)	74 (67.3%)	3 (2.7%)	0.261	0.88
rs28363149	N	Del/Del	Del/Ins	Ins/Ins	χ^2 df=2	*P*
Comparison group	253	228 (90.1%)	23 (9.1%)	2 (0.8%)		
Affected males[1]	110	94 (85.5%)	15 (13.6%)	1 (0.9%)	1.714	0.42
EX15 VNTR	N	9/9 & 9/10	10/10	other/other	χ^2 df=2	*P*
Comparison group	251	102 (40.6%)	138 (55.0%)	11 (4.4%)		
Affected males[1]	110	47 (42.7%)	59 (53.6%)	4 (3.6%)	0.209	0.90

[1]One affected individual was randomly chosen from each family

Table 3.5: FBAT of rs2975226, I8 VNTR, rs28363149 and EX15 VNTR allele transmissions and HBAT of haplotype transmissions derived from these polymorphisms at the *SLC6A3* locus in the TEST families[1]

Marker Locus and alleles	# Fam	Observed	Expected	Z	*P*
rs2975226					
A[2]	74	172.0	172.5	-0.1	0.94
T	74	130.0	129.5	0.1	0.94
I8 VNTR					
5-repeat[2]	52	72.0	70.5	0.3	0.79
6-repeat	50	133.0	134.5	-0.3	0.79
rs28363149					
Ins[2]	26	34.0	29.5	1.2	0.23
Del	26	72.0	76.5	-1.2	0.23
EX15 VNTR					
9-repeat[2]	65	88.0	86.5	0.2	0.81
10-repeat	67	179.0	182.5	-0.6	0.58
Haplotypes[3]	# Fam	Observed	Expected	Z	*P*
A-5-Ins-9[4]	0				
A-5------9	27	31.5	33.5	-0.5	0.60
5-Ins-9[4]	2				
A-5	34	40.5	41.5	-0.2	0.82
5-Ins[4]	3				
Ins-9[4]	4				
5------9	44	60.0	57.0	0.6	0.56

[1]All affected individuals were included in the analyses
[2]Allele associated with decreased gene expression and/or protein availability
[3]Marker order of haplotype comparisons: rs2975226 - I8 VNTR - rs28363149 - EX15 VNTR
[4]Insufficient number of families were informative to determine haplotype transmission

3.1.1.2.2.3 Genotype-Phenotype Associations

QTDT analyses did not find an association between rs2975226 A, I8 VNTR 5-repeat, rs28363149 Ins and EX15 VNTR 9-repeat alleles or haplotypes derived from these alleles with ADI-R subdomain scores in reciprocal social interaction, communication and repetitive and stereotyped behaviours (data not shown) with the exception of an association of the I8 VNTR 5-repeat – EX15 VNTR 9-repeat (5-9) haplotype with greater impairments in communication (P=0.022; not significant following FDR correction).

3.1.1.2.2.4 Haplotype-based Findings

I next performed analyses on haplotypes derived from several marker combinations spanning the *SLC6A3* locus. Because of the variable LD seen between markers (Figure 2.2) and low rs28363149 Ins allele frequency (<10%), I examined haplotypes from all four markers as well as rs2975226 - I8 VNTR – EX15 VNTR haplotypes which excluded rs28363149. In addition, because Greenwood *et al.* (2002) found two haplotype block regions within the *SLC6A3* locus, one spanning the promoter region to intron 6 and the other spanning from intron 8 to exon 15, I performed haplotype analyses on haplotypes derived from I8 VNTR and EX15 VNTR with and without rs28363149. Lastly, because it is unclear whether I8 VNTR is part of the 3' haplotype block region, I also used a 2-marker sliding haplotype window. As shown in Table 3.5, haplotypes predicted to be associated with decreased gene expression or protein binding were not preferentially transmitted (P=0.55-0.82) in my cohort of male-only affected sib-pair families (TEST).

Haplotype frequency comparisons between affected males and the comparison group were not significant for any of the hypothesized risk haplotypes (data not shown).

Permutation analyses comparing haplotypes of any of my marker combinations between cohorts were not significant (*P*=0.20-0.74).

3.1.1.2.3 Findings in Mothers

I examined single marker allele and haplotype frequencies between mothers from my affected male family cohort and comparison group taking into consideration that alleles associated with either decreased expression or increased expression may be present in mothers. There were no differences found in rs2975226 A/T, I8 VNTR 5-repeat or 6-repeat, rs28363149 Del/Ins and EX15 VNTR 9-repeat or 10-repeat allele frequencies (*P*=0.20-0.98) between TEST mothers and the comparison group (data not shown). Haplotype case-control comparisons did not identify any haplotypes associated with mothers of affected sons that may suggest altered *SLC6A3* function (data not shown). Haplotype permutation tests between mothers and the comparison group were not significant (*P*=0.73-0.82).

3.1.1.3 *DRD1*

3.1.1.3.1 Linkage Disequilibrium of Polymorphisms at *DRD1* Locus

LD measures (D') between markers showed high LD in both the comparison group (D'>0.8) and the parents of affected children (D'>0.9), with greater LD observed in the parents (Figure 2.3).

3.1.1.3.2 Findings in Affected Males

3.1.1.3.2.1 Case-control Comparisons

All three markers were in HWE in the study populations (*P*=0.10-0.65). Increased frequencies of the common alleles of rs265981 C (*P*=0.007) and rs4532 A (*P*=0.013) were observed in affected males (72.2% and 71.0%, respectively) relative to the comparison group (62.5% and 61.9%, respectively), both of which were significant following corrections for multiple comparisons (Table 3.6). The rs265981 CC (*P*=0.030) and rs4532 AA (*P*=0.036) genotype frequencies were increased in affected males (54.6% and 52.3%, respectively) relative to the comparison group (40.9% and 38.8%, respectively), but these findings were not significant following FDR-based corrections (data not shown).

3.1.1.3.2.2 Family-based Association Tests

In family-based association analyses, preferential transmission of rs265981 C alleles (*P*=0.040) was found in TEST families which remained significant following FDR correction. Over-transmission of rs4532 A (*P*=0.038) was also observed but was not significant following corrections for multiple comparisons (Table 3.7).

3.1.1.3.2.3 Genotype-Phenotype Associations

Multivariate QTDT for single markers showed strong evidence of association between each marker and the combined effect of the three ADI-R subdomains, social interaction, nonverbal communication and stereotypies (*P*=0.0027-0.0065), all of which were significant following FDR-based corrections (Table 3.8).

Table 3.6: Marker allele frequencies at the *DRD1* locus in the comparison group and males with ASD from the TEST families

		Allele				FDR
rs265981	N	C	T	χ^2 df=1	P^1	threshold[2]
Comparison group	880	550 (62.5%)	330 (37.5%)			
Affected males[3]	216	156 (72.2%)	60 (27.8%)	7.152	**0.007**	0.014
rs4532	N	A	G	χ^2 df=1	*P*	
Comparison group	876	542 (61.9%)	334 (38.1%)			
Affected males[3]	214	152 (71.0%)	62 (29.0%)	6.233	**0.013**	0.021
rs686	N	T	C	χ^2 df=1	*P*	
Comparison group	884	555 (62.8%)	329 (37.2%)			
Affected males[3]	216	150 (69.4%)	66 (30.6%)	3.347	0.067	0.043

[1]P-values less than 0.05 are in bold and P-values which remain significant following false-discovery rate (FDR) corrections for multiple comparisons are underlined
[2]P-value ≤ FDR threshold is significant
[3]One affected individual was randomly chosen from each family

Table 3.7: FBAT of rs265981, rs4532 and rs686 allele transmissions at the *DRD1* locus in the TEST families[1]

Marker Locus and alleles	# Fam	Observed	Expected	Z	P^2	FDR threshold[3]
rs265981						
C	64	168.0	154.5	2.1	**0.040**	0.040
T	64	92.0	105.5	-2.1	**0.040**	
rs4532						
A	63	167.0	153.5	2.1	**0.038**	0.030
G	63	91.0	104.5	-2.1	**0.038**	
rs686						
T	70	180.0	167.0	1.9	0.054	0.050
C	70	104.0	117.0	-1.9	0.054	

[1]All affected individuals were included in the analyses
[2]P-values less than 0.05 are in bold and P-values which remain significant following false-discovery rate (FDR) corrections for multiple comparisons are underlined
[3]P-value ≤ FDR threshold is significant

Table 3.8: Multivariate QTDT of single markers and QTDT of rs265981, rs4532 and rs686 C-A-T haplotypes at the *DRD1* locus in the TEST families[1]

Combined ADI-R Subdomains	Multivariate QTDT				
	Marker	# Fam	χ^2 df = 3	P^2	FDR threshold[3]
Social Interaction, Nonverbal Communication & Stereotypies	rs265981	29	12.290	**<u>0.0065</u>**	0.025
	rs4532	30	12.560	**<u>0.0057</u>**	0.017
	rs686	32	14.189	**<u>0.0027</u>**	0.0083

ADI-R Subdomain	QTDT						
	Haplotype	# Fam	Observed	Expected	Z	P^2	FDR threshold[3]
Social Interaction	C-A-T	49	2523.2	2268.6	2.1	**0.039**	0.033
Nonverbal Communication	C-A-T	30	559.6	481.8	1.9	0.061	0.042
Stereotypies	C-A-T	49	784.9	713.4	1.8	0.072	0.050

[1]All affected males were included for QTDT analyses
[2]P-values less than 0.05 are in bold and P-values which remain significant following false-discovery rate (FDR) corrections for multiple comparisons are underlined
[3]P-value ≤ FDR threshold is significant

3.1.1.3.2.4 Haplotype-based Findings

Based on FDR-corrected findings in case-control comparisons and family-based tests including QTDT, rs265981 C was identified as a putative risk allele associated with autism. Haplotype analyses was performed to determine whether a risk haplotype containing these three markers showed greater evidence of association. The rs265981 C – rs4532 A – rs686 T (C-A-T) haplotype was found at a significantly higher frequency in affected males relative to the comparison group (149/220 or 67.7% *versus* 498/874 or 57.0%, P=0.004), which was significant following corrections for multiple comparisons. However, family-based tests showed transmission of the C-A-T haplotype was not significant following FDR correction (P=0.022; data not shown) and QTDT analysis showed no significant association of the haplotype with the three ADI-R subdomains following corrections for multiple comparisons (P=0.039-0.072)(Table 3.8). Thus, these findings show that the rs265981 C allele itself is associated with autism in TEST families.

3.1.1.3.3 Findings in Mothers

In addition to my finding of a putative DRD1 risk allele in males with autism, separate analyses using ETDT showed that over-transmission of the rs265981 A allele (21 transmitted, 8 untransmitted) was from mothers to affected children (ETDT: χ^2=5.828, df=1, P=0.016) while no significant paternal transmission was found (20 transmitted, 18 untransmitted; χ^2=0.105, df=1, P=0.75). Although an increased frequency of rs265981 C alleles was found in the mothers of autistic sons relative to the comparison group (69.6% *versus* 62.5%, P=0.052), the finding was not significant; however, a significantly

increased frequency of the C-A-T haplotype was found in mothers compared to the comparison group (65.4% *versus* 57.0%) which was significant following FDR corrections (*P*=0.025).

3.1.1.4 *DRD2*

3.1.1.4.1 Linkage Disequilibrium of Polymorphisms at *DRD2* Locus

High D' measures of LD were observed between rs1079597 and both rs1800498 and rs1800497 with slightly lower LD observed between rs1800498 and rs1800497. Low LD was found between rs1799732 and rs1079597 and rs1800497, with higher LD seen between rs1799732 and rs1800498 (Figure 2.4). The extent of LD was comparable between parents of affected children and the comparison group with the exception that lower LD was seen between rs1799732 and rs1079597 (0.04 *versus* 0.34) and rs1799732 and rs1800497 (0.07 *versus* 0.29) in parents versus the comparison group.

3.1.1.4.2 Findings in Affected Males

3.1.1.4.2.1 Case-control Comparisons

All four markers were in HWE in the comparison and TEST cohorts with the exception of rs1799732 and rs1800498 in affected males (*P*=0.009 and *P*=0.012, respectively); these markers were in HWE in the parents from these families. As shown in Table 3.9, an increased frequency of the rs1800498 TT genotype (*P*=0.007) was observed in affected males (43.4% *versus* 28.7% in the comparison group) which remained significant following FDR correction. No significant differences in genotype frequencies

Table 3.9: Marker genotype frequencies at the *DRD2* locus in the comparison group and males with ASD from the TEST families

		Genotype			χ^2 (df=2)		FDR
rs1799732	N	Ins/Ins	Del/Ins	Del/Del		P[1]	threshold[2]
Comparison group	238	188 (79.0%)	46 (19.3%)	4 (1.7%)			
Affected males[3]	109	89 (81.7%)	16 (14.7%)	4 (3.7%)	2.253	0.32	0.025
rs1079597	N	G/G	A/G	A/A	χ^2 (df=2)	P	
Comparison group	244	168 (68.9%)	71 (29.1%)	5 (2.0%)			
Affected males[3]	105	70 (66.7%)	30 (28.6%)	5 (4.8%)	1.944	0.38	0.038
rs1800498	N	T/T	C/T	C/C	χ^2 (df=2)	P	
Comparison group	244	70 (28.7%)	130 (53.3%)	44 (18.0%)			
Affected males[3]	106	46 (43.4%)	38 (35.8%)	22 (20.8%)	9.790	**0.007**	0.013
rs1800497	N	C/C	T/C	T/T	χ^2 (df=2)	P	
Comparison group	245	164 (66.9%)	69 (28.2%)	12 (4.9%)			
Affected males[3]	107	65 (60.7%)	35 (32.7%)	7 (6.5%)	1.333	0.51	0.050

[1]P-values less than 0.05 are in bold and P-values which remain significant following false-discovery rate (FDR) corrections for multiple comparisons are underlined
[2]P-value ≤ FDR threshold is significant
[3]One affected individual was randomly chosen from each family

of the other three markers were seen between cases and the comparison group (*P*=0.32-0.51)(Table 3.9).

3.1.1.4.2.2 Family-based Association Tests

As shown in Table 3.10, family-based tests using FBAT showed that the rs1800498 T allele was over-transmitted to affected males (*P*=0.0003; significant following FDR correction) while no evidence of preferential allele transmission was found for the other three markers (*P*=0.16-0.94).

3.1.1.4.2.3 Genotype-Phenotype Associations

Because the rs1800498 T allele was associated with susceptibility to ASDs in TEST families, I used QTDT to determine whether the extent of impairments in the core behaviours was more pronounced in affected males with the rs1800498 T risk allele. Table 3.11 shows that the risk allele was associated with more severe impairments in reciprocal social interaction (*P*=0.0002), verbal communication (*P*=0.0004), and repetitive and stereotyped behaviours (*P*=0.0021) and these findings remained significant following corrections for multiple comparisons.

3.1.1.4.2.4 Haplotype-based Findings

HBAT was performed to determine whether the rs1799732 - rs1079597 - rs1800498 - rs1800497 haplotype was more strongly associated with autism than rs1800498 alone. The rs1799732 Ins - rs1079597 G - rs1800498 T - rs1800497 C (Ins-G-T-C) haplotype, which consists of the major alleles of all four markers, was over-transmitted to affected

Table 3.10: FBAT of rs1799732, rs1079597, rs1800498 and rs1800497 allele transmissions at the *DRD2* locus in the TEST families[1]

Marker Locus and alleles	# Fam	Observed	Expected	Z	P^2	FDR threshold[3]
rs1788732						
Ins	31	90.0	84.0	1.4	0.16	0.030
Del	31	38.0	44.0	-1.4	0.16	
rs1079597						
G	56	149.0	148.0	0.17	0.86	0.040
A	56	71.0	72.0	-0.17	0.86	
rs1800498						
T	73	185.0	160.0	3.6	**0.0003**	0.010
C	73	115.0	140.0	-3.6	**0.0003**	
rs1800497						
C	63	171.0	170.5	0.08	0.94	0.050
T	63	89.0	89.5	-0.08	0.94	

[1]All affected individuals were included in the analyses
[2]P-values less than 0.05 are in bold and P-values which remain significant following false-discovery rate (FDR) corrections for multiple comparisons are underlined
[3]P-value ≤ FDR threshold is significant

Table 3.11: QTDT of rs1800498 alleles at the *DRD2* locus in the TEST families[1]

ADI-R Subdomain	rs1800498	# Fam	Observed	Expected	Z	P^2	FDR threshold[3]
Social Interaction	T	56	2909.0	2452.5	3.7	**0.0002**	0.017
	C	56	1557.0	2013.5	-3.7	**0.0002**	
Verbal Communication	T	46	1364.0	1103.5	3.6	**0.0004**	0.033
	C	46	666.0	926.5	-3.6	**0.0004**	
Stereotyped Behaviours	T	56	876.0	754.5	3.1	**0.0021**	0.050
	C	56	502.0	623.5	-3.1	**0.0021**	

[1]All affected males were included for QTDT analyses
[2]P-values less than 0.05 are in bold and P-values which remain significant following false-discovery rate (FDR) corrections for multiple comparisons are underlined
[3]P-value ≤ FDR threshold is significant

males but with a P-value (*P*=0.0009; data not shown) slightly higher than that observed with rs1800498 T alone (*P*= 0.0003), showing that preferential transmission of these haplotypes is driven solely by the rs1800498 T allele.

3.1.1.4.3 Findings in Mothers

Separate analyses using ETDT showed that significant over-transmission of the rs1800498 T allele was not from mothers (21 transmitted, 11 untransmitted; χ^2=3.125, df=1, *P*=0.077) but was from fathers to affected sons (26 transmitted, 12 untransmitted; χ^2=5.158, df=1, *P*=0.023). No differences in rs1800498 T allele or rs1800498 TT genotype frequencies were found between mothers (*P*=0.69 and *P*=0.26, respectively) or fathers (*P*=0.90 and *P*=0.65, respectively) compared to the comparison group (data not shown).

3.1.1.5 *PPP1R1B*

3.1.1.5.1 Linkage Disequilibrium of Polymorphisms at *PPP1R1B* Locus

D' measures showed high LD (D'>0.9) between markers in the comparison group and parents of the affected children (Figure 2.5).

3.1.1.5.2 Findings in Affected Males

3.1.1.5.2.1 Case-control Comparisons

All three markers were in HWE in the comparison group (data not shown). However, none of the markers, rs1495099, rs907094 and rs3764352, were in HWE in the cohort of affected individuals (*P*=0.008, *P*=0.033 and *P*=0.033, respectively) but all markers were

in HWE in parents (data not shown). As shown in Table 3.12, the minor allele frequencies of all three markers, rs1495099 C, rs907094 C and rs3764352 G, were increased in the cohort of affected males relative to my comparison group (P=0.001, P=0.014 and P=0.021, respectively). The findings on the genotypes of these three polymorphisms were similar. The rs1495099 CC (P=0.001), rs907094 CC (P=0.010) and rs3764352 GG (P=0.007) genotype frequencies were increased in affected males (22.0%, 14.5% and 14.5%, respectively) relative to the comparison group (9.9%, 6.9% and 6.7%, respectively)(Table 3.13). All case-control comparisons were significant following corrections for multiple comparisons.

3.1.1.5.2.2 Family-based Association Tests

I next asked whether the rs1495099 C, rs907094 C and rs3764352 G alleles were over transmitted using a recessive model. A recessive model was applied based on the significantly increased homozygous rs1495099 CC, rs907094 CC and rs3764352 GG genotype frequencies found in affected males (Table 3.13). As shown in Table 3.14, family-based association analyses and FDR-based corrections for multiple comparisons showed significant over-transmission of rs1495099 C (P=0.00092) but not of rs907094 C (P=0.11) or rs3764352 G (P=0.09).

3.1.1.5.2.3 Genotype-Phenotype Associations

To determine whether the rs1495099 C allele was associated with the degree of impairments in the core behaviours of autism, QTDT under a recessive model was performed. As shown in Table 3.15, this allele was associated with higher ADI-R

Table 3.12: Marker allele frequencies at the *PPP1R1B* locus in the comparison group and males with ASD from the TEST families

		Allele				FDR
rs1495099	N	G	C	χ^2 df=1	P[1]	threshold[2]
Comparison group	868	619 (71.3%)	249 (28.7%)			
Affected males[3]	218	131 (60.1%)	87 (39.9%)	10.269	**<u>0.001</u>**	0.017
rs907094	N	T	C	χ^2 df=1	P	
Comparison group	868	662 (76.3%)	206 (23.7%)			
Affected males[3]	220	150 (68.2%)	70 (31.8%)	6.061	**<u>0.014</u>**	0.042
rs3764352	N	A	G	χ^2 df=1	P	
Comparison group	868	658 (75.8%)	210 (24.2%)			
Affected males[3]	220	150 (68.2%)	70 (31.8%)	5.339	**<u>0.021</u>**	0.050

[1]P-values less than 0.05 are in bold and P-values which remain significant following false-discovery rate (FDR) corrections for multiple comparisons are underlined
[2]P-value ≤ FDR threshold is significant
[3]One affected individual was randomly chosen from each family

Table 3.13: Marker genotype frequencies at the *PPP1R1B* locus in the comparison group and males with ASD from the TEST families

		Genotype				FDR
rs1495099	N	C/C	C/G & G/G	χ^2 (df=1)	P[1]	threshold[2]
Comparison group	434	43 (9.9%)	391 (90.1%)			
Affected males[3]	109	24 (22.0%)	85 (78.0%)	11.813	**<u>0.001</u>**	0.0083
rs907094	N	C/C	C/T & T/T	χ^2 (df=1)	P	
Comparison group	434	30 (6.9%)	404 (93.1%)			
Affected males[3]	110	16 (14.5%)	94 (85.5%)	6.605	**<u>0.010</u>**	0.033
rs3764352	N	G/G	A/G & A/A	χ^2 (df=1)	P	
Comparison group	434	29 (6.7%)	405 (93.3%)			
Affected males[3]	110	16 (14.5%)	94 (85.5%)	7.151	**<u>0.007</u>**	0.025

[1]P-values less than 0.05 are in bold and P-values which remain significant following false-discovery rate (FDR) corrections for multiple comparisons are underlined
[2]P-value ≤ FDR threshold is significant
[3]One affected individual was randomly chosen from each family

Table 3.14: FBAT of rs1495099, rs907094 and rs3764352 allele transmissions under a recessive model at the *PPP1R1B* locus in the TEST families[1]

Marker Locus and alleles	# Fam	Observed	Expected	Z	P^2	FDR threshold[3]
rs1495099						
G	52	42.0	43.8	-0.4	0.72	0.043
C	34	39.0	26.3	3.3	**0.00092**	0.0071
rs907094						
T	52	46.0	48.3	-0.5	0.66	0.036
C	24	25.0	19.8	1.6	0.11	0.029
rs3764352						
A	54	48.0	49.8	-0.3	0.73	0.050
G	26	27.0	21.3	1.7	0.09	0.021

[1]All affected individuals were included in the analyses
[2]P-values less than 0.05 are in bold and P-values which remain significant following false-discovery rate (FDR) corrections for multiple comparisons are underlined
[3]P-value ≤ FDR threshold is significant

Table 3.15: QTDT and multivariate QTDT of rs1495099 C alleles under a recessive model at the *PPP1R1B* locus in the TEST families[1]

ADI-R Subdomain	QTDT						FDR threshold[3]
	rs1495099	# Fam	Observed	Expected	Z	P^2	
Social Interaction	C	19	480.0	283.5	3.2	**0.0016**	0.038
Nonverbal Communication	C	10	108.0	52.3	2.8	**0.0046**	0.050
Stereotyped Behaviours	C	19	142.0	79.8	3.4	**0.00072**	0.025
Combined ADI-R Subdomains	Multivariate QTDT						FDR threshold[3]
	rs1495099	# Fam	χ^2 df = 3			P^2	
Social Interaction, Nonverbal Communication & Stereotypies	C	10	18.118			**0.00042**	0.013

[1]All affected individuals were included for QTDT analyses
[2]P-values less than 0.05 are in bold and P-values which remain significant following false-discovery rate (FDR) corrections for multiple comparisons are underlined
[3]P-value ≤ FDR threshold is significant

domain scores (more severe problems) in affected males for social interaction (*P*=0.0016), nonverbal communication (*P*=0.0046), and stereotyped behaviours (*P*=0.00072) with strong evidence of association shown by multivariate QTDT between rs1495099 C and the combined effect of all three ADI-R subdomains (*P*=0.00042). These findings were all significant following FDR-based corrections for multiple comparisons.

3.1.1.5.2.4 Haplotype-based Findings

HBAT was then performed to determine whether haplotypes derived from the inclusion of rs907094 and rs3764352 showed greater evidence of association compared to the single marker rs1495099 findings using a recessive model. The rs1495099 C - rs907094 C - rs3764352 G (C-C-G) haplotype was not significantly over-transmitted to affected males (*P*=0.031; not significant following FDR corrections) compared to that observed with rs1495099 C alone (*P*=0.00092), showing that over-transmission is driven solely by the rs1495099 C allele.

3.1.1.5.3 Findings in Mothers

Frequencies of rs1495099 C alleles and rs1495099 CC genotypes were not significantly different (*P*=0.19 and *P*=0.39, respectively) between mothers from TEST (33.2% and 12.7%, respectively) compared to the comparison group (28.7% and 9.9%, respectively).

3.1.2 Gene-gene Findings

Because single-gene analyses of families from TEST showed evidence of association of *DRD1*, *DRD2* and *PPP1R1B* with affected sons and evidence of association of *TH* and *DRD1* in mothers, logistic regression using HelixTree was performed to test for evidence of gene-gene interactions of DA-related genes and ASDs in TEST families.

3.1.2.1 Gene Interaction Findings in Affected Males

Evidence for DA-related gene interactions were found between *DRD1* rs265981, *DRD2* rs1800498 and *PPP1R1B* rs1495099 (P=0.0094) in affected males relative to the comparison group accounting for 4.7% of the variance (adjusted R^2) between cohorts. Significant interactions were also found between *DRD1* and *DRD2* (P=0.012), *DRD1* and *PPP1R1B* (P=0.0095), and *DRD2* and *PPP1R1B* (P=0.0035) between individuals with ASDs and the comparison cohort. All comparisons were significant following FDR-based corrections for multiple comparisons.

3.1.2.2 Gene Interaction Findings in Mothers

No significant interaction between *TH* HUMTH01 and the *DRD1* C-A-T haplotype was found in mothers from TEST compared to the comparison group (P=0.28).

3.2 Replication Study

The second stage of this study was as a replication study and consisted of testing positive gene findings in TEST families in three additional affected male-only family cohorts (REPLICATION 1, REPLICATION 2 and REPLICATION 3).

3.2.1 Replication Findings in Affected Males

3.2.1.1 *DRD1*

In the first stage of my study I found a risk allele, rs265981 C, in the *DRD1* gene associated with affected males from families of TEST. This polymorphism was genotyped in three replication family cohorts and was in HWE in all three cohorts (P=0.38-1.0). As shown in Table 3.16, case-control analyses did not find an increased frequency of the rs265981 C allele in affected males from REPLICATION 1 or REPLICATION 2 (64.3% and 65.7% *versus* 62.5% in the comparison cohort). An increased (P=0.035) frequency of this allele was found in affected males from REPLICATION 3 families (73.8%) but the finding was not significant following FDR-based corrections for multiple comparisons (Table 3.16).

FBAT analysis did not show over-transmission of the rs265981 C allele in any of the replication cohorts (P=0.16-0.58)(Table 3.17) and I found using QTDT analysis of available ADI-R data that the rs265981 C allele was not associated with impairments in social interaction (P=0.44 and P=0.97, respectively) and nonverbal communication (P=0.86; insufficient number of families for determination in REPLICATION 2), and more severe stereotypies (P=0.73 and P=0.66, respectively) in affected individuals from REPLICATION 1 and REPLICATION 2 (data not shown).

Table 3.16: Rs265981 allele frequencies at the *DRD1* locus in the comparison group and males with ASD from the REPLICATION families

	Allele					FDR
rs265981	N	C	T	χ^2 df=1	*P*[1]	threshold[2]
Comparison group	880	550 (62.5%)	330 (37.5%)			
Affected males						
Replication 1[3]	126	81 (64.3%)	45 (35.7%)	0.150	0.70	0.050
Replication 2	204	134 (65.7%)	70 (34.3%)	0.722	0.40	0.033
Replication 3	88	65 (73.8%)	23 (26.1%)	4.459	**0.035**	0.017

[1]P-values less than 0.05 are in bold and P-values which remain significant following false-discovery rate (FDR) corrections for multiple comparisons are underlined
[2]P-value ≤ FDR threshold is significant
[3]One affected individual was randomly chosen from each family

Table 3.17: FBAT of rs265981 allele transmissions at the *DRD1* locus in the REPLICATION families

Family cohort[1]	rs265981	# Fam	Observed	Expected	Z	*P*
Replication 1	C	37	74.0	70.5	0.7	0.48
	T	37	78.0	81.5	-0.7	0.48
Replication 2	C	66	51.0	48.5	0.6	0.58
	T	66	81.0	83.5	-0.6	0.58
Replication 3	C	33	20.0	24.5	-1.4	0.16
	T	33	46.0	41.5	1.4	0.16

[1]All affected individuals were included in the analyses

3.2.1.2 *DRD2*

Of the four markers examined in the TEST family cohort, I identified an association of the rs1800498 polymorphism at the *DRD2* locus to autism susceptibility in affected males. To further examine the nature of this association, I genotyped this polymorphism in my replication cohorts of affected males and found an increased frequency of the TT genotype (P=0.003) in affected individuals (51.0%) from REPLICATION 3 which was significant following FDR corrections (Table 3.18). No differences in rs1800498 TT genotype frequencies were found between affected individuals from REPLICATION 1 (P=0.24) or REPLICATION 2 (P=0.073) compared to the comparison cohort. This marker was in HWE in all replication cohorts (P=0.24-0.29).

FBAT analysis of the rs1800498 T allele did not identify over-transmission to affected males in families from REPLICATION 1 (P=0.92), REPLICATION 2 (P=0.66) or REPLICATION 3 (P=0.19)(Table 3.19). QTDT analyses showed that the rs1800498 T allele was not associated with greater impairments in ADI-R measures of social interaction, (P=0.30 and P=0.79, respectively), communication (P=0.050 and P=0.41, respectively) or more severe stereotypies (P=0.18 and P=0.85, respectively) in affected individuals from REPLICATION 1 and REPLICATION 2 (data not shown). Although it was associated with improved verbal communication in affected males (P=0.050) in REPLICATION 1, this finding was not significant following FDR corrections.

Table 3.18: Rs1800498 genotype frequencies at the *DRD2* locus in the comparison group and males with ASD from the REPLICATION families

	Genotype						FDR
rs1800498	N	T/T	C/T	C/C	χ^2 df=2	*P*[1]	threshold[2]
Comparison group	244	70 (28.7%)	130 (53.3%)	44 (18.0%)			
Affected males							
Replication 1[3]	65	20 (30.8%)	28 (43.1%)	17 (26.2%)	2.836	0.24	0.050
Replication 2	103	42 (40.8%)	43 (41.7%)	18 (17.5%)	5.223	0.073	0.033
Replication 3	49	25 (51.0%)	22 (44.9%)	2 (4.1%)	11.888	**0.003**	0.017

[1]P-values less than 0.05 are in bold and P-values which remain significant following false-discovery rate (FDR) corrections for multiple comparisons are underlined
[2]P-value ≤ FDR threshold is significant
[3]One affected individual was randomly chosen from each family

Table 3.19: FBAT of rs1800498 allele transmissions at the *DRD2* locus in the REPLICATION families

Family cohort[1]	rs1800498	# Fam	Observed	Expected	Z	*P*
Replication 1	T	37	80.0	80.5	-0.1	0.92
	C	37	74.0	73.5	0.1	0.92
Replication 2	T	67	76.0	74.0	0.4	0.66
	C	67	58.0	60.0	-0.4	0.66
Replication 3	T	28	40.0	36.0	1.3	0.19
	C	28	16.0	20.0	-1.3	0.19

[1]All affected individuals were included in the analyses

3.2.1.3 *PPP1R1B*

In the first stage of my study, I found evidence of association of rs1495099 with autism susceptibility. This polymorphism was subsequently genotyped in three replication family cohorts and was in HWE in all three cohorts (*P*=0.38-0.93). As shown in Table 3.20, there were no differences in rs1495099 C allele or rs1495099 CC genotype frequencies between affected males from any of the replication cohorts compared to the comparison group (*P*=0.45-0.98).

Family-based tests under a recessive model showed that the rs1495099 C allele was not preferentially transmitted to affected sons in REPLICATION 1 (*P*=0.41), REPLICATION 2 (*P*=0.37) or REPLICATION 3 (*P*=0.74) families (Table 3.21). There was no evidence of association of the rs1495099 C allele to ADI-R measure of autism severity in either REPLICATION 1 (*P*=0.56-0.80) or REPLICATION 2 (*P*=0.44-0.52) families (data not shown).

3.2.1.4 Gene-gene Findings

Because there were no major findings of the *DRD1*, *DRD2* and *PPP1R1B* genes in affected sons from my replication cohorts, gene-gene analyses were not performed.

3.2.2 Replication Findings in Mothers

Two genes, *TH* and *DRD1* showed evidence of association in mothers from TEST. The HUMTH01 polymorphism at the *TH* locus was not genotyped in the replication cohorts because our laboratory discontinued radioactivity-based procedures prior to the

Table 3.20: Rs1495099 allele and genotype frequencies at the *PPP1R1B* locus in the comparison group and males with ASD from the REPLICATION families

	Allele				
rs1495099	N	G	C	χ^2 df=1	*P*
Comparison group	868	619 (71.3%)	249 (28.7%)		
Affected males					
Replication 1[1]	130	91 (70.0%)	39 (30.0%)	0.095	0.76
Replication 2	194	133 (68.6%)	61 (31.4%)	0.583	0.45
Replication 3	98	70 (71.4%)	28 (28.6%)	0.001	0.98

	Genotype				
rs1495099	N	C/C	C/G & G/G	χ^2 df=1	*P*
Comparison group	434	43 (9.9%)	391 (90.1%)		
Affected males					
Replication 1[1]	65	6 (9.2%)	59 (90.8%)	0.029	0.86
Replication 2	97	10 (10.3%)	87 (89.7%)	0.014	0.91
Replication 3	46	3 (6.5%)	43 (93.5%)	0.550	0.46

[1]One affected individual was randomly chosen from each family

Table 3.21: FBAT of rs1495099 C allele transmissions under a recessive model at the *PPP1R1B* locus in the REPLICATION families

Family cohort[1]	rs1495099	# Fam	Observed	Expected	Z	*P*
Replication 1	C	14	7.0	9.0	-0.8	0.41
Replication 2	C	23	10.0	8.0	0.9	0.37
Replication 3	C	11	3.0	3.5	-0.3	0.74

[1]All affected individuals were included in the analyses

replication stage of my lab work and thus, my replication study of findings in mothers is solely *DRD1*.

3.2.2.1 *DRD1*

I examined three polymorphisms in the TEST family cohort and identified a putative risk haplotype (C-A-T) at the *DRD1* locus which was associated with mothers of affected sons. To determine the extent of this association in families with ASDs, I genotyped these three markers in mothers from replication family cohorts and found no significant differences in C-A-T haplotype frequencies between mothers from REPLICATION 1 (P=0.57), REPLICATION 2 (P=0.21) or REPLICATION 3 (P=0.042) compared to the comparison cohort after corrections for multiple comparisons (data not shown).

3.2.2.2 Gene-gene Findings

Because a single gene was investigated in mothers from replication cohorts, gene-gene analyses were not performed.

Chapter 4

Discussion

The purpose of my study was to determine whether dopamine-related genes were significant factors in the etiology of ASDs in males from families with only affected males using a Test-Replication Study design. I found evidence for association of the *DRD1*, *DRD2* and *PPP1R1B* genes with autism in affected males from MPX families in the TEST cohort. Furthermore, I found that polymorphisms in the *TH* and *DRD1* genes were more common in mothers in these families and that putative risk alleles in *DRD1* and *DRD2* were preferentially transmitted from mothers and fathers respectively, to affected sons in the TEST cohort. To examine the scope of these findings in affected males with autism and their mothers, I performed a replication study using three additional male-only MPX and SPX replication cohorts using only the markers that showed a significant association in the TEST families. No evidence for association of these markers was found in affected sons or their mothers from REPLICATION 1, REPLICATION 2 or REPLICATION 3 cohorts.

Replication studies in autism are useful because they provide a means to test the validity of findings from one family cohort and to determine the applicability of these findings to other families with ASDs. The lack of replication in my study may mean that 1) there is no role for DA-related genes in autism susceptibility and my findings in TEST families are a false positive or 2) the contrasting findings between TEST and REPLICATION cohorts indicate differences between these families.

4.1 Replication Studies

False positive findings are a valid and significant concern in complex human genetics and disease, and can occur in linkage, candidate gene and whole-genome association studies. However, I feel that my findings in individuals from TEST families represent evidence for association with autism in these families for three reasons. 1) I used multiple approaches in the analysis of my data and my findings are consistently identified in case-control comparisons, family-based tests of allele transmission and genotype-phenotype associations using measures of autism severity. 2) I used FDR to correct for multiple comparisons which is important because the greater the number of comparisons the higher the chances of false positive findings. 3) I found consistent evidence for association of not one but three genes in the same pathway with autism in affected individuals from TEST families. Although the risk for false positives cannot be eliminated, I have minimized the risk and thus, believe that my findings in TEST families are evidence for association of DA-related genes with autism in that family cohort.

The finding that polymorphisms identified in the TEST cohort are not associated with autism in families from the replication cohorts suggests that the genetic etiologies of these family cohorts are different. If there are different genetic contributions in these cohorts then those genetic differences may be reflected by phenotypic differences between affected individuals from TEST and REPLICATION families. To test this hypothesis, I used clinical, medical and physical information available on affected individuals in TEST and REPLICATION families and a modified version of a clinical dysmorphology checklist developed by de Vries *et al.* (2001) to assign dysmorphology scores to affected individuals for phenotype comparisons. There are four dysmorphology

measures: prenatal growth delay, postnatal growth abnormalities, craniofacial dysmorphic features, and non-craniofacial dysmorphism and congenital anomalies. Prenatal growth delay includes percentile indication (≥2 standard deviations) of low birth weight while postnatal growth abnormalities included percentile indicators of short or tall stature, microcephaly and macrocephaly. Craniofacial dysmorphic features include nasal, ear and eye abnormalities (e.g. hypertelorism – an increased distance between eyes) and examples of congenital anomalies and non-craniofacial dysmorphism includes cardiac, gastrointestinal, genitourinary, skin and joint anomalies (e.g. pes planus – absent foot arch). It is important to note that this information was not available on all affected children, especially those from the replication families. Comparisons between affected individuals (N=112) from TEST families and affected individuals (N=25) pooled from REPLICATION 1 and REPLICATION 2 families showed phenotypic differences. A greater proportion of systemic anomalies (P=0.00009) were found in affected males from TEST families (71.4%) compared to affected males from REPLICATION families (56.0%) while a greater number of affected individuals from REPLICATION (28.0%) had postnatal growth anomalies (P=0.000005) compared to affected individuals from TEST families (11.6%). These findings are significant following FDR corrections and, although there was information on only a small number of individuals from the REPLICATION families, I believe that phenotypic differences among the families account for the differences obtained in the genetic findings.

Traditional replication studies test only those markers identified in the TEST set of families in the replication cohort. Often, replication studies fail to reproduce the findings in the test cohort (Gorroochurn *et al.*, 2007). If the lack of replication is because

of differences between families (i.e. different genetic etiologies), then other approaches to testing the scope of genetic findings in families with ASDs are warranted. One approach is to test different family cohorts using all polymorphisms in a gene of interest. This approach allows one to detemine whether a given gene is important generally and across cohorts. For example, although there is no evidence for association of *DRD1* rs265981 or *DRD2* rs1800498 in my replication families, other variants at these loci (i.e. *DRD1* rs4532 or *DRD2* rs1799732) may contribute to autism susceptibility in the replication families. Similarly, there may be evidence for association of polymorphisms in *TH* or *SLC6A3* with autism in the replication families; however I did not test these because my study was a strict replication study.

Another approach would be to identify a 'phenotypic signature' unique to TEST families using measures of dysmorphology, performance on tests of executive functions, measurements of HVA levels, etc. and to use this phenotypic signature to select families from REPLICATION 1, REPLICATION 2 and REPLICATION 3 which share similar phenotypic characteristics. Such matching of families may be a better replication study than testing only those markers that showed a significant association in TEST families in a separately recruited family cohort.

4.2 Test Study

Thus, although there may be differences between REPLICATION and TEST families, I feel that my results on the TEST families are important and provide a framework for understanding the role of DA-related genes in susceptibility to ASDs. Our model for the involvement of the DA pathway in determining some of the core deficits of ASDs is

based on earlier results implicating the *DBH* gene as a maternal effect locus and is based on the hypothesis that autism susceptibility is determined by a combination of fetal susceptibility genes and fetal gender as well as maternal effects including genetic factors (Robinson *et al.*, 2001).

4.2.1 Contribution of Dopamine-related Genes to Autism in Affected Males

4.2.1.1 *TH*

I examined two polymorphisms, HUMTH01 which is associated with DA levels, and rs2070762 at the *TH* locus to determine whether there was evidence for association of HUMTH01 10-repeat alleles or HUMTH01 10-repeat-rs2070762 T haplotypes with autism susceptibility. Individuals homozygous for the 10-repeat allele have been found to have increased levels of HVA, an indirect measure of DA, compared to those heterozygous for this allele (Wei *et al.*, 1997). As shown in Table 3.1, there were no differences in the frequencies of the 10-repeat allele or genotypes containing this allele and no increased transmission of this allele to affected males (P=0.11-0.69). No association (P=0.17-0.94) between alleles of either polymorphism with ADI-R measures of reciprocal social interaction, communication and stereotypies was found using using QTDT. Furthermore, haplotype analyses which included case-control comparisons, HBAT and haplotype-QTDT, did not show evidence for association between haplotypes containing the HUMTH01 10-repeat allele (10-T) and autism or measures of autism severity in affected individuals (P=0.10-0.87).

My findings do not support the hypothesis that variants at the *TH* locus contribute to autism susceptibility in males with autism in the TEST cohort. Gillberg and

Svennerholm (1987) and Martineau *et al.* (1994) found increased levels of HVA, which is a direct indicator of DA levels, in children with autism in CSF and urine, respectively. Although I did not measure DA or HVA levels in the TEST families, if they do, in fact, have increased levels of these molecules, then an interpretation for my findings would be that abnormal DA levels are not because of changes in *TH* expression or enzyme function but indirectly by changes in autoreceptor function. Because of a lack of HVA measurements in affected individuals, the nature of the association between alleles or haplotypes of my two markers and DA levels in the TEST cohort is not known. However, based on these findings I conclude that the *TH* gene is not a major susceptibility locus for ASD in males from this cohort.

4.2.1.2 *SLC6A3* (Dopamine Transporter Gene)

I examined four polymorphisms at the *SLC6A3* locus and based on functional studies (Table 1.1), hypothesized that rs2975226 A, I8 VNTR 5-repeat, rs28363149 Ins and EX15 VNTR 9-repeat alleles, or haplotypes derived from these alleles, would be risk alleles for ASD. There were no differences in the frequencies of any of these alleles (Table 3.3) and no increased transmission of these alleles to affected males (Table 3.5)(P=0.22-0.94). Haplotype-based analyses using HBAT did not show evidence for preferential haplotype transmision of any marker combinations (Table 3.5) and I did not find an association using QTDT analysis between single marker alleles or haplotypes with more severe impairments in social interaction, communication and repetitive behaviours. Furthermore, haplotype permutation tests between affected male and comparison cohorts were not significant (P=0.20-0.74).

These findings do not support the hypothesis that *SLC6A3* polymorphisms associated with decreased gene expression or dopamine transporter availability contribute to risk of autism susceptibility in affected males in the TEST cohort. Thus, similar to my findings in the *TH* gene, I conclude that the *SLC6A3* gene is not a major susceptibility locus in autism in these families.

4.2.1.3 *DRD1*

I identified a risk allele, rs265981 C, that was significantly increased in affected males relative to the comparison group (*P*=0.007)(Table 3.6) and was over-transmitted in these families (*P*=0.040)(Table 3.7) following FDR adjustments for multiple comparisons. Multivariate QTDT identified a significant association of rs265981 (*P*=0.0065) with the combined effect of all three subdomain scores (Table 3.8) and the rs265981 C allele was associated with an increased risk for ASD with an odds ratio (OR) of 1.56 [95% confidence interval (CI): 1.2-2.0] (i.e. (156/60)/(550/330)). The OR is the ratio of the ratio of risk allele/non-risk allele in the affected cohort (i.e. 156/60) *versus* the comparison cohort (i.e. 550/330)(reviewed by Bewick *et al.*, 2004). An OR >1.3 is considered significant in complex, polygenic conditions when several loci of small to moderate effect are involved. In the case of *DRD1*, individuals with the rs265981 C allele are almost 60% more likely to have autism, further supporting my findings from case-control comparisons and family-based tests of a role for the *DRD1* gene with autism.

The rs265981 polymorphism is not predicted to affect the amino acid sequence of the dopamine D1 receptor, as it is located within the 5' untranslated first exon of the gene (Figure 2.3)(reviewed by Wong *et al.*, 2000). I performed *in silico* analyses using

PupaSuite (Conde *et al.*, 2004, 2005; available at http://pupasuite.bioinfo.cipf.es/), to determine whether this SNP may affect putative transcription factor binding sites, intron-exon boundaries, exon splicing enhancers or triplex-forming sequences, and found that the C allele of rs265981 is predicted to result in the loss of an exon splice enhancer. The *DRD1* gene has two promoters which result in differential expression of two transcripts – one containing both exons 1 and 2 and one having only the second exon (Minowa *et al.*, 1992, 1993; Lee *et al.*, 1996). The longer of the two transcripts is present at approximately twice the level of the shorter transcript in human caudate tissue and SK-N-MC cells (Lee *et al.*, 1996) and the longer transcript has a half-life of 1 hour in cultured cells, compared to a half-life of 1.8 hours for the shorter transcript (Lee *et al.*, 1996). Thus, the rs265981 C allele could affect *DRD1* mRNA abundance and stability, ultimately affecting receptor distribution and/or availability. Although microarray studies using either post-mortem brain samples (Purcell *et al.*, 2001) or lymphoblastoid cell lines (Baron *et al.*, 2006; Hu *et al.*, 2006) from individuals with autism have not identified changes in total *DRD1* gene expression, the predicted loss of a putative exon splice enhancer in exon 1 may alter the proportion of long-to-short transcripts in affected males from TEST families with the rs265981 C allele. Further studies are needed to determine whether the predicted function of the C allele, and/or other variants in LD with this allele, does indeed affect the proportions and/or the half-lives of the two *DRD1* transcripts.

The positive association of the *DRD1* allele with autism in males extended to the genotype-phenotype associations, with significant findings for rs265981 C in multivariate QTDT (Table 3.8). Two brain regions which have dense D1 receptor distribution are the amygdala and striatum, and both have been found to be associated with the core deficits

seen in persons with ASDs. Functional neuroimaging studies of individuals with ASDs have shown decreased amygdala activation during participation in ToM tasks (Baron-Cohen *et al.*, 1999; reviewed by Di Martino & Castellanos, 2003). Structural MRI and post-mortem neuroanatomical studies have revealed enlarged amygdalar volumes, as well as decreased cell size and increased cell packing density in the amygdalae of affected individuals (Brambilla *et al.*, 2003; reviewed by Bauman & Kemper, 2005). Similarly, there is evidence for striatal dysfunction, which is implicated in the pathophysiology of stereotypies, in individuals with autism. A structural magnetic resonance imaging (MRI) study of adult males with autism and matched controls showed increased caudate volumes in affected individuals, a finding that was confirmed in a replication study that also demonstrated a correlation between caudate volume and measures of the ADI-R repetitive behaviour subdomain (Sears *et al.*, 1999). Although there is no direct evidence for altered amygdalar or striatal dopamine D1 receptor function or availability in autism, a possible interpretation of my findings is that the rs265981 C allele affects D1 receptor density in these brain regions as a result of the predicted effects of the rs265981 C allele on the relative abundance of long and short *DRD1* transcripts or D1 receptor function from other functional variants in LD with this allele. Furthermore, the predicted effects of the *DRD1* rs265981 C allele on the relative amounts of the two *DRD1* transcripts also has implications regarding PFC function and working memory. Based on the inverted-U relationship between D1 receptor function and working memory performance in the PFC, the relative amounts of the two *DRD1* mRNA transcripts could have an effect on dopamine D1 receptor density and result in non-optimal (higher or lower) receptor levels thus leading to impairments in working memory performance.

4.2.1.4 *DRD2*

I found an increased frequency of the DRD2 rs1800498 TT genotype (*P*=0.007) in affected males (43.4%) compared to the comparison group (28.7%)(Table 3.9) and the rs1800498 T allele was over-transmitted to affected children in TEST families (*P*=0.0003)(Table 3.10). Both findings were significant following FDR-based corrections for multiple comparisons. Because the frequency of the T allele in the mothers (53.7%) and fathers (54.8%) did not differ compared to the comparison group (55.3%)(*P*=0.69 and *P*=0.90, respectively), the observed over-transmission of the rs1800498 T allele reflects distorted transmission and not increased allele frequencies in parents. Additional support for segregation distortion at rs1800498 stems from my finding that this marker is not in HWE in affected males (*P*=0.012) although it is in equilibrium in the comparison group and parents (data not shown). Finally, the rs1800498 risk allele was associated with more severe impairments in social interaction (*P*=0.0002), verbal communication (*P*=0.0004) and stereotyped behaviours (*P*=0.0021) in affected males in TEST families (Table 3.11) and the rs1800498 TT genotype was associated with an increased risk for ASD with an OR of 1.9 [95% CI: 1.5-2.5](i.e. individuals with autism are almost twice as likely to have the rs1800498 TT genotype (46/60) compared to indivduals from the comparison cohort (70/174)).

My highly significant findings with rs1800498 are in contrast to my non-significant findings with rs1079597 despite a maximal D' measure of 1.0 between these two polymorphisms (Figure 2.4). Although D' is a common measure of LD it has a tendency to overestimate the degree of LD between polymorphisms (Ardlie *et al.*, 2002). Thus to better determine the extent of LD between rs1800498 and rs1079597 I calculated

r^2, a more robust measure of LD, and found an r^2 of 0.28, which shows that these variants are not in absolute LD but much lower LD than indicated by measures of D'. Because functional analyses of rs1800498 have not been reported and *in silico* analyses performed using PupaSuite (Conde *et al.*, 2004, 2005; available at http://pupasuite.bioinfo.cipf.es/) did not identify any putative functional role for rs1800498, my findings may reflect the functional effects of unidentified risk variants in LD with rs1800498. The rs1800498 polymorphism was found to be in higher LD with rs1799732, a functional variant (Arinami *et al.*, 1997), than with either rs1079597 or rs1800497 in my comparison group and parents from TEST families (Figure 2.4), with similar findings reported in a European-American cohort (N=83)(Gelernter *et al.*, 1998). Comparisons between family-based tests of rs1800498 and rs1799732 alleles considered separately (P=0.0003 and P=0.16, respectively) and haplotypes containing alleles from both rs1800498 and rs1799732 show that the observed over-transmission in families is derived from rs1800498, and not because of the rs1799732 polymorphism (data not shown). However, only 31 families are informative for rs1799732 compared to 73 families for rs1800498, so I cannot determine from these findings whether alleles from the functional variant rs1799732 are contributing as a risk factor for autism susceptibility in TEST families. Additional families informative at both loci are needed to determine whether this functional variant or another functional polymorphism in LD with rs1800498 is responsible for the increased risk in autism. If so, then functional analyses and sequencing of the *DRD2* gene in affected individuals with this risk haplotype are required.

The QTDT results support an association of the *DRD2* locus with autism. The rs1800498 T allele was associated with more severe impairments in the core symptoms of autism, social interaction (P=0.0002) and communication (P=0.0004), and greater stereotypic behaviours (P=0.0021)(Table 3.11), in the large TEST cohort of affected males. A role for the *DRD2* gene in autism susceptibility is suggested by the fact that antipsychotic medications, which prevent dopamine D2 receptor activation, improve the core symptoms of ASDs. Troost *et al.* (2005) measured the long-term effects of risperidone in 36 children with ASDs using the Aberrant Behavior Checklist and found improvements in subscales of social withdrawal (P<0.001), stereotypy (P=0.047) and inappropriate speech (P=0.033). Similarly, another 6-month study used risperidone and reported improvements in the Vineland Adaptive Behaviour Scale domains of communication, daily living skills and socialization (all P<0.01) in 48 affected children (Williams *et al.*, 2006) while a double-blind, 12-week, placebo-controlled study of risperidone in adults with ASDs found a reduction in repetitive behaviours as measured by the Yale-Brown Obsessive Compulsive Scale (Y-BOCS)(McDougle *et al.*, 1998).

In addition, dopamine D2 receptor and *DRD2* mRNA is found in PFC, hippocampus, amygdala and striatum (Levey *et al.*, 1993; Meador-Woodruff *et al.*, 1996), brain regions which show either neuroanatomical abnormalities in post-mortem brains of individuals with autism (reviewed by Bauman & Kemper, 2005) or volumetric differences in affected individuals as measured using MRI (Sears *et al.*, 1999; Hollander *et al.*, 2005). Postsynaptic D2 receptors and presynaptic D2 autoreceptors are involved in the DAergic modulation of cognitive and emotional processes that are impaired in individuals with autism. For example, Floresco *et al.* (2006) found that reducing D2

receptor activation in the PFC of rats caused impairments in cognitive flexibility while Kellendonk *et al.* (2006) found that over-expression (i.e. over-activity) of striatal D2 receptors in mice caused impairments in working memory. Thus, functional polymorphisms which affect receptor availability (i.e. altered gene expression) may contribute to the cognitive impairments found in individuals with autism (Reed, 2002; Tsuchiya *et al.*, 2005). Alternatively, polymorphisms in the *DRD2* gene may affect D2 receptor function or availability on DAergic neurons thus contributing to impairments because of changes in presynaptic DA synthesis and release. Guarraci *et al.* (2000) and Greba *et al.* (2001) found that over-activation of D2 presynaptic autoreceptors in mesoamygdalic DAergic neurons in the VTA of rats impaired emotional processing while Montoya *et al.* (2008) found that systemic administration of apomorphine at doses which stimulate dopamine D2 autoreceptors caused impairments in episodic memory in healthy volunteers. Individuals with autism are impaired in episodic memory (Crane & Goddard, 2008) and emotional processing (Gaigg & Bowler, 2007). In addition, support for a role of the D2 receptor in stereotypies stems from the finding that *Drd2* -/- mice exhibit greater stereotypies when administered D1 agonists compared to wild type mice (Glickstein & Schmauss, 2001). Although microarray studies (Purcell *et al.*, 2001; Baron *et al.*, 2006; Hu *et al.*, 2006) have not identified changes in *DRD2* gene expression in individuals with autism, I interpret my findings as reflecting functional variants in LD with the rs1800498 T allele cause changes in either D2 receptor expression or function in these brain regions or that there are changes in autoreceptor function in DAergic projections to these subcortical structures.

4.2.1.5 *PPP1R1B (DARPP-32)*

I examined three polymorphisms at the *PPP1R1B* locus and identified the rs1495099 C allele as a recessive risk allele for susceptibility to ASDs in male-only families from the TEST cohort. As shown in Tables 3.12 and 3.13, the C allele and CC genotype frequencies were increased in affected males (39.9% and 22.0%) relative to the comparison group (28.7% and 9.9%, both comparisons P=0.001) and family-based association tests using FBAT with a recessive model showed distorted allele transmission with over-transmission of this allele in TEST families (P=0.00092)(Table 3.14). All findings were significant following correction for multiple comparisons. Further support that this over-transmission reflects altered transmission stems from the observation this marker is not in HWE in affected males (P=0.008), although it is in equilibrium in the comparison group and parents (data not shown). This allele was associated with greater impairments in social interaction (P=0.0016) and nonverbal communication (P=0.0046), and more severe stereotyped behaviours (P=0.00072), core features of ASDs. There was significant association of the rs1495099 C allele with the combined effects of all three subdomain scores using multivariate QTDT (P=0.00042)(Table 3.15). Finally, the rs1495099 CC genotype was associated with an increased risk for ASD with an OR of 2.6 [95% CI=1.9-3.6](i.e. (24/85)/(43/391)).

Since rs1495099 is located in intron 2, it is unlikely that it affects the amino acid sequence of the DARPP-32 protein (Figure 2.5). I performed *in silico* analyses using PupaSuite (Conde *et al.*, 2004, 2005; available at http://pupasuite.bioinfo.cipf.es/) and found no predicted functional effects for rs1495099, but suggest that this marker may be in LD with functional variants at or near the *PPP1R1B* locus and that additional work is

required to identify and characterize polymorphisms and determine the haplotype structure of this locus. For example, I performed *in silico* analyses for all SNPs (dbSNP Build 124) at *PPP1R1B* and identified two variants that may affect *PPP1R1B* expression and mRNA processing while Meyer-Lindenberg *et al.* (2007) identified a 7-marker haplotype which was associated with increased mRNA expression in post-mortem human brain. Although this haplotype did not include the rs1495099 polymorphism (or either of the variants identified by PupaSuite) it does illustrate the need for additional work in the characterization of this gene.

The QTDT findings showed an association of a risk allele at *PPP1R1B* with the core symptoms of autism. The rs1495099 C allele is associated with greater impairments in social interaction (P=0.0016) and nonverbal communication (P=0.0046) as well as more severe repetitive behaviours (P=0.00072) in affected males from TEST families (Table 3.15). DARPP-32 mediates the downstream effects of dopamine receptor activation and thus has an important role in the modulation of DA-related processes which are abnormal in individuals with autism. Unlike dopamine receptors, which can be studied using systemic or local administration of receptor agonists or antagonists, DARPP-32 is found in the cytoplasm of DAceptive neurons and thus there are few studies which have examined its role in DA-modulated processes and behaviours. Hotte *et al.* (2006) found that administration of D1 receptor antagonists in mice caused deficits in working memory which coincided with decreased levels of pDARPP-32 in the PFC. Deficiencies in working memory (Bennetto *et al.*, 1996; Steele *et al.*, 2006) and impairments in reversal learning (Coldren & Halloran, 2003) are found in individuals with autism. The OFC mediates reversal learning in rats and humans (McAlonan &

Brown, 2003; Hornak *et al.*, 2004) and both *Drd2* -/- mice and *Ppp1r1b* -/- mice exhibit impairments in reversal learning compared to wild-type mice (Heyser *et al.*, 2000; Kruzich *et al.*, 2006). In addition, the role of DARPP-32 in mediating the DA-related changes to neuronal excitability necessary for memory and learning is shown in a study by Calabresi *et al.* (2000) who were unable to induce long-term potentiation (LTP) and long-term depression (LTD), two forms of synaptic plasticity, in striatum of *Ppp1r1b* -/- mice. DA has a role in synaptic plasticity in the striatum (Centonze *et al.*, 2001; Calabresi *et al.*, 2007) and amygdala (Bissiere *et al.*, 2003), subcortical structures that are important in regulating emotional behaviours (Hare *et al.*, 2005; Peper *et al.*, 2006) and are implicated in the pathophysiology of repetitive behaviours (Bachevalier, 1994; Canales & Graybiel, 2000). Researchers using imaging and neuroanatomical methods have found abnormalities in these structures in individuals with autism. Sears *et al.* (1999) used structural MRI and found increased caudate volumes in affected males with autism compared to matched controls, a finding that was confirmed in a replication study that also demonstrated a correlation between caudate volume and measures on the ADI-R repetitive behaviour subdomain. Ashwin *et al.* (2006) used fMRI and found individuals with autism had decreased amygdalar activation during emotional processing of fearful stimuli while Nacewicz *et al.* (2006) found decreased amygdalar volumes in individuals with autism. Smaller amygdala volume was correlated with greater impairments in nonverbal communication. Although there is no direct evidence for altered amygdalar or striatal DARPP-32 function in individuals with autism or changes in *PPP1R1B* gene expression either in lymphoblastoid cell lines (Baron *et al.*, 2006; Hu *et al.*, 2006) or post-mortem brain samples (Purcell *et al.*, 2001) from affected individuals, one possible

interpretation of my findings is that functional variants in LD with the rs1495099 C allele in affected males homozygous for this allele cause changes either in *PPP1R1B* expression or DARPP-32 function in these brain regions, conferring risk to autism susceptibility in male-only affected sib-pair families.

4.2.1.6 Interactions between Dopamine-related Genes and Risk for ASD

I found evidence for gene-gene interactions between *DRD1* rs265981, *DRD2* rs1800498 and *PPP1R1B* rs1495099 in affected males from the TEST cohort relative to the comparison group. Significant interactions were found between *DRD1* and *PPP1R1B* (P=0.0095), *DRD2* and *PPP1R1B* (P=0.0035) as well as a three-way interaction among all loci (P=0.0094). As described in previous single-gene sections (4.2.1.3 to 4.2.1.5), the dopamine D1 receptor, dopamine D2 receptor and DARPP-32 have important roles in the DAergic modulation of processes and behaviours that are abnormal in individuals with autism. My findings of interactions between these genes likely reflect the fact that many of the processes mediated by DA involve both dopamine D1 and D2 receptors, and that DARPP-32 is critical for the downstream postsynaptic effects of both D1 and D2 receptor activation. For example, the gene-gene interaction found between *DRD1* rs265981 and *PPP1R1B* rs1495099 may reflect changes in the density or function of dopamine D1 receptors and DARPP-32 protein levels in the PFC that contribute to the working memory impairments seen in individuals with autism. The interaction found between *DRD2* rs1800498 and *PPP1R1B* rs1495099 may reflect differences in D2 receptor and DARPP-32 function in the amygdala which contributes to the deficits in emotional processing found in affected individuals. Alternatively, the interaction

between *DRD2* and *PPP1R1B* may reflect changes in striatal function leading to impairments in executive functions such as planning. Similarly, my findings in interactions between all three genes may reflect changes in D1 receptor, D2 receptor and DARPP-32 densities or function in PFC and OFC, thus contributing to the impairments in attentional set-shifting and reversal learning respectively, seen in individuals with ASDs. This three-way gene interaction may also reflect changes in striatal function which leads to the abnormalities in gait found in children with autism or contributes to the development of stereotypies. Furthermore, brain regions such as the OFC, amygdala and striatum have multiple roles and thus, disruptions in the DAergic modulation of these regions may lead to impairments in more than one process or behaviour. For example, the OFC is critical for reversal learning and social behaviour, the amygdala is required for emotional processing and ToM, while the striatum is involved in executive and motor functions: all of these functions are impaired in individuals with autism. However, the true pathophysiological contributions of the interactions between *DRD1* rs265981, *DRD2* rs1800498 and *PPP1R1B* rs1495099 in affected males from the TEST cohort are not known because the role of these polymorphisms in gene expression or protein function remain to be determined. Thus, functional studies of these variants or polymorphisms in LD with these variants as well as phenotypic studies that examine, for example, working memory, emotional processing, planning, gait, and reversal learning, on affected individuals are required in order to understand their neurobiological contribution to ASDs in this cohort.

4.2.2 Dopamine-related Genes and Maternal Risk for having a Son with ASD

Our model of autism susceptibility includes maternal effects as a contributing factor (Robinson *et al.*, 2001). Therefore, I examined polymorphisms at the *TH*, *SLC6A3*, *DRD1*, *DRD2* and *PPP1R1B* loci to determine whether specific gene variations were more common in mothers of affected children compared to a comparison cohort. I found a decreased frequency of the *TH* HUMTH01 10-repeat allele (P=0.007) and an increased frequency of the *DRD1* C-A-T haplotype (P=0.025) in mothers compared to the comparison group. I also found that over-transmission of *DRD1* rs265981 A alleles and *DRD2* rs1800498 T alleles were from mothers (P=0.016) and fathers (P=0.023) respectively, to affected children. These findings were significant following corrections for multiple comparisons. No evidence was found for an association of markers in the *SLC6A3* and *PPP1R1B* genes with a risk to mothers or fathers of having sons with autism (data not shown).

4.2.2.1 *TH*

Following the finding of a decreased frequency of 10-repeat alleles in mothers, I examined the frequency of 10-repeat containing genotypes (i.e. 10/10, 10/other and other/other). This comparison was made because it is difficult to intepret functional effects in the context of a decreased frequency of a specific allele *versus* an increased frequency of the remaining alleles, since there is insufficient data on the effects of some of the HUMTH01 alleles on DA levels. Although Wei *et al.* (1997) found that individuals homozygous for the 10-repeat allele had increased HVA levels compared to heterozygous individuals, measurements of levels relative to each genotype have not been

done. As anticipated with the finding of a decreased frequency of 10-repeat alleles in mothers, I found fewer mothers (6.5%) were homozygous for the 10-repeat allele compared to the comparison group (13.8%). I also found an increased frequency of mothers homozygous for other alleles (54.2%) compared to the comparison group (40.1%)(P=0.026). Although Wei *et al.* (1997) performed additional comparisons with individuals homozygous for some of the other alleles, changes in HVA levels are not known for certain homozygotes because few individuals with those genotypes were available for study (N=2 to N=11 for each of the other homozygous groups). Nevertheless, the findings of Wei *et al.* (1997) suggests that although mothers are not predicted to have increased HVA levels because of the low frequency of the 10-repeat allele, they may have altered HVA levels because of functional contributions by other alleles at the HUMTH01 polymorphism.

HVA levels are used as an indirect measure of DA levels (Wei *et al.*, 1997) and it is tempting to speculate on the effects of altered HVA and thus, altered DA, in processes and behaviours in mothers of affected sons. For example, decreased levels of HVA have been reported in individuals with depression (Reddy *et al.*, 1992; Mitani *et al.*, 2006). Interestingly, an increased prevalence of depression has been reported in mothers (N=79) of children with autism compared to the comparison group (N=59)(Micali *et al.*, 2004). Decreased levels of midbrain DA in female rats are associated with changes in maternal behavour (Hansen *et al.*, 1991; Champagne *et al.*, 2004). Additional studies are required to determine the significance of different HUMTH01 alleles and other polymorphisms at the *TH* locus on DA synthesis and to better characterize the mothers' phenotypes including measurements of HVA levels.

4.2.2.2 *DRD1*

The finding of an increased frequency of the *DRD1* C-A-T haplotype in mothers from TEST families may reflect a maternal effect or a phenotype present in the mothers of boys with autism. For example, an increased prevalence of anxiety has been reported in mothers of children with autism (Murphy *et al.*, 2000), and parents from families with either autism or obsessive compulsive disorder (OCD) share similar impairments in executive functions (Bartz & Hollander, 2006; Delorme *et al.*, 2007). On the other hand, the *DRD1* haplotype in mothers may be related to mother-infant interactions. Strathearn *et al.* (2008) found that brain regions which have DAergic cell groups (i.e. VTA) or regions which receive dense DAergic innervation (i.e. NAc and striatum) are activated in first-time mothers, suggesting a role of DA in maternal behaviours in humans. Although there is no direct evidence for a role of the D1 receptor in human social behaviours, Stolzenberg *et al.* (2007) found that interactions between female rats and their pups was modulated by D1 receptors. Thus one possibility is that the *DRD1* haplotype or a functional variant in LD with this haplotype may modulate maternal behaviours in mothers of children with autism. Although clinical information is not available on the mothers, it is of interest that the *DRD1* gene has an upstream estrogen-response element and transcribes two transcripts. As mentioned, the longer of the two transcripts is present at approximately twice the level of the shorter transcript and has a shorter half-life in human caudate tissue (Lee *et al.*, 1996). Transient co-transfection and serial 5' deletion experiments using the human *DRD1*-expressing neuroblastoma cell line SK-N-MC have shown that estrogen increases transcription of the longer DRD1 transcript (Lee &

Mouradian, 1999). Because estrogen plays a role in stress and PFC-dependent working memory (Shansky *et al.*, 2004), as well as maternal behaviour (Leckman & Herman, 2002), it will be of interest to determine whether the *DRD1* C-A-T haplotype results in altered regulation or function of the estrogen-response element or the level of expression of the long *DRD1* transcript and what its impact is on cognitive functions such as working memory, maternal behaviours or obsessive compulsive or anxiety-related behaviours.

4.2.2.3 Parent-of-Origin Effects of *DRD1* and *DRD2* Alleles on Development

The increased transmission of the *DRD1* rs265981 A allele from mothers (P=0.016), and of the *DRD2* rs1800498 T allele from fathers (P=0.023), to affected sons suggests that imprinting effects of these genes are also important risk factors for ASDs. A role for imprinting has been proposed for several brain-related disorders, including autism (Petronis, 2000; Jiang *et al.*, 2004). Furthermore, there is evidence of imprinting of genes from neurotransmitter pathways in ASDs. For example, the 5-hydroxytryptamine receptor 2A (*HTR2A*) gene in the serotoninergic pathway, another autism candidate pathway, was found to be expressed only in human fibroblast tissue cultures having a maternal allele (Kato *et al.*, 1998) and to be polymorphically imprinted in brains, with monoallelic expression in 4 of 18 brains and biallelic expression in the remaining 14 (Bunzel *et al.*, 1998).

There is evidence supporting a role for both maternal and paternal imprinting in embryonic development. Studies using nuclear transfer to create mouse embryos diploid for either maternal (gynogenetic) or paternal (androgenetic) chromosomes found that

neither androgenetic nor gynogenetic embryos develop normally, but androgenetic embryos show greater development of extraembryonic tissues (Barton *et al.*, 1984) compared to gynogenetic embryos, which show very poor extraembryonic tissue development but more developed embryos (Surani *et al.*, 1984). The dopamine D1 and D2 receptors are expressed in human placentae (Yanagawa *et al.*, 1997; Vaillancourt *et al.*, 1998) and fetal brains (Brana *et al.*, 1996, 1997). Placental D2 receptors are involved in DA-mediated inhibition of placental lactogen release (Petit *et al.*, 1993), a hormone which is required for fetal development and growth (Handwerger & Freemark, 2000), whereas placental D1 receptors are involved in DA-mediated stimulation of placental opiod release (Stratakis *et al.*, 1996). There is evidence for a role for both dopamine D1 and D2 receptors in brain development. Dopamine D2 receptors expressed in fetal brain induce neurite outgrowth and axon elongation while dopamine D1 receptors inhibit neurite outgrowth in cortical neuron differentiation (Reinoso *et al.*, 1996), with the opposite effects found in striatal neuron differentiation (Schmidt *et al.*, 1996). There is no evidence for parent-of-origin-specific DNA methylation at the *DRD1* or *DRD2* loci in either human placentae or fetal brains suggestive of imprinting (Shen *et al.*, 2006) and a review of the 'imprinted gene and parent-of-origin effect' database (Glaser *et al.*, 2006; available at http://igc.otago.ac.nz/home.html) did not yield any evidence supporting imprinting at either locus. The possibility remains, however, that imprinting of these genes may occur during a very narrow developmental period or in a specific subpopulation of brain cells, as has been demonstrated for the *Ube3a* gene in mice (Yamasaki *et al.*, 2003). It should be noted that the evidence presented for parent-of-

origin effects in ASDs is based on one marker in each of the two genes and thus, more markers are needed to confirm the finding.

4.3 Limitations and Next Steps

As mentioned previously, a limitation of this study is the lack of functional information on the *DRD1* rs265981, *DRD2* rs1800498 and *PPP1R1B* rs1495099 polymorphisms and of variants in LD with these markers. My study found evidence for association of polymorphisms at these loci with autism susceptibility and thus my results contribute to the findings from other studies that support a role for DA in autism in affected males. However, because the function of variants in these loci are not known, it is difficult to determine the neurobiological contribution of these variants to the pathophysiology of the condition. Although *in silico* analysis may identify putative functional effects (i.e. *DRD1* rs265981) it does not replace laboratory assays which quantitatively show changes in gene expression or protein function. Knight *et al.* (2003) has developed an assay for G-protein coupled receptors in transformed insect cell lines, demonstrating its applicability to the study of the dopamine D1 receptor. Such an assay would be well-suited for functional studies of dopamine receptors and a strength of the assay is that more than one receptor subtype (i.e. *DRD1* and *DRD2*) can be expressed and measured in a single transfected cell line.

A second limitation of this study is the lack of endophenotypic information on affected boys and their mothers. QTDT analyses showed association of variants at DA-related genes with autism severity but one is unable to further refine the association of the genotype with a DA-mediated phenotype in individuals because of the limited

information available on them. Thus, measures of planning, cognitive flexibility and emotional processing, performance on tests of learning and memory, biochemical measures of HVA and hormones including prolactin, oxytocin and vassopressin as well as information on sleeping and waking, gait pattern, blood pressure, appetite and body weight in individuals from TEST families, are critical in order to understand the contribution of polymorphisms in DA-related genes with specific phenotypic characteristics in ASDs in this cohort. Furthermore, as mentioned in section 4.1, additional phenotypic information would allow the identification of a 'phenotypic signature' that could be used to distinguish family cohorts and aid in genetic replication studies.

A next step in this work would include the study of other DA-related genes in TEST families. Because my findings suggest that genes involved in neurotransmission are associated with autism susceptibility in affected males, I would examine polymorphisms in *DRD3, DRD4* and *DRD5* genes using single-gene analyses and tests for gene-gene interactions to further characterize the 'genetic signature' of this family cohort together with phenotypic measures of executive functions, gait pattern and blood pressure.

4.4 Summary of Discussion

This thesis describes a genetic study of five genes involved in the synthesis or function of dopamine. I found evidence of association of polymorphisms in the *DRD1, DRD2* and *PPP1R1B* loci with autism susceptibility in affected males from a large multiplex family cohort having only affected males (i.e. no affected females). In the same cohort, I found

specific polymorphisms in the *TH* and *DRD1* gene were less and more frequent, respectively, in the mothers of these affected children. These findings were not seen in three additional family cohorts. However, comparisons on measures of dysmorphology suggest that there are phenotypic differences between the TEST and REPLICATION families, likely reflecting different genetic etiologies.

The dopamine D1 receptor, dopamine D2 receptor and DARPP-32 have important and varied roles in the DA pathway which modulates a variety of processes impaired in individuals with autism including executive functions, learning, memory and emotional processing. However, to better understand the contribution of DA-related genes in affected males and their mothers, functional studies of the polymorphisms and phenotypic studies of the families are required and will contribute towards the eventual identification of culprit genes in autism.

The identification of genes in autism will lead to improved risk assessment and earlier diagnosis, improved treatment outcomes including pharmacological interventions with fewer side-effects, and overall better quality-of-life for individuals with ASDs and their families.

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Appendix A

A.1 Justification to Study *ARPP-21*

The cAMP-regulated phosphoprotein (M_r 32kDa) is encoded by the *ARPP-21* gene and has a role in mediating intracellular signaling of DA in DAceptive neurons. Following phosphorylation by cAMP-mediated activation of protein kinase A (PKA) (Hemmings *et al.*, 1989; Girault *et al.*, 1990), ARPP-21 binds to calmodulin and prevents calmodulin-mediated activation of downstream target proteins such as protein phosphatase 2B (PP2B) (Rakhilin *et al.*, 2004). PKA and PP2B phosphorylate and dephosphorylate DARPP-32, respectively, the phosphorylated state of which largely determines the post-synaptic effects of dopamine D1 and D2 receptor activation via PP1 (reviewed by Svenningsson *et al.*, 2004). Comparisons between levels of dopamine D1 receptor agonist-mediated stimulation of PKA-dependent phosphorylation, and dopamine D2 receptor agonist-mediated stimulation of PP2B-dependent dephosphorylation, of DARPP-32 in striatal slices from wild-type and *Arpp-21*-/- mice showed that ARPP-21 modulates DARPP-32 responses to D1 and D2 receptor activity (Rakhilin *et al.*, 2004).

The *ARPP-21* gene consists of 15 exons and maps to 3p22.3 (http://www.ncbi.nlm.nih.gov; GeneID 10777). To date, *ARPP-21* has not been studied as a candidate gene in autism. In this study, I examined three polymorphisms in the *ARPP-21* gene. Because the functional contribution of these polymorphisms is not known, I do not have a hypothesis that specific alleles will be associated with autism susceptibility.

A.2 Materials and Methods

A.2.1 Subjects

The cohorts used in this study were described previously in section 2.1. I used the TEST cohort of families as well as the comparison cohort of 253 individuals. Deviations from HWE were found for markers in the *ARPP-21* gene for the 190 samples obtained from Guthrie spots and were excluded from analyses.

A.2.2 Marker Selection, Amplification and Genotyping

Three intronic polymorphisms, rs3821447 A/G, rs2278758 G/A and rs921822 G/A, were genotyped at the *ARPP-21* locus (Figure A.1). These variants were chosen from the NCBI dbSNP Build 121 database from the Human Genome Project (available at http://www.ncbi.nlm.nih.gov/SNP/snp_summary.cgi) under the criteria that these markers span the *ARPP-21* locus and have MAFs of approximately 20%. These criteria were used because there were no known functional SNPs in the coding region of the *ARPP-21* gene and marker selection for this study was performed prior to the availability of HapMap data for the identification of htSNPs. The following primers were used to amplify the *ARPP-21* rs3821447 A/G, rs2278758 G/A and rs921822 G/A polymorphisms: rs3821447 F 5'- GAATGACAGACCCTGGGAGA-3' and R 5'- ACACCTTATTTCTCTGCACCAA-3'; rs2278758 F 5'- TGCAAGTTGAAGGCATGAAC-3' and R 5'- TCCCTGGAATTGACTGATTTCT-3'; and rs921822 F 5'- TGCAGCCAAATTTAGTATTTTACTG-3' and R 5'- CAGATCAAGAGTTTTATTTTGTTTTTG-3'. PCR reactions were carried out using 5ng of template DNA in 3µl reaction volumes. Amplification of rs3821447 included

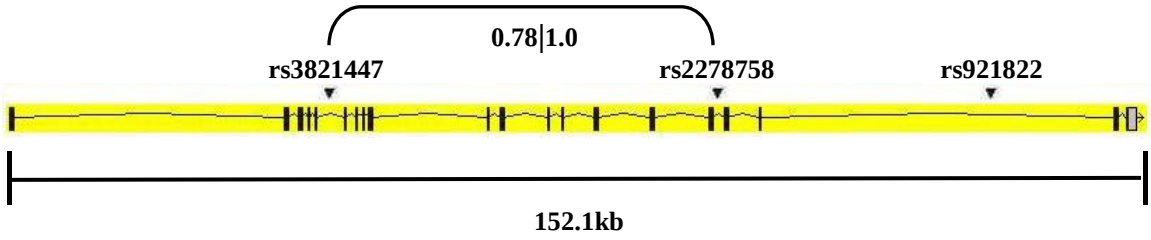

Figure A.1: Illustration of the *ARPP-21* locus. A schematic showing gene structure, marker positions and measures of linkage disequilibrium between rs3821447 and rs2278758 listing D' of the comparison group (N=245) followed by D' of parents from TEST families (N=216). Rs921822 was excluded from analyses. Legend: ■ exon; ▬ intron; ▭ untranslated region.

1mM $MgCl_2$, whereas rs2278758 and rs921822 used 1.5mM of $MgCl_2$. Cycling conditions were: 94ºC for 5 min, cycles of 94ºC for 30 sec, 55ºC (rs921822) or 57ºC (rs2278758) or 58ºC (rs3821447) for 50 sec, 72ºC for 50 sec, followed by a final extension at 72ºC for 10 min., with 32, 33, and 35 cycles for rs2278758, rs921822 and rs3821447, respectively. The rs3821447 (A allele cut; G allele uncut), rs2278758 (G allele cut; A allele uncut) and rs921822 (G allele cut; A allele uncut) amplicons were digested with 0.3U MfeI, 0.3U HpyCH4IV and 0.5U BsrI (New England Biolabs, Pickering, ON, Canada) respectively, and all digestion products were separated on 2% agarose gels and visualized using ethidium bromide and UV illumination. All results were independently assessed by two persons.

A.2.3 Statistical Analyses

Analyses were performed as described in section 2.3.

A.3 Results

Of the 3 polymorphisms selected for this study, rs921822 failed quality control standards and was not included in the analyses. Prior to population-based case-control comparisons, rs3821447 and rs2278758 were tested for deviations from HWE and both SNPs were in HWE in affected individuals and controls (data not shown). As shown in Table A.1, there were no significant differences in rs3821447 allele (P=0.26) or rs2278758 allele (P=0.72) frequencies in affected males compared to the comparison group and no preferential transmission of alleles at either locus to affected individuals in TEST families (P=0.88-1.0)(Table A.2). There was no association of specific alleles at

Table A.1: Marker allele frequencies at the *ARPP-21* locus in the comparison group and males with ASD from the TEST families

		Allele			
rs3821447	N	A	G	χ^2 df=1	*P*
Comparison group	486	331 (68.1%)	155 (31.9%)		
Affected males[1]	218	139 (63.8%)	79 (36.2%)	1.281	0.26
rs2278758	N	G	A	χ^2 df=1	*P*
Comparison group	498	435 (87.3%)	63 (12.7%)		
Affected males[1]	220	190 (86.4%)	30 (13.6%)	0.132	0.72

[1]One affected individual was randomly chosen from each family

Table A.2: FBAT of rs3821447 and rs2278758 allele transmissions at the *ARPP-21* locus in the TEST families

Marker Locus and alleles	# Fam	Observed	Expected	Z	*P*
rs3821447					
A	68	168.0	167.0	0.2	0.88
G	68	106.0	107.0	-0.2	0.88
rs2278758					
G	48	141.0	141.0	0	1.0
A	48	61.0	61.0	0	1.0

[1]All affected individuals were included in the analyses

either the rs3821447 or rs2278758 locus, with phenotypic measures from the ADI-R using QTDT in affected males: reciprocal social interaction (P=0.83; P=0.91), communication (P=0.77; P=0.84) and repetitive stereotyped behaviours (P=0.97; P=0.87) (data not shown). To determine whether haplotypes containing markers from both loci were more informative than single markers, haplotype frequencies in affected individuals and controls were estimated for haplotype case-control comparisons and HBAT was performed. There were no significant differences in haplotype frequencies between affected individuals and the comparison group or preferential haplotype transmission to affected males from TEST families (data not shown).

Frequencies of rs3821447 and rs2278758 alleles were not significantly different (P=0.44 and P=0.33, respectively) between mothers from TEST families compared to the comparison group.

A.4 Discussion

No conclusion regarding association of the *ARPP-21* gene with autism in affected males or mothers from TEST families can be made because only two polymorphisms were studied in a gene which is over 150kb in size (Figure A.1). A major limitation at the time of marker selection when I did these studies was the lack of information on SNPs at the locus and thus, polymorphism selection was restricted. Using current HapMap data, I found that the *ARPP-21* gene has nine haplotype blocks and that a total of 19 htSNPs are required to perform an association study, demonstrating that my marker coverage at this locus was inadequate and no valid conclusions can be made regarding the role of the *ARPP-21* gene in autism.

Appendix B

A total of 79 male-only affected sib-pair families from the Autism Genetics Resource Exchange (AGRE) were included as part of the TEST cohort. The AGRE family identification numbers are:

AU0012	AU0106	AU0203	AU0289	AU0419
AU0017	AU0114	AU0207	AU0293	AU0427
AU0018	AU0120	AU0208	AU0298	AU0432
AU0022	AU0122	AU0212	AU0299	AU0438
AU0025	AU0139	AU0215	AU0305	AU0450
AU0028	AU0140	AU0230	AU0311	AU0465
AU0030	AU0145	AU0240	AU0314	AU0489
AU0037	AU0148	AU0242	AU0328	AU0493
AU0043	AU0149	AU0247	AU0336	AU0501
AU0048	AU0150	AU0250	AU0346	AU0545
AU0053	AU0175	AU0253	AU0349	AU0553
AU0055	AU0177	AU0254	AU0361	AU0556
AU0073	AU0179	AU0258	AU0362	AU0561
AU0081	AU0183	AU0259	AU0370	AU0599
AU0089	AU0193	AU0284	AU0381	AU0608
AU0095	AU0200	AU0287	AU0383	