THE GENETICS OF AUTISM/PDD:

AN INVESTIGATION OF SEROTONIN, NOREPINEPHRINE AND DOPAMINE TRANSPORTER GENES IN THE ETIOLOGY OF AUTISM AND THE RELATED PERVASIVE DISORDERS

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Abstract

Autism is a pervasive developmental disorder (PDD) characterized by behavioral abnormalities which include: lack of social relationships, delayed or deviant language development, motor stereotypes and preoccupation with repetitive movements or activities. Twin and family studies have suggested that a strong genetic component exists for this condition, however, attempts to identify the genes involved have failed. Current investigations into the etiology and pathophysiology of many neuropsychiatric disorders implicate the involvement of neurotransmitter systems. Previous studies from this laboratory have identified possible maternal effect loci that in conjunction with alleles of fetal susceptibility genes could explain many of the complex features of the etiology and genetics of autism/PDD.

This study involves the analysis of monoamine (biogenic amine) transport systems in relation to autism. Markers linked to and within the serotonin, norepinephrine and dopamine transporter genes were used for assessing concordance or allele sharing among affected sib pairs and allele and genotype distributions among first-degree relatives and affected children. The presence of linkage with autism was observed for all three candidate genes. Significant levels of identity by descent (IBD) allele sharing were observed among affected sibling pairs for the serotonin transporter (Mean statistic; t_2=2.27, $p<0.05$), dopamine transporter (t_2=1.20, $p<0.05$) and norepinephrine transporter (t_2=1.64, $p<0.05$). A separate analysis of each microsatellite locus revealed a trend towards increased maternal concordance. Symmetry between transmitted and non-transmitted alleles was noted at microsatellite and VNTR loci when transmission

disequilibrium of alleles was analyzed. Application of the transmission disequilibrium test (TDT) to STin2/5HTTLPR haplotypes demonstrated association to the 12 copy (304 bp) allele of STin2 and the long variant of the functional polymorphism, 5HTTLPR. Mothers of two affected children showed STin2 (SERT intronic VNTR) genotype frequency differences when compared to female controls (χ^2= 20.97, d.f.=11, p=0.0099). Significant differences between parental allele frequencies and population-based control frequencies tested for all microsatellite loci were observed. Correlation between those sib pairs sharing two alleles IBD for SERT loci and mothers with a del/del genotype at the DβH locus (associated with low serum DβH) supports the interaction between this locus and the maternal effect genes. Susceptibility, conferred by biogenic amine transporters, SERT, NET and DAT1, is supported by linkage and allelic association observed for all three genes. Correlation between concordance for SERT markers and maternal genotype at the DβH locus lends support to a maternal effect / fetal susceptibility model of autism. The presence of a possible parent-of-origin effect is also indicated by the presence of increased maternal concordance at microsatellite loci linked to SERT, NET and DAT1.

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Chapter 1:

GENERAL INTRODUCTION

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Autism is considered the most severe form of a spectrum of disorders called pervasive developmental disorders (PDDs). Autism (PDD) is characterized by abnormal behavior with respect to the lack of formation of interpersonal relationships, delayed or deviant language development, resistance to change, repetitive motor stereotypes, such as rocking, and preoccupation with repetitive activities [Frith et al., 1991]. The onset of symptoms is usually apparent in infancy or early childhood. No dysmorphic features have been associated with autism. Autistic individuals display varying degrees of mental impairment (or mental retardation) [Bailey et al., 1996]. Clinical heterogeneity also includes variation in severity, number and characteristics of symptoms [Rapin, 1997]. The DSM-IV [APA 1994] characterizes the many subtypes of PDD, some of which are classified under the **B**roader definition of **A**utistic **P**henotype (BAP). Less severe and more prevalent of the PDDs include Asperger syndrome and atypical autism. Rarer forms include: Rett syndrome (restricted to females) characterized by severe neurological impairment and microcephaly, and, childhood disintegrative disorder (Heller's syndrome) which, by definition, affects normal infants that undergo massive regression between the ages of 2 and 10 years to an acquired autistic state [DSM IV, APA 1994].

The Biological Basis of Autism/PDD

The etiology of autism is believed to be heterogeneous in nature, encompassing both genetic and environmental risk factors. Epidemiological and family studies have provided little support for a correlation between environmental causal factors such as social class, economic status or geographic location. Autism affects approximately 4-10 out of every 10000 live births and affects predominantly males with a sex ratio of 4:1 males to females [Ciaranello and Ciaranello, 1995]. The belief that autism is organic (versus psychogenic) in nature stems from the multitude of reported causal factors, including environmental factors, that may lead to brain damage. Cases of the disorder have been attributed to both congenital and prenatal factors, such as prenatal viral infection with congenital rubella syndrome and cytomegalovirus [Ciaranello and Ciaranello, 1995], and perinatal factors involving biological impairment at the time of birth, such as anoxia or trauma [Folstein and Rutter, 1977]. There have also been a significant number of reported incidences of the disorder in children exposed to cocaine, alcohol and anticonvulsants *in utero*[Davis et a.l, 1992; Harris et al., 1995; Aronson et al., 1997; Williams and Hersh, 1997]. In addition, it is of note that obstetric complications linked to the genesis of the disorder are under significant genetic influence and could be the result of fetal abnormality [Bailey et al., 1995].

Several studies have found medical conditions that occur more commonly among autistic subjects. In an investigation of Scandinavian autistic individuals, 33% suffered from epileptic seizures, 45% showed some major electroencephalogram (EEG)

abnormality, a major cerebral spinal fluid (CSF) protein abnormality was seen 4%, and 20% had a neurogenic hearing deficit [reviewed by Frith et al., 1991]. EEG abnormalities, investigated in autistic individuals suffering from epilepsy, seemed to be generated from the temporal and phylogenetically older regions of the brain indicating possible structural brain damage in these areas [Olssen et al., 1988].

A Genetic Etiology of Autism/PDD

Although it is clear that some cases of autism can be attributed to externally applied risk factors, there is strong evidence to suggest the involvement of genetic factors responsible for autism does exist. Several studies have found greater concordance for the disorder among monozygotic (MZ) than dizygotic (DZ) twins. Reported concordance varies from 91% to 36% among MZ twins versus 30% to 0% among DZ twins, depending on inclusion criteria used for the study [Folstein and Rutter, 1977; Ritvo et al., 1985; Steffenburg et al, 1989; Bailey et al., 1995]. The disorder is also found to be more prevalent among non-twin siblings of autistic individuals, with a documented risk of 2-6% [Smalley et al., 1988]. This may not seem overly significant, however, when compared to the prevalence of the disorder, the risk to siblings is 60-100 times greater than that of the general population [Ciaranello and Ciaranello, 1995]. Pooling this information gives an estimated heritability of 80-90% for autism [Smalley et al., 1988]. The high phenotypic concordance observed among affected siblings is suggestive of familiality and possibly an underlying genetic contribution to the disorder [Spiker et al., 1994; Szatmari et al., 1996]. Other evidence implying a genetic etiology is the incidence of cytogenetic abnormalities

found in association with autism [Hebebrand et al., 1994; Anneren et al., 1995; Flejter et al., 1996; Shaffer et al., 1996; Swillen et al., 1996; Upadhyaya et al., 1996; Cook et al., 1997a; Michealis et al., 1997; Monaghan et al., 1997] and significant co-occurrence of autism with other disorders with known autosomal and sex chromosome linkage. Associated disorders include tuberous sclerosis [Gillberg and Dennis, 1987; Gillberg et al., 1994; Hunt and Dennis, 1987], Fragile X syndrome [Brown et al, 1982; Brown et al., 1986; Gillberg et al., 1988; Reiss and Freund, 1992], Angelman syndrome [Steffenburg et al., 1996; Cook et al., 1998] and untreated phenylketonuria (PKU) [Rutter et al., 1990]. The absence of a higher incidence of organic, pre-, peri- and post-natal antecedents or cerebral lesions in autistic individuals with epilepsy is compatible with a hypothesis of a genetically determined neurochemical abnormality that may underlie the relationship between the two disorders [Rossi et al., 1995].

Family history data have revealed significant aggregation of related neuropsychiatric disorders such as major depressive disorder and social phobias in first degree relatives of autistic individuals (37.5%), having onset prior to the birth of the affected autistic individual compared to 11.1% in controls [Smalley, McCracken and Tanquay, 1995]. Motoric tics and obsessive-compulsive disorder (OCD) are also seen more frequently in relatives of autistic individuals [Bolton et al., 1998]. Family assessment also shows clustering of cognitive deficits, abnormal social functioning and communication deficits in non-affected relatives of autistic probands, implying that the genetic liability for autism confers a risk of autistic-like symptoms to other family members [August et al., 1981; Minton et al., 1982; Piven et al., 1997a].

Although the evidence for genetic involvement is convincing, no clear mode of inheritance has been observed. Genetic models have to take into account the unusual sex ratio, variable phenotype, the prevalence of the disorder in siblings and high estimated heritability, which cannot be explained by simple Mendelian or single gene modes of inheritance. Current hypotheses include: (1) the possibility of an X-linked gene, which has been discounted as a mechanism working in isolation due to the observation by Hallmayer et al. [1996] of pedigrees suggesting male-to-male transmission of the disorder; (2) transmission of an autosomal gene with reduced penetrance in females (to account for the unusual sex ratio); (3) a genetically heterogeneous disorder with both autosomal and sex-linked transmission and; (4) a polygenic disorder, which consists of the simultaneous occurrence of several genes of small effect that combine with environmental factors [Hallmayer et al., 1996a]. Computation of likelihood estimates for different genetic models performed by Jorde and colleagues [1991] and Pickles et al. [1995] did not support a major susceptibility locus but favored a multifactorial / polygenic threshold model of inheritance.

Several lines of evidence are consistent with the expectations of a model of which the combined effects of multiple susceptibility loci (and environmental condition) acting in a graded fashion either increase or decrease an individual's liability to render that individual more or less likely to develop the disorder [Falconer, 1965]. The observed sibling risk of 2-6% [Smalley et al., 1988] is consistent with the predicted rate under multifactorial models ($q^{1/2}$, where q=population frequency) [Emery, 1986]. The incidence of the disorder among sibs born subsequent to the first-born autistic increases to 8.6%

[Jorde et al., 1990], presumably due to an increased mean liability in the form of an increased number of abnormal genes segregating in a family and/or an elevation in shared environment. Recurrence risk to more distant relatives of the proband declines more rapidly than would be expected under a unifactorial model [Pickles et al., 1995]. The rate with which twins are considered concordant for the disorder is dependent on the inclusion criteria used by researchers. For example, Bailey et al. [1995] reported concordance levels of 60% among MZ twins and 0% among DZ twins for autism. However, when concordance was re-examined under a broader autistic phenotype, these levels increased to 92% for MZ twins and 10% for DZ twins. Such findings suggest that vulnerability to autism is expressed in relatives of autistic probands as a sub-threshold level of impairment that does not qualify under current inclusion criteria for autistic disorder. The aforementioned aggregation of neuropsychiatric disorders sharing characteristics with autism in families with an autistic individual provides additional support for the theory of multifactorial inheritance and possibly the mutual pathogenesis of such disorders.

Two liability thresholds can account for the existence of a skewed sex ratio. Greater genetic loading is likely necessary for the less affected sex to develop the disorder and thus recurrence risks to relatives of the less affected sex are probably greater, since the mean liability of their relatives is higher. Ritvo et al. [1989] reported a nonsignificant inflated frequency of the disorder among relatives of first-born autistic female (14%) versus 7% recurrence rate for relatives of first-born autistic male. It has been noted as well that when females are affected with the disorder that they are usually more severely affected [Tsai et al., 1983].

The proposition of a multifactorial model does not contradict the possibility of an X-linked locus. Several scans for a susceptibility gene on the X-chromosome have revealed some evidence for minor gene effects [Hallmayer et al., 1996b; Petit et al., 1996]. An association between the fragile X syndrome (Xq27) and autism has been well documented [Brown et al., 1982, 1986; Blomquist et al., 1985; Cohen et al., 1991; Gillberg et al., 1988; Meryash et al., 1982; Piven et al., 1991; Reiss and Freund, 1992; Watson et al., 1984]. The frequency of the fragile X cytogenetic marker among autistic males, as estimated from pooled studies, is 8% (48/613) [reviewed by Smalley et al., 1988]. However, due to the significant overlap of behavioral symptoms expressed by autistic individuals and fragile X affected individuals, these findings could largely be due to misclassification of the individual. A study of three folate sensitive, fragile sites located between Xq27 and Xq28 in boys affected with autism failed to find any trinucleotide repeat expansion or pre-mutation size alleles, nor was there increased concordance for alleles among affected sibling pairs indicating that mutations in these genes do not account for the autistic/PDD phenotypes observed in the affected males [Holden et al., 1996].

Genetic Dissection of a Complex Disorder

Complex modes of inheritance require more robust, powerful methods of investigation as etiological heterogeneity, ambiguous phenotype possibly leading to diagnostic difficulties and unclear mode of inheritance render conventional LOD score

based methods of linkage analysis inappropriate and ineffective [Risch, 1990a]. Such complex disorders benefit from nonparametric linkage analyses such as the Affected Pedigree Member (APM) and Affected Sib Pair (ASP) methods, which circumvent obstacles of varied behavioral phenotype and misspecification of the model of susceptibility to disease [Risch 1990b; Holmans and Craddock, 1997]. These methods do not require specification of a model for disease susceptibility. Detection of linkage in the context of APM and ASP designs utilize deviations of observed distributions of allele sharing (or identity by descent, IBD) relative to that which would be expected based on independent segregation for different degrees of relatedness [Blackwelder and Elston, 1985; Weeks and Lange, 1988]. The use of affected relative pairs, versus population based studies, has the advantage of minimizing the probability of phenocopies (autistic phenotype in individuals due to causes not associated with susceptible genotype) and, therefore, more apt to be informative for linkage. These methods also have the advantage of efficiency with respect to ascertainment, as it is easier to collect small nuclear families than a large multigenerational, multiaffected pedigree needed for LOD score analysis. APM and ASP methods do not correct for reduced power to detect multiple susceptibility genes either acting independently, additively and/or epistatically, therefore, ASP/genome-scanning strategies used by many researchers today are subject to criticism due to the probability of false-positive and false-negative results and limited success. In combining candidate genes, chosen on the premise of considerable physiological and etiological relevance, with the ASP approach, the efficiency of study is greatly increased and risk of false-positives decreased.

Power to detect linkage is dependent on many factors. A certain degree of power is obtained from the estimation of relative risk (λ_R), the recurrence risk to a relative of the index case compared to the risk in the general population [Risch, 1990a]. The probability of a relative being affected with the disorder indicates the strength of the genetic component as it is calculated relative to population incidence which presumably takes into account those environmental components that would be shared by the affected population as a whole.

Marker selection is an important variable for successful linkage analysis. Distance between marker loci and critical susceptibility genes and low polymorphic information content (PIC), or heterozygosity, of a marker locus can decrease the power to detect linkage. The ability to establish identity by descent allele sharing between affected sib pairs or affected relative pairs is contingent upon the parents or intervening relatives being informative, or heterozygous, for alleles at the marker locus. Increased distance will increase the recombination frequency between marker and gene, and decreased marker polymorphism effectively decreases the number of informative meioses. Therefore, highly polymorphic markers (>80% heterozygosity) tightly linked (θ~0) to candidate genes should be selected to maximize the power of the research scheme [Risch, 1990c]. Currently, mapping efforts of the Human Genome Project are aiming to identify short tandem repeat sequences (STRs) for every 1 cM interval of the human genome in order to aid the efforts of disease gene mapping. Many of these DNA segments are highly polymorphic di-, tri- and tetra- nucleotide repeats more commonly referred to as microsatellites. Other polymorphic markers found less ubiquitously throughout the

genome are variable number of tandem repeat loci (VNTRs), sequences with larger repeat units ranging from nine to 100 base pairs per unit. These repeat elements can reach lengths of greater than 20 kilobase pairs (Kb) and have a substantially greater number of alleles leading to very high PIC values. The number of markers tested for regions under investigation can increase the informativeness of the test population. It is valid to consider the next closest marker tested when the closest marker is non-informative for IBD, thus effectively avoiding decreasing the sample size when the markers being used are less than 100% polymorphic (PIC value < 1). The disadvantage to individual analysis of multiple markers is the issue of multiple testing. Type 1 error rate predicts that one of every 20 independent trials will result in a significant finding by chance, hence a nominal significance level of $p<0.05$. When multiple non-independent tests are performed the probability of observing significance by chance increases. Therefore, the significance level required for a finding to be deemed statistically significant is divided by the number of non-independent tests that are performed, or in this case, the number of linked loci tested. However, if marker information is pooled, and therefore, treated as a haplotype, correction is not required.

Another important consideration in selecting an appropriate research design is the degree to which the putative disease susceptibility locus contributes to the etiology of the disease [Risch, 1990b]. Under a multifactorial / polygenic threshold model of inheritance the effect of a susceptibility locus can be theoretically quantified to represent the total amount of genetic variance that can be accounted for by that locus. Susceptibility genes of weak to moderate effect account for approximately 10% of the total genetic variance

and may not be a factor in the etiology of all of the families tested. A susceptibility locus may also be insufficient for the development of the disorder in the absence of other genetic or environmental factors. Thus, genes of weak effect are very difficult to detect unless measures are taken to increase the probability of observing the presence of linkage.

Sample size is a major factor in determining the power to detect linkage. The number of individual families required to detect the presence of linkage, if it exists, largely depends on the other parameters affecting power. Approximately 100 families are required to achieve 80% power, or sufficient power to reject the null hypothesis of no linkage, under the assumption of PIC=1, 0% recombination, 100% penetrance, 10% genetic effect and relative risk to sibs 50 times that of the risk to the general population.

Statistical power to detect quantitative trait loci of weak to moderate effect can also be obtained through the use of association studies. Tests of allelic association are increasingly being used to complement linkage strategies. Successful identification of genes for multiple sclerosis [Tienari et al., 1993; Sawcer et al., 1996], Huntington's disease [Skraastad et al., 1992], cystic fibrosis [Weir, 1989] and insulin dependent diabetes mellitus (IDDM) [Bennett et al., 1995] were identified using such strategies. Allelic association refers to correlation between the presence of a particular phenotype and a marker allele, represented by allele and/or genotype frequency differences between the affected population and control populations. The origin of association can be attributed to linkage disequilibrium between a marker locus and a disease susceptibility locus. Alternatively, association can occur if the marker itself encodes a functional polymorphism having direct physiological effects that contribute to the phenotype. In order to detect

linkage disequilibrium, the marker must be very close to the disease locus, since alleles quickly return to linkage equilibrium if the marker and disease locus are separated by more than 0.5-1cM [Xiong and Guo, 1997]. Determination of linkage disequilibrium requires that appropriately matched control groups be used. Ethnic stratification of populations can contribute to false-positive findings, thus, methods using family-based controls are recommended to eliminate this possibility. The transmission disequilibrium test (TDT) examines the degree of symmetry between those alleles transmitted and not transmitted to affected offspring, effectively using the nontransmitted alleles as controls [Spielman, McGinnis and Ewens, 1993]. The TDT can also be applied to any type of genotypic data in both single affected- and multi-individual affected- families [Speilman and Ewens, 1996; Cleves, Olsen and Jacobs, 1997]. The TDT is beneficial in cases of affected sib pair analysis due to the inability to directly compare the affected population to population-based controls. Comparisons of parental allele frequencies are used to represent those of the affected population because sampling from affected sibs is invalid due to lack of independence. However, comparison to well-matched population based controls can reveal evidence of generalized genetic liability differences between the families with autistic probands and controls. Ethnically diverse test populations can diminish the ability to identify linkage disequilibrium. Alleles associated with a disorder in one family may differ from an associated allele in another that differs in ancestral origin. Thus, ideally, the use of functional polymorphisms is recommended where possible. This is only possible in the event that researchers use a candidate gene approach and that such functional polymorphisms have been identified.

In combining candidate gene investigation with linkage and association strategies one is still faced with the problem of etiological heterogeneity, which can obscure biological clues to an underlying physiological condition existing in the affected population complicating the selection of candidate genes. In the case of autism/PDD there are several lines of evidence to suggest the involvement of biogenic amine systems and directly implicate biogenic amine transporters as potential susceptibility genes to autism/PDD.

Evidence Supporting Monoamine Systems in the Etiology of Autism/PDD

The use of pharmacological therapies targeting monoamine systems, including noradrenergic, dopaminergic and serotonergic systems, have demonstrated the ability to ameliorate some of the behavioral symptoms of autism [Campbell et al., 1978; Anderson et al., 1984; Gilman and Tuchman, 1995; McDougle et al., 1996(a); Zuddas et al., 1996] suggesting that imbalances or dysregulation of one or all of these systems may be involved in the elucidation of the disorder. Noradrenergic agents (beta blockers and α_2 agtonists), such as propanadol and clonidine, are used to combat aggressive and explosive behavior. Haloparidol, thioridazine, pimozide and chloropromazine, all dopamine receptor blockers, are prescribed to control aggressive, destructive, self-injurious and stereotypic behavior. In fact, the stereotypies seen in patients with autism are not unlike those observed in patients suffering from tardive dyskinesia, an antipsychotic-induced movement disorder experienced by individuals taking these neuroleptic drugs for long periods of time. Selective serotonin reuptake inhibitors (SSRIs), serotonin agonists and antidepressants that target the serotonergic system are used to treat perseverative behaviors (overly

narrow range of interests), obsessive behavior, aggressiveness, flatness of affect and attention deficit disorders [reviewed by Rapin, 1997; Sloman, 1991]. Anticonvulsants are often used when epileptiform EEG activity or visible seizures accompany autism. It is often the case that drugs prescribed for particular symptomatic treatment aggravate other behaviors. Stimulants, methylphenidate and amphetamines, commonly decrease hyperactivity and inattention, but, tend to worsen irritability, stereotypies, and can induce self-injurious behavior absent prior to treatment [Gilman and Tuchman, 1995]. Evidence of improvement for some symptoms and exacerbation of others and the necessity for multiple agents correcting for neurochemical imbalance indicate the complexity of interplay between the biochemicals that evoke this disorder. Involvement of monoamine systems in other neuropsychiatric disorders and behavioral disturbances related to autism is also suggested by clinical, biochemical and pharmacological evidence [Schildkraut, 1965, Rogness et al., 1992; Kuchel, 1994].

The connectivity of monoaminergic neurons and the various brain abnormalities observed in neuroanatomical examination of autistic subjects gives clues to the importance of these neurochemical systems in the pathogenesis of autism. The vast majority of noradrenergic neurons originate in the locus coeruleus, supplying cerebellar Purkinje cells, the cerebral cortex and the thalamus [Kuffler, Nichols and Martin, 1984]. Control of attention and cognitive function are attributed to areas fed by noradrenergic neurons. Four separate laboratories noted reduction in the numbers of Purkinje neurons in all of a total of 12 cases ranging from 35-50%, and in one case, 95% [Bauman, 1991; Courchesne et al., 1991; Courchesne, 1997] The lack of pathology in surrounding dependent structures

points to an onset of abnormality during embryogenesis. The cardiovascular abnormalities and elevated heart rate and blood pressure observed by Cohen and Johnson [1977] in autistic subjects have been attributed to hyperarousal of the brainstem caused by dysregulation of the noradrenergic system. This system also directly modulates dopaminergic, serotonergic, endogenous opioid and neurohormonal activity. Cell bodies of the dopaminergic neurons lie in the substantia nigra. Injury, damage or dysregulation of this structure leads to movement disorders. The serotonergic system, originating in the raphe nucleus, has ubiquitous connections throughout the brain. Projections of these systems include the limbic system, which plays an important role in the regulation of memory and emotion. Increase in neuron-packing density (but, not in number or size of neurons) in the hippocampus and amgydala and other limbic regions were seen in autopsies performed on autistic subjects [Courchesne, 1997]. Bilateral temporal lobectomies that encompassed removal of the amygdala and medial hippocampus produced "autistic-like" behaviors in a previously violently aggressive rhesus monkey. After operation the animal displayed Kluver-Bucy Syndrome (KBS) characterized by hyperexploratory behavior (hyperactive), hyperorality (the tendency to interact with objects in a peculiar way, smelling or tasting inanimate, non-food items) and failure to understand words and phrases previously understood [Gualtieri, 1991]. Although, changes observed in the monkey resemble some of the manifestations of autism, this animal model fails to address aberrant language use and formation, motoric stereotypies, and qualitative impairment of social interaction, symptoms key to the diagnosis of autistic disorder. Furthermore, it is difficult to anticipate that any animal model could truly mimic

autism, given the difficulties in determining deficits in communication. Serotonergic disturbances have been linked to disinhibition of aggression, sleep disorders, depression and anxiety [Siever et al., 1991]. Other regions with reported abnormality have included the posterior fossa [Courchesne, 1994], brainstem [Rodier et al., 1996] and corpus callosum [Piven et al., 1997b]. A number of the observed neuropathological irregularities found consistently among independent researchers are congruent with the symptomology seen in autism and the hypothesized multifactorial model is supported by the presence of many different aberrant regions in different subjects.

Due to their involvement in the central nervous system processes which appear to be compromised in autistic individuals, the biogenic amines currently being studied in relation to autism include serotonin (5-HT) and the catecholamines, dopamine (DA) and norepinephrine (NE) [Rogeness et al., 1992 and Dolan and Grasby, 1994]. Investigations of these neurotransmitters in relation to autism have consistently shown elevated levels of serotonin (5-HT) in whole blood and platelet-rich plasma [Schain and Freedman, 1961, Boullin et al., 1970; Ritvo et al., 1970; Cohen et al., 1974; Cohen et al., 1977; Hanley et al., 1977; Anderson et al., 1987; Anderson 1987; Cook et al., 1988; Leventhal et al., 1990; Cook et al., 1993; Herault et al., 1994; Martineau et al., 1994] and increased platelet uptake of 5-HT in autistic individuals when compared to "normal" and neuropsychiatrically disturbed, age-matched children [Cook et al., 1993; Cohen et al., 1977]. Peripherally produced blood serotonin may not truly correlate with that which is synthesized in the central nervous system (CNS) because it does not cross the blood-brain barrier [Gilman and Tuchman, 1995]. The use of agents that decrease plasma and brain

serotonin, such as fenfluramine, fail to consistently produce the expected positive clinical results [Gilman and Tuchman, 1995]. Dietary depletion of the serotonin precursor, tryptophan, has been associated with an unexpected intensification of behavioral symptoms [MacDougle et al., 1996(b)]. Therefore, it seems efforts to treat elevations of 5-HT have failed to correct the underlying physiological abnormality giving rise to hyperserotonemia in individuals affected with autism/PDD.

One method employed to analyze CNS 5-HT turnover is the determination of levels of accumulation of the primary metabolite of 5-HT, hydroxyindolacetic acid (5-HIAA) following oral administration of probenecid, a benzoic acid derivative which blocks the release of the dopamine metabolite, homovanillic acid, and 5-HIAA [Cohen et al., 1977]. The presence of lower CSF 5-HIAA in a relatively small sample (N=10) of autistic children compared to age-matched, nonautistic, psychotic children (N=10) is interesting but not necessarily contradictory to the aforementioned studies [Cohen et al., 1977]. The uncontrolled independent variable is the levels of the enzyme in the oxidative degradation pathway leading to the production of 5-HIAA, monoamine oxidase A (MAOA). High levels of brain serotonin could still exist in an environment of MAOA disturbance. Lower 5-HIAA levels could be attained in the presence of low MAOA activity and normal or high serotonin levels depending on the respective concentrations of the interacting substrates. Significant difference in 5-HIAA concentration did not extend to the comparison of autistic children to "normal" controls introducing the potential issue of an inappropriate control population. Given the theory of neuropathological relatedness among various psychiatric conditions it seems unwise to use children that the authors even

describe as being "autistic-like" in such studies. More direct analysis of serotonin in the CNS of autistic children was performed by Chugani et al. [1997] through positron emission tomography (PET) detection of α–[^{11}C] methyl-L-tryptophan ([^{11}C]AMT), a tracer for the measurement of serotonin synthesis. Decreased 5-HT synthesis in the frontal cortex and thalamus was observed in all seven boys. The decrease was isolated in the left hemisphere of five of the seven boys and in the right hemisphere in the remaining two boys. This asymmetry was conserved in the observation of increased serotonin synthesis in the contralateral dentate nucleus of all seven cases. The three regions demonstrating altered serotonin synthesis are connected via the dentatothalamocortical pathway. Modulation of synthesis may relate to the innervation of the dentate nucleus by Purkinje neurons, earlier described to be sparse in number in autistic individuals. The thalamic ventral lateral nucleus mediates communication between the dentate nucleus and Broca's language area, motor cortex and supplementary motor cortex residing in the prefrontal cortex [Chugani et al., 1997] providing an interesting link between the pathology and symptomology. The use of internal family-based controls (nonautistic siblings) allows for greater confidence that this finding is specific to autistic individuals, as opposed to related neuropsychiatric disorders. However, comparisons to non-autistic sibs with the potential of sub-threshold liability for the disorder could conceal differences, which may be more apparent upon comparison to the general population. Findings of alterations in focal serotonin synthesis indicate that methods analyzing metabolites and blood do not approach the level of sensitivity required to detect the complexities of biogenic amine systems. The need to replicate this level of study in greater numbers of subjects is clear.

Some researchers have also found results that indicate abnormal binding and efflux of 5-HT across platelets of autistic children [Schain and Freedman, 1961; Boullin et al., 1970]. Genetic determinism of serotonin uptake is common for both neurons and platelets, through transporter proteins expressed by both, validating the use of platelets to serve as a peripheral model of serotonin uptake, but not storage, release and concentration as platelets do not synthesize serotonin. Uptake of serotonin by transport proteins also represents a potential mechanism for hyperserotonemia. Even opposing inconsistencies between research findings of those investigating serotonin involvement in autism do not minimize the importance of hyperserotonemia as the most consistent biomarker for the disorder. It is estimated that it exists in approximately 40% of the autistic population [Ritvo et al., 1970; Hanley et al., 1977; Herault et al., 1994] with levels 18-137% over levels recorded in various control groups [reviewed by Anderson, 1987]. In addition, it seems hyperserotonemia extends to siblings and parents of autistic individuals such that hyperserotonemia in the proband increases the likelihood of the condition in first degree relatives by 2.4 times [Leventhal et al., 1990].

Reports of altered plasma norepinephrine (NE), epinephrine (E) and decreased dopamine β hydroxylase (DβH) (the enzyme responsible for the catalysis of the conversion of dopamine to norepinephrine) have been associated with autistic individuals [Lake et al., 1977; Anderson, 1987; Herault et al., 1994; Martineau et al., 1994]. Indicators of MAOA activity signal trends towards lower levels of degradation by this enzyme [Martineau et al., 1994; Cohen et al., 1977]. However, conclusions based on these findings fall under the same constraints as those mentioned in the discussion of

serotonin research. Peripheral measures of NE, DA and their respective metabolites may be poor reflections of synthesis and activity in the CNS.

Molecular Genetic Studies in Autism/PDD

Linkage and association studies of the genes controlling the biochemical markers discussed above have not yielded much success. This may be partly due to the lack of power to detect a gene effect by virtue of the study design. Restriction Fragment Length Polymorphisms (RFLPs) were used to analyze allele frequencies in population-based control studies performed by Martineau et al. [1994] and Herault et al. [1994]. Typing of tyrosine hydroxylase (TH) (involved in the synthesis of dopamine, see Appendix A), dopamine-β-hydoxylase (DβH), a dopamine receptor (DRD3), tryptophan hydroxylase (TpH) (involved in the synthesis of 5-HT, see Appendix B) and c-Harvey-Ras (HRAS) (a gene involved in the regulation of neural growth and differentiation during development) revealed an association for a HRAS allele [Herault et al., 1994]. Replication of these and other biochemically and cytogenetically relevant candidate genes using repeat polymorphisms and an affected sib pair (ASP) strategy did not find increased concordance for alleles of the marker linked to HRAS. However, a predominant decrease in the expected rate of informativeness for mothers at the DBH locus was found [Robinson, 1996]. No evidence of linkage or association was observed for markers linked to the serotonin receptor, 5HT2, DRD2, TH, catechol-o-methyltransferase (COMT) (an enzyme involved in the breakdown of catecholamines, see Appendix A and B), Prader-Willi syndrome region, $GABA_A$ receptor B3 and the tuberous sclerosis locus (TSC2). Further

analysis of the DβH finding exposed the reason behind the high proportion of homozygosity responsible for the lack of informativeness. The microsatellite used to analyze DβH is localized to the 5' flanking region of the gene. This CA repeat polymorphism contains a subset of alleles with a 19 bp (base pair) deletion. Mothers of male multiplex families (those with more than one autistic male child) had a marked increase in the frequency of a 257 bp DβH allele (which contains the 19 bp deletion) compared to control populations (0.385 vs. 0.159, published frequencies; 0.385 vs. 0.224, tested Canadian controls). Furthermore, the frequencies of all deletion alleles were increased in the mothers (0.654 vs. 0.390, published frequencies; 0.654 vs. 0.455, tested Canadian controls). Allele frequency differences were, as a whole, significantly different ($p<0.001$) using the likelihood-ratio (L^2) test. Transmission of these 19 bp deletion alleles did not seem to be increased to affected sibs, however, determination of this was hindered by the relatively small sample size. Assays of the levels of serum DβH in these women showed an excellent correlation between genotype and mean serum DβH activity ($F=5.45$, $df=59$, $p<0.01$). The presence of a deletion allele correlated with decreased enzyme activity. The location of this microsatellite and correlation with enzyme activity is suggestive of a role for this polymorphism in the transcriptional regulation of the DβH gene. Low DβH activity would result in altered levels of dopamine (DA) and decreased production of norepinephrine (NE) and epinephrine (E) (see Appendix A) [Gary and Robertson, 1994].

DA, NE and 5-HT are tied together by a common degradative enzyme, monoamine oxidase-A (MAO-A)(see Appendices A and B), encoded on the X-chromosome. A number of different alleles exist for MAO-A, which result in alterations in the level of enzyme activity [Berry, Juorio and Paterson, 1994]. Similar to the previous study, findings of decreased heterozygosity for two polymorphisms linked to MAOA were observed in the same multiplex (MPX) mothers [Schutz, 1998]. Allelic distributions of the MAOA CA dinucleotide repeat polymorphism and MAOA VNTR compared to a random sample of the Canadian population (Guthrie spots from newborns) were significantly different (χ^2=24.14, df=10, p<0.0014; χ^2=16.27, df=14, p<0.0398, respectively). The B2 allele of the MAOA VNTR, was found far more often in MPX mothers than expected based on control frequencies (0.437 vs. 0.261). A 122 bp allele of the MAOA CA dinucleotide, found in the first intron of the MAOA gene, was also more frequent compared to controls (0.375 vs. 0.158). In fact, the B2-122 bp haplotype occurred significantly more often than would be expected from the observed frequencies of the respective alleles in the mothers (χ^2=44.3, p<<<0.001) evidence for the presence of linkage disequilibrium of these markers in the MPX mothers. Again, as in the latter discussion of DβH, transmission of alleles to the affected sib pairs does not appear to be altered in any way. The increased haplotype frequency may be representative of linkage disequilibrium with a mutation affecting the regulation of transcription of the gene, allowing for a gain or loss in the function or production of the enzyme, reflected in the activity level of the enzyme. Variants with a 30-fold and 100-fold differences in activity level in the MAOA gene have been isolated [Hotamisligil and Breakefield, 1991; Tivol et

al., 1996]. Investigations of the potential relationship between the functional polymorphisms and the B2-122 bp haplotype have yet to be performed on this population.

The Maternal Effect Model

The absence of greater transmission of those DβH and MAOA alleles found in excess in mothers, to their affected offspring is suggestive of an important role for maternal genotype in the etiology of autism. We have proposed a maternal effect model for autism in which maternal genotype contributes to the phenotype of the child, such that altered maternal serum levels of biogenic amines under the control of DβH and MAOA create a stressful environment for the developing fetus [Schutz et al., 1998].

Transgenic mouse studies lend support to the hypothesis that normal fetal development requires the appropriate regulation of monoamine systems including enzymes involved in catecholamine synthesis. Tyrosine hydroxylase (TH) catalyses the conversion of tyrosine to dopa (a catecholamine precursor)(Appendix A) [Martineau et al., 1994]. Transgenic mouse studies have found that 90% of fetuses homozygous for a nonfunctional TH allele die in utero, indicating the absolute requirement of this enzyme and its resultant catecholamines (including DA, NE and E) in the normal development of the fetus [Zhou, Quaife and Palmiter, 1995]. Additional support for the importance of DA, NE and E is found in mouse studies performed by Thomas, Matsumoto and Palmiter [1995], demonstrating the necessity of DβH and thus, NE, for mouse fetal development. In DβH (-/+) heterozygous mothers the majority of DβH (-/-) homozygous fetuses died in utero. A

few of the DβH (-/-) fetuses did survive, presumably due to the transfer of the necessary catecholamines across the placenta to provide the fetus with these factors for normal fetal development. This hypothesis was based on the absence of any surviving DβH (-/-) fetuses when they were carried by homozygous mothers [Thomas, Matsumoto and Palmiter, 1995].

Monoamines, 5-HT and DA have well documented roles as neural morphogens, important early in embryogenesis for neural differentiation [Lauder and Krebs, 1978; Lauder and Krebs, 1984; Fiszman et al., 1991; Todd, 1992]. Altered levels of neural morphogens have been shown to have teratogenic effects consistent with those observed in autism [Lauder, 1988]. Recent studies have found that exogenous monoamines in combination with growth factors are required for the successful initiation of differentiation of monoaminergic neurons in the fetal brain. Thus, fetal neuronal development and differentiation represents a paradoxical situation where by the ability for these neurons to synthesize their respective neurotransmitters requires differentiation of the neuron, which necessitates exposure to the same neurotransmitters they are differentiated to produce [Du and Iacovitti, 1995]. The study concluded that the maternal serum represents a possible source of these biogenic amines, such that these factors in the maternal serum cross the placental membrane and freely permeate the fetal brain [Saunders and Mollgard, 1984; Lossinsky et al., 1986]. Therefore, neurotransmitters in maternal circulation and the proper functioning of transporters located in the placenta are critical in mediating fetal development. Previously mentioned neuroanatomical abnormalities, found with magnetic

resonance imaging (MRI) and post-mortem examinations, are also consistent with disturbed embryological brain development prior to 30 weeks gestation.

The maternal effect model acting alone in pathogenesis does have difficulties accounting for some of the unusual characteristics of the disorder. First, the altered gender distribution would not be expected, however, there have been some researchers that have alluded to an increased risk of pre-, peri- and neonatal insult among male concepti [Knobloch and Pasamanick, 1975; Folstein and Rutter, 1977]. One would also expect a greater than observed general population incidence of the disorder given the respective DβH and MAOA allele frequencies conferring the proposed maternal effect. Alternatively, autism does exist in families in which the mother does not carry the vulnerability genotype. If altered intra-uterine environment was the only causal factor, one would expect a more equivalent level of concordance among MZ and DZ twins. If we invoke the presence of a maternal effect it must act in conjunction with fetal susceptibility factors to account for inconsistencies that the maternal effect model alone cannot explain. This led us to hypothesize that a possible factor for fetal susceptibility in the etiology of autism could be related to monoamine (biogenic amine) transport systems in the placenta (a tissue representing fetal genotype) which could act alone or in conjunction with the maternal effect loci.

The Maternal Effect / Fetal Susceptibility Model

Altered DβH, MAO-A and 5-HT levels in the mothers of multiplex (MPX) families [Robinson, 1996; Schutz, 1998; Leventhal et al., 1990] exacerbated by normal pregnancy-induced changes [Gilbert, 1994; Ostensen, 1995] lead to abnormal levels of monoamines DA, NE, E and 5-HT in the maternal serum. This, in conjunction with altered regulation, tissue-specific expression or substrate affinity of biogenic amine transporters normally expressed in the placenta, may result in altered fetal exposure to those factors in the maternal serum. The potential for abnormal brain development is clear, as these neural morphogens act in a dose-dependent manner [Lauder, 1988]. Therefore, the combination of maternally induced compromised intra-uterine environment and fetal genetic susceptibility in the form of altered transport of biogenic amines within the developing fetal brain or from maternal circulation could result in the pervasive developmental disorder, autism. The existence of an autosomal locus linked to the disorder, along with the maternal effect model, could account for the frequency of the disorder in the general population, as well as the difference in concordance rates among MZ and DZ twins.

Biogenic Amine Transport Proteins

The biogenic amine family of transporters consisting of SERT, NET and DAT1 (serotonin, norepinephrine and dopamine transporters, respectively) share considerable sequence and structural homology [reviewed in Amara and Kuhar, 1993]. Common properties shared by SERT, NET and DAT1 (and their gene products, SLC 6A4, SLC

6A5 and SLC 6A3) include twelve transmembrane domains (TMDs) (each encoded by a single exon), termination of N and C termini intracellularly, a large extracellular loop between the third and fourth TMDs which confers substrate specificity, and dependence on Na^+ and Cl^- ions for transport activity [Kuhar et al., 1990; Giros and Caron, 1993; Lesch et al., 1994; Porzgen, Bonisch and Bruss, 1995].

NE, DA and 5-HT transport systems function in the CNS to aid in the reuptake of their respective neurotransmitters into the presynaptic neuron, terminating the action of the neurotransmitters by removing them from the synaptic cleft. The 5-HT and NE transporters have been localized to only a few cell types of non-neuronal origin. Interestingly, the brush border membrane of the placenta represents one of the few nonneuronal tissues which exhibits SERT and NET expression and biogenic amine transport activity [Cool et al., 1991; Ramamoorthy et al., 1992; Ramamoorthy et al., 1993(a); (b); Lesch et al., 1994]. The brush-broader syncytiotrophoblast represents the first barrier between fetal and maternal circulation. Orientation of the membrane (facing maternal circulation) allows SERT and NET to clear biogenic amine substrates from the intervillous space, aiding in the prevention of premature vasoconstriction and transplacental transfer of substrates to the developing embryo [Schroeter and Blakely, 1996]. Although the dopamine transporter is not expressed in placental tissue, the NET and SERT proteins show considerable substrate specificity and affinity for dopamine as well as their own ligands [Gordon and Olverman, 1978; Ramamoorthy et al., 1992; Faraj et al., 1994].

Pharmacological studies have also revealed that these transporters are the primary sites of action of therapeutic antidepressants, as well as cocaine and amphetamines [Kuhar et al., 1990; Ramamoorthy et al., 1992; Giros and Caron, 1993; Jayanthi et al., 1993; Ramamoorthy et al., 1993(a); (b); Watanabe et al., 1993; Lorang et al., 1994; Ronnekleiv and Naylor, 1995; Schloss and Betz, 1995; Qian et al., 1995; Ramamorrthy et al., 1995]. It is interesting to note that one of the possible mechanisms associated with developmental neurotoxicity induced by cocaine during pregnancy includes impairment of monoamine transplacental transfer [MacGregor et al., 1987; Olsen, 1995]. As mentioned earlier, a number of cases of autism have resulted from cocaine abuse during pregnancy [Davis et al., 1992; Harris et al., 1995].

SERT, NET and DAT1 have also been implicated in the pathophysiology of neuropsychiatric disorders, such as schizophrenia [Stahl et al., 1983; Dean et al., 1996; Inada et al., 1996], bipolar disorder [Risch and Nemeroff, 1992; Nemeroff et al., 1994; Owens and Nemeroff, 1994; Hadley et al., 1995; Kelsoe et al., 1996 (a); Kelsoe et al., 1996 (b); Souery et al., 1996], Alzheimer's disease [Tejani-Butt et al, 1993], Tourette syndrome [Comings et al., 1996] and Obsessive-Compulsive disorder (OCD) [Weizman et al., 1992; Altemus et al., 1996] due to pharmacological evidence to suggest their involvement. Mutations in the biogenic amine transporter genes could predispose an individual to psychiatric disorders by effectively increasing or decreasing the activity of a neurotransmitter by altering its availability to the post-synaptic neuron [Gelernter, Pakstis and Kidd, 1995]. Alternatively, mutations in functionally important regions, such as those that are critical to substrate transport, could render the transporter less effective [Bruss et

al., 1993; Porzgen, Bonisch and Bruss, 1995]. Alterations in gene regions that are responsible for the regulation of transcription could dysregulate expression of the proteins, resulting in a decrease or increase in the numbers or tissue-specific expression of the transporters.

The SERT gene, localized to chromosome 17q 11.2-q12 [Gelerneter, Pakistis and Kidd, 1995], contains two variable number of tandem repeat (VNTR) polymorphisms. One, a functional polymorphism, is located in the 5' untranslated region (5'-UTR) of the gene, referred to as the 5-HT transporter linked polymorphic region (5-HTTLPR)[Heils et al., 1996]. The second, a VNTR located in the second intron of the gene, referred to as the serotonin transporter intronic polymorphic region (STin2)[Lesch et al., 1994]. The 5-HTTLPR corresponds to nucleotide positions -1,416 to -1,397. Allelic variants of this GC-rich, 20-23 bp core repeat appear to influence promoter activity such that the shorter allele, corresponding to a deletion of 44 bp's spanning repeats 6-8, leads to impairment of promoter repression, and thus, up regulation of gene transcription [Heils et al., 1996]. Association studies have linked this variant to anxiety-related traits [Lesch et al., 1996]. STin2 consists of a 17 bp core repeat with 10 and 11 copy alleles followed by an AP-1 motif, a putative transcription factor binding site for heterodimer c-*fos*/c-*jun* [Lesch et al., 1994]. This tandem repeat DNA polymorphism may also play a role in transcriptional regulation of SERT. Repeat length variation could therefore contribute to altered SERT expression and or function. Substantiation of this theory has yet to be performed. Investigation of this putative regulatory element in a population of individuals with

affective disorder revealed two additional alleles corresponding to 9 and 13 copies of the core repeat [Ogilvie et al., 1996].

Cook et al. [1997b] have studied these SERT polymorphisms in a population of families with a single autistic proband. The transmission disequilibrium test (TDT) was used to determine whether a particular allele for each of the variants, was being transmitted more often than non-transmitted alleles. Significant association with the short allele of the promoter variant (TDTχ^2=4.69, df=1, p=0.030) establishes this gene as a strong candidate for fetal susceptibly to autism.

A variable 40 bp repeat located in the 3' untranslated region (UTR) of DAT1 provides an intragenic marker to aid in the investigation of this gene (Vandenbergh et al., 1992). Haplotype relative risk (HRR) analysis of 49 family trios (father, mother and affected child) has revealed an association between the 480 bp (10 copy) allele of the DAT1 VNTR and attention-deficit hyperactivity disorder (ADHD) (χ^2=7.51, df=1, p=0.006) [Cook et al., 1995]. A separate study reported an increased frequency of the DAT1 VNTR 10/10 genotype in ADHD and autistic subjects when compared to population-based controls [Comings et al., 1996]. In addition, Comings et al. [1996], also noted association of this genotype with the presence of symptoms and behavioural traits associated with pervasive developmental disorders. These results indicate the potential for DAT1 involvement in behavioral and psychiatric disorders further justifying investigation of this gene in relation to autism.

This study entails the investigation of biogenic amine transport genes as potential fetal susceptibility loci. Linkage and association strategies are used in combination with the affected sib pair design using microsatellite loci, VNTRs and functional polymorphisms linked to and within these candidate genes.

Information from microsatellite loci linked to each of the biogenic amine transporter loci will be pooled to examine segregation patterns of those regions containing the SERT, NET and DAT1 genes. Identity by descent (IBD) allele sharing between affected siblings will be examined through genotype determination of 49 pairs of affected siblings and their parents. Under the null expectation of no linkage the expected proportions of 0, 1 and 2 alleles shared IBD would be 0.25, 0.50 and 0.25, respectively. Detection of significant deviation from the expected proportion of concordant alleles among sib pairs, using the mean (t_2) statistic [Blackwelder and Elston, 1985], indicates the presence of linkage between the polymorphic marker and disease susceptibility locus. Separate analysis of maternal and paternal concordance will be conducted to effectively increase the number of informative families, as IBD allele sharing can only be established in the event that both parents are informative. Assuming independent segregation of alleles, one would expect 50% concordance among sib pairs. Determination of significant deviations of observed concordance from expected values of concordance, indicating the presence of linkage, are ascertained using the chi-square (χ^2) statistic. Concordance for DAT1 and SERT intragenic polymorphisms was not analyzed due to predominantly biallelic systems providing very low PIC values.

Allelic association is examined for intragenic polymorphisms including the DAT1 VNTR located in the 3' UTR region of the gene, the intronic VNTR of SERT (STin2) and SERT functional promoter variant (5HTTLPR), in addition to the highly polymorphic microsatellite loci chosen on the basis of their high PIC values and close proximity to the candidate genes. Biallelic and multiallelic tests of transmission disequilibrium (TDT) are applied to determine the extent of symmetry between alleles transmitted in common to affected sibling pairs and non-transmitted alleles. This approach avoids the confounding effect of ethnic stratification by using non-transmitted alleles as the "control population" However, due to the potential of genetic loading among relatives of affected individuals under the hypothesized multifactorial / threshold model of inheritance, parental allele and genotype frequencies will also be compared to tested and published frequencies to ascertain whether linkage disequilibrium is present in this population. Assessment of the appropriateness of population-based control comparisons is also considered. Testing of multiple sub-populations from a large sample of random male and female Canadian newborns validates the use of such populations for comparisons to test populations. Monte Carlo simulation [Reap software; Roff and Bentzen, 1989] will be used to determine the extent of population differences for multiallelic TDT testing and population-based control comparisons.

Due to the proposed fetal susceptibility / maternal effect model for the etiology of autism/PDD, genotypic correlation between maternal genotype at the DβH locus and IBD allele sharing at SERT, DAT1 and NET will also be examined to determine whether there is any interdependence between maternal genotype at maternal effect loci and fetal genetic

vulnerability, represented by affected sib pair concordance for regions encompassing the transporter genes.

CHAPTER 1:

Analysis of Markers for Biogenic Amine Transporters in Sib Pairs Affected with Autism/PDD

(Formatted for submission to *American Journal of Medical Genetics*)

INTRODUCTION

Autism is a pervasive developmental disorder (PDD) which affects 4-10 individuals for every 10000 in the general population with 4:1 male to female ratio [Smalley et al, 1991] Symptoms of the disorder, characterized by repetitive motoric activities, extreme resistance to change, delayed acquisition and abnormal use of language and impaired social interaction can appear in infancy, usually by three years of age. Autistic individuals display a considerable range in clinical presentation with respect to the number and severity of symptoms present. PDDs represent a wide range of related disorders, of which the most severe is autism [DSM-IV; APA, 1994].

Autism and the spectrum of PDD disorders show strong familial tendencies. The relative risk to siblings of affected individuals is 60-100 times greater than the observed general population incidence [Ciaranello and Ciaranello, 1995]. Support for underlying genetic factors for autism/PDD is also demonstrated by an increased incidence of concordance for the disorder among monozygotic vs. dizygotic twins [Folstein and Rutter 1977; Ritvo et al., 1985; Steffenburg et al., 1989] in addition to the presence of

association to disorders of known genetic etiology such as tuberous sclerosis, neurofibromatosis, Fragile X syndrome and untreated phenylketonuria (PKU) [Hunt and Dennis, 1987; Reiss and Freund, 1992; Rutter et al., 1994].

Although a strong genetic component to the disorder is demonstrated, single gene models of inheritance fail to account for the low sib risk, considerable clinical heterogeneity within families, unusual sex ratio and co-occurrence of autism/PDD with other genetic disorders which suggest etiological heterogeneity. Etiological factors encompassing multiple genetic susceptibility determinants and environmental contributions better explain these characteristics.

The candidate gene strategy employed in this study was aided by biochemical and pharmacological studies, which pointed to a number of genes that could contribute to the etiology of autism/PDD. Abnormalities in the biochemical profile among autistic individuals [Anderson, 1987; Martineau et al., 1994] and amelioration of symptoms upon pharmacological targeting of the same biochemical systems have indicated that biogenic amine systems, including dopamine, norepinephrine and serotonin, are strongly associated with autism/PDD. Thus the genes involved in these pathways are candidates for liability to autism/PDD. Previous results from our laboratory on the dopamine beta hydroxylase [DBH; Robinson et al., in prep] and monoamine oxidase A [MAOA; Schutz et al. (a), in prep] genes, two genes encoding enzymes involved in the metabolism of the monoamines (Appendices A and B), have implicated abnormal monoamine levels in maternal serum as contributing factors in the etiology of autism/PDD. It is therefore important to examine

some of the other genes involved in this pathway for possible involvement in the genetics of these disorders.

We have analyzed genotypes at biogenic amine transporter loci in the etiology of autism. The genes encoding the transporters for serotonin (SERT), norepinephrine (NET1) and dopamine (DAT1) are expressed in their respective neuronal systems at synaptic clefts, where the role of their gene products is the termination of neurotransmission via the reuptake of neurotransmitter from the synapse into the presynaptic neuron for recycling or degradation. The transporter proteins are also expressed in a variety of other tissues, where they function to remove neurotransmitters from the bloodstream. Alterations in the function of one or more of these transporters could result in abnormal synaptic transmission leading to autistic behaviour, or in the altered serum transmitter levels observed in autistics and their relatives [Anderson, 1987]. The genes encoding all three of the biogenic amine transporters have been mapped and cloned, with NET located in 16q12.2 [Bruss et al., 1993], DAT1 in 5p15.3 [Vandenbergh et al., 1992] and SERT in 17q11.2 [Gelernter et al., 1995]. The three transporters are members of a family of symporters exhibiting similar functionality and structural characteristics [Melikian et al., 1994]. These transporters exhibit some affinity for the other biogenic amines in addition to their own substrate; for example, NET has a higher affinity for DA than for NE, but is classified as a NE transporter on the basis of its pharmacological characteristics [Ramamoorthy et al., 1992]. Dopaminergic and noradrenergic transport is also mediated by the serotonin transporter [Faraj et al., 1994].

The recent mapping of these transporter genes allows the use of linked polymorphic loci and intragenic polymorphisms in genetic studies of these genes in the etiology of autism. We have studied the degree of allelic concordance at genetic loci near these transporter genes, in 49 pairs of siblings affected with autism/PDD, with the prediction of significantly increased allele sharing between sibs at these loci if the linked transporter genes are involved in the etiology of autism/PDD in these families. Family-based and population-based control comparisons were also performed to investigate the possibility of allelic association.

MATERIALS AND METHODS

The families and inclusion criteria used in this study have been described previously [Szatmari et al., 1996]. Questionnaires were sent out to members of the Autism Society of Canada and Southern Ontario children's mental health and social service agencies to ascertain families in which there were siblings of an autistic proband that qualified as having PDD. Families with more than one PDD affected child were also identified among those utilizing services of the clinics at Chedoke-McMaster Hospitals. Several interview and observation-based tests were performed to aid in diagnosis of multiplex families. Family History interview for developmental disorders [Folstein and Rutter, 1991], Autism Diagnostic Observation Schedule (ADOS) [Lord et al., 1989], Autism Diagnostic Interview (ADI) [Le Couteur et al., 1989], Autism Behavioral Checklist (ABC) [Krug et al.,1980], Vineland Adaptive Behavior Scales (VABS) [Sparrow et al., 1984] and various age-appropriate tests to assess intelligence were

applied to provide a diagnostic profile of probands and their affected sibling(s). Subjects were also assessed by three independent clinicians (psychiatrists and psychologists) to provide accurate diagnosis of multiple incidence families [Szatmari, personal communication]. In total, 37 male:male, 11 male:female and 1 female:female multiple incidence families were included in the study (10 families were provided by Dr. C. Schwartz). Sibships concordant under a broader autistic phenotype were included for study due to the assumptions under a multifactorial / threshold mode of inheritance, such that genetic liability for the disorder at a sub-threshold level could phenotypically manifest as a qualitatively similar syndrome, characterized by the related PDDs. Genetic determinants for the etiology of the disorder would then be common for children expressing similar phenotypes.

Canadian control populations composed of anonymous male and female neonates were obtained through the Ontario Ministry of Health. Guthrie cards containing heel prick blood spots taken for the purpose of newborn PKU (phenylketonuria) testing were gathered from undisclosed locations throughout Ontario to provide a representative sample of the Canadian population from which the affected population was derived.

Genomic DNA was extracted from peripheral lymphocytes of affected individuals and all available parents using a phenol/chloroform extraction method. In some cases, genomic DNA was obtained from dried blood (Guthrie) spots [Holden et al., 1996].

Markers and their respective primers used for PCR analysis of concordance for the three regions containing transporter genes are listed in Table 1. Primers were obtained from Research Genetics (Huntsville, Alabama) and MOBIX Facility (McMaster

University, Hamilton, Ontario). The DAT1 gene contains a variable number of tandem repeats (VNTR) polymorphism in its 3' untranslated region [Vandenbergh et al., 1992] allowing us to directly assess concordance of alleles at this gene. More highly variable microsatellite loci linked to DAT1, D5S117 and D5S417 were examined as well. The functional polymorphism (5HTTLPR) [Heils et al., 1996] and intronic VNTR (STin2) [Lesch et al., 1994] of the SERT gene were also analyzed. The highly polymorphic loci, D16S398, D16S389, D16S451, D17S932, D17S933, D17S250 and THRA-1, linked to NET1 and SERT, were used to examine identity by descent (IBD) sharing of alleles for these two genes.

Polymerase chain reaction (PCR) amplification of alleles at D16S398, D16S389, D16S451, D17S932, D17S933, D17S250, THRA-1, D5S417 and D5S117 were carried out using 25-50 ng template DNA in 10 μl volumes in thin-walled amplification tubes in a Perkin-Elmer Cetus 400 or 480 thermal cycling apparatus. Reactions contained 1X PCR buffer (BRL; 20 mM Tris HCl, 50 mM KCl), 4X200 mM dNTPs, 1.5-2.0 mM $MgCl_2$, 1.0 μg bovine serum albumin (BSA; Pharmacia Biotech), 2.0 pmol cold forward primer, 1.6 pmol cold reverse primer, 0.25 pmol γ-^{33}P ATP end-labelled reverse primer and 0.5-0.75 U Taq Polymerase (BRL). Amplification conditions comprised an initial denaturation stage at 94°C for 3 min, succeeded by cycling at 94°C for 30 sec (denaturation), 53-58°C for 30 sec-1 min (annealing), 72°C for 1 min (extension), for 28 cycles, followed by an additional final extension at 72°C for 10 min. To amplify from blood spots, five additional cycles of amplification were used. PCR products were subsequently electrophoresed on 6% denaturing polyacrylamide gels and visualized overnight by autoradiography. PCR

reactions for the DAT1 VNTR were carried out as above, with the omission of primer end-labelling. Amplification were performed in 20 µl reaction volumes, with 5 pmol of each primer and 35 cycles with an annealing temperature of 68°C. Electrophoresis was performed on 2% agarose gels and visualized by ethidium bromide staining and ultraviolet illumination. For the PCR analysis of the SERT promoter variant, 5HTTLPR, 50 ng of template were used in a 20 µl reaction volume, with 200 µM each of dATP, dCTP and dTTP, 100 µM each of dGTP and 7'-deaza-dGTP (7'-deaza-2'-deoxyguanosine 5'-triphosphate; Pharmacia Biotech), 1X PCR buffer, 0.75 units of Taq Polymerase, 1.5 mM $MgCl_2$, 2.0 µg BSA, 2 pmol cold forward primer, 1.6 pmol cold reverse primer, 0.25 pmol of γ-^{33}P ATP end-labelled reverse primer. Products were separated on 6% denaturing polyacrylamide gels following 28 cycles of amplification at 94°C for 30 sec, 61°C for 30 sec, 72°C for 1 min. Separation of 5HTTLPR amplification products on 2% agarose was also successful. The same cycling parameters and reaction conditions were used except, 10 pmol of each primer, 100 ng of template and 1.0 U of Taq Polymerase were required. The analysis of SERT intronic VNTR (STin2) was carried out using 150 mM dGTP: 50 mM 7'-deaza-dGTP, 200 mM of each of dATP, dCTP and dTTP, 1 X PCR Buffer, 1.5 mM $MgCl_2$, 2.0 µg BSA, 2 pmol cold forward primer, 1.6 pmol cold reverse primer, 0.25 pmol of γ-^{33}P ATP end-labelled reverse primer, 1.0 U Taq Polymerase (BRL). Amplification parameters included 28 cycles of 94°C for 30 sec, 53°C for 30 sec, 72°C for 1 min. Product separation and visualization was performed as above. Allele sizes were assigned by comparison to a sequencing ladder (Cycle Sequencing Ladder; Perkin Elmer) or

molecular weight standard (50 bp ladder; Pharmacia) and consistency of scoring was maintained by inclusion of individual size standards.

The sharing of alleles identical by descent (IBD) was assessed for each sibling pair. Establishment of 0, 1 or 2 alleles shared IBD required that both parents be informative for allelic segregation. Chromosomal regions were treated as haplotypes (by pooling microsatellite concordance information) to determine if there was any evidence of non-independent segregation of regions containing the candidate genes. Therefore, IBD scoring was assessed first by using the closest microsatellite marker and then those located at a greater distance from the gene were used if one or both parents were non-informative at the locus closer to the gene. Microsatellite locus distance from the transporter genes was derived from genetic mapping studies [Anderson et al., 1993; Gelernter et al., 1993; Gyapay et al., 1994; Shen et al., 1994; Doggett et al., 1995; Gelernter, Pakstis and Kidd, 1995; Gelernter et al., 1995; Rahman et al., 1996; Genome Database; NCBI Database] and the observation of the expected decreasing concordance rates with increasing distance from the gene. Recombination events observed between microsatellite loci were assessed to confirm the order of loci along the chromosome. The order of loci is consistent with the lack of double recombination events (for order and distance of markers proximal to the respective transporter genes refer to Appendix C). The proportions of 0, 1 and 2 alleles shared IBD for each chromosomal region were compared to the expected proportions (0.25, 0.50, 0.25, respectively) using the mean (t_2) statistic (Blackwelder and Elston, 1985). Concordance for paternal and maternal alleles was also assessed separately for each locus, and compared to the 50% concordance expected assuming independent

segregation, using the chi-square (χ^2) statistic. Adjustment of the nominal significance level, p=0.05, was carried out to correct for the use of multiple loci [sequential Bonferroni correction; Holm, 1979; Rice, 1988]. Due to the lack of independence of the markers analyzed at each candidate gene, application of this correction factor is required. However, when pooling information from each of the markers to analyze the chromosomal region as a haplotype, the markers no longer exist as separate, non-independent factors, and therefore, do not require correction. Bonferroni correction is not required for the testing of multiple candidate genes, as each represents a separate and independent hypothesis with respect to fetal susceptibility to autism/PDD. SERT intragenic polymorphisms were not assessed for concordance as 5HTTLPR and STin2 are primarily biallelic systems with low PIC values.

Biallelic and multiallelic transmission disequilibrium tests were performed for all loci (as appropriate) for detection of the presence of allelic association. As an additional measure of association, parental allele, genotype and haplotype frequencies were also compared to population-based Canadian controls and published control frequencies (where Canadian controls where unavailable).

RESULTS

Genotypes were determined for all members of the 49 multiplex families (MPX) at nine microsatellite loci, and three intragenic polymorphisms. Allelic phenotypes are characteristic to each locus, as seen in Figures 1-8. Illustration of the method of concordance scoring is provided in Figures 1-5. Pedigrees along the top of each

autoradiograph are aligned with individuals to portray familial relationships. Amplification products of microsatellite locus, D17S933 (Fig. 1), shows the presence of extra bands, referred to as "stutter" or "shadow" bands, two base pairs smaller and fainter than the actual products of the alleles, which appear as the most intense bands. The size difference between alleles and shadow bands is apparent upon comparison to the pUC 18 sequence ladder. Ideally, amplification of polymorphic repeat sequences should produce a singe band for every allele. However, for the majority of loci, several bands are seen per allele. These shadow bands arise as a result of Taq polymerase slippage during DNA replication or secondary structure of DNA [Miller and Yuan, 1997]. The presence of shadow bands can interfere with genotyping, unless strict criteria for scoring are established. The most common misinterpretation in assigning genotypes is caused by the overlapping of alleles and shadow bands. Heterozygous individuals with alleles spaced by two base pairs create a skewed pattern of band intensity such that shadow bands of the larger allele will combine with the second, smaller allele to create a band of greater intensity than the larger allele. An example of this phenomenon is depicted in Fig. 1. Individual 1 of Family 1 could easily be misinterpreted as homozygous if band intensity was ignored. The segregation of alleles in this family also supports these band intensity considerations. In the event that the relative band intensities were indeterminate or indecipherable by the human eye, Image Quant software line graph analysis was utilized to quantify band intensity. Similar examples are provided in Figures 2 (D17S250) and 3 (D16S398). Amplification of products for D17S250, represented by Figure 2, shows six of the 14 alleles observed for this locus. Family 1 depicted in Figure 2 demonstrates the difficulties encountered when

information from one parent is missing. The father of these affected boys was unavailable for genotyping. It is apparent that this sib pair inherited the same size allele from their father; however, when the full genotype of the father is unavailable one can not assume concordance due to the possibility of homozygosity. Five of the total 12 alleles observed for D16S398 are depicted in Figure 3. The genotypes of Family 3 provide an example of allelic overlap. Both parents and children are heterozygous for the 188 bp and 190 bp alleles. In this situation, it is impossible to determine whether the affected sibs inherited the same alleles from each parent; therefore, a score of non-informative (NIm and NIp) is given for each parent. The appearance of a 1 bp and 2 bp stutter pattern is illustrated in Figures 4 (D17S932) and 5 (D5S117). This 1 bp stutter is caused by the ability of Taq Polymerase to add an extra base at the end of amplified products in a template dependent manner [Ginot et al., 1996]. Both figures show alleles with two equally intense bands, separated by one base pair, representing the actual allele and allele plus one base pair. The fact that the bands appear equally intense signifies that the two products are amplified in equal proportions. Stutter bands are visible for both the allele and artifactual larger allele. The affected sib pair from Family 1 (Figure 4, D17S732) demonstrates sharing of two IBD alleles. The parental origin of alleles inherited in common by the two children can clearly be deciphered. Eight of the nine alleles found for this locus are visible in Figure 4. In Family 3 of Figure 5, the father was unavailable for study, however, his genotype can be deciphered because the children are discordant for his alleles. Because the two unaffected siblings confirm the paternal genotype, this case can be scored as discordant. Biasing towards paternal discordance is of concern in the event that paternal genotype can not be

directly analyzed. Thus, events of paternal discordance can not be scored as such, if concordance is not an equally probable observational event. It is hypothetically possible in this case to observe paternal concordance, among the affected sib pair, if the unaffected siblings demonstrate that the father was heterozygous. Therefore, paternal discordance can be assigned because there is the equal possibility of determining concordance. Family 6 of Figure 5 demonstrates an example in which paternal concordance or discordance can never be assigned due to paternal unavailability.

Single bands at 406 bp and 450 bp represent amplification of the two alleles of the functional promoter variant of SERT (5HTTLPR, Figure 6). Heterozygous individuals all have an extra band at approximately 500 bp. This consistent phenomenon may be due to heteroduplex structures from the two alleles. Biasing of allelic amplification can also be seen in this figure. Individuals 3 and 4 of Family 2 demonstrate biasing of opposite alleles signifying this phenomenon is template-dependent, as opposed to allele-dependent. Individual 1 of Family 3 represents an extreme example of allelic biasing; the long allele and 500 bp band are faintly visible, however, confirmation of the genotype was provided by re-amplification of the sample. The use of 7' -deaza - dGTP alleviated some of the PCR allelic biasing, but did not abolish the extra band seen in heterozygotes. Efforts to eliminate this band were not continued as the characteristic presence of the band in heterozygotes aided in the determination of genotype under the still present conditions of allelic biasing possibly caused by reduced template quality or secondary structure of the DNA [Mutter and Boynton, 1995]. The consistent appearance of an extra, larger band showing size variability with different heterozygotes was also observed in heterozygotes

of the DAT1 VNTR (Figure 7). Alleles corresponding to 3, 7 and 8 copies of the 40 bp core repeat (200 bp, 360 bp and 400 bp, respectively), shown in this figure, are among the rarer alleles of this locus, with allele frequencies less than or equal to 0.01 depending on the population sampled [Vandenbergh et al., 1992]. Amplification of alleles of the STin2 locus demonstrated a considerable extent of allelic biasing which was corrected upon addition of 7'-deaza-dGTP The four alleles observed at this locus, corresponding to 9, 10, 11 and 12 copies (253 bp, 270 bp, 287 bp and 304 bp) of the 17 bp repeat motif were characterized by a single band (Fig. 8). Only three of the four alleles observed at this locus are shown in this figure. The 11 copy allele was only observed in the control population.

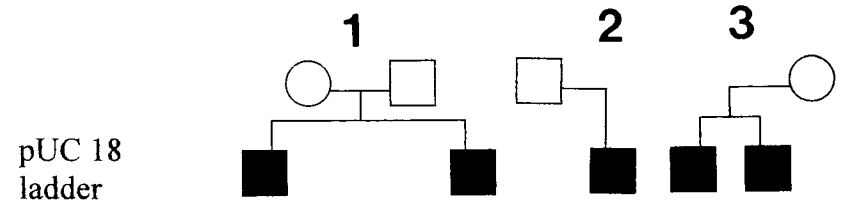
The concordances for alleles for the families at all loci studied are shown in Appendix D. Concordance of both maternal and paternal alleles is shown separately. Summary of maternal and paternal concordance is presented in Table 2. A trend towards increased concordance over independent segregation (50%) was observed in four instances: 1) maternal alleles at D16S451, linked to NET (χ^2=4.76, 1 df, p=0.03); 2) maternal alleles at THRA-1, linked to SERT (χ^2=3.10, df=1, p=0.08); 3) maternal alleles at D5S117, linked to DAT1 (χ^2=4.83, df=1, p=0.03) and 4) maternal alleles at D5S417, linked to DAT1 (χ^2=4.83, df=1, p=0.03). Although three of these observations are significant at $p<0.05$, correction for multiple testing requires that significance levels be adjusted for the number of non-independent tests applied or, in this case, the number of non-independent loci tested for each of the transporter genes. Significance for maternal

concordance fails to survive correction; however, the presence of a trend for all three loci is striking, suggesting the possibility of paternal imprinting at these loci. Concordance for paternal alleles at these loci was not significantly increased. However, the limited sample size prohibits the exclusion of increased paternal concordance at these loci. Similarly, increased concordance cannot be statistically ruled out at the other loci tested, due to reduced power given the number of informative families in the sample. Analysis of pooled data for IBD allele sharing revealed the presence of linkage. The proportion of sib pairs sharing two alleles was in excess of expected values for SERT (t_2=2.27, $p<0.05$), DAT1 (t_2=1.20, $p<0.05$) and NET (t_2=1.64, $p<0.05$); Table 3. Assessment of association in multiallelic markers using non-transmitted parental alleles for controls (Appendix E), summarized in Table 4, shows asymmetry of alleles transmitted from mothers at D16S389 (T_{mh}=19.39, df=9, p=0.0011). Evidence of allelic association was demonstrated when: paternal THRA-1 allele frequencies were compared to combined (male and female) controls (χ^2=35.44, df=14, p=0.0089); maternal and paternal allele frequencies were compared to published controls for D16S389 (χ^2=22.99, df=12, p=0.0162 and χ^2=30.63, df=12, p=0.0008, respectively); maternal D16S398 allele frequencies were compared to female controls (χ^2=17.85, df=10, p=0.0404); and maternal allele frequencies were compared to combined controls at D5S417 (χ^2=24.14, df=9, p=0.0068) (Table 5, allele frequencies available in Appendix C). Parental allele frequencies differences at THRA-1, D16S389 and D5S417 demonstrate statistically significant evidence for the existence of linkage disequilibrium at SERT, NET and DAT1, respectively following Bonferroni

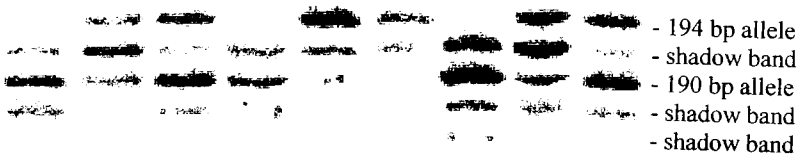
correction (Holm, 1979). Examination of maternal genotype frequencies at STin2 (Table 6, Appendix F) revealed a significant increase in the 12/12 genotype with respect to population-based controls (χ^2=16.07, df=4, p=0.0005). Statistically significant differences in STin2 / 5HTTLPR haplotype frequencies (Table 6) were observed when the same populations were compared (χ^2=20.97, df=11, p=0.0099). Parental 5HTTLPR allele and genotype distributions did not deviate from those observed in controls (Table 6). Family-based control comparisons do not indicate distorted allelic transmission to affected sib pairs at STin2 (Table 4) or 5HTTLPR (Table 7) (T_{mh} χ^2=2.14, df=2, p=0.41 and TDTχ^2=4.89, df=2, p>0.05). Separate analysis of maternal and paternal (versus combined parental) transmitted alleles at STin2 is significant, although this result must be interpreted with caution as only five fathers and four mothers were informative for the segregation analysis. Combining the parental alleles allows for inclusion of a greater number of meioses. Meioses are still informative in the event that both parents are heterozygous for the same alleles and of the two affected offspring, one is homozygous and the other heterozygous. In this case, offspring must have inherited the same allele from *one* of the identically heterozygous parents; however, it is impossible to assess which parent gave the same allele to the two children. Thus, a greater number of families can be examined when assessing combined parental contribution; however, in light of the increased maternal concordance seen for various loci it is still necessary to also evaluate parents separately. It also appears that the 12/L STin2 / 5HTTLPR haplotype is transmitted more often to both affected offspring (TDTχ^2=10.12, df=5 p=0.0019) (Table 8).

Figure 1: Amplification of Alleles of Microsatellite Locus D17S933 (linked to SERT) from Genomic DNA of Autistic Sib Pairs and Their Parents.

Allele sizes were determined by comparison to the pUC 18 sequence ladder. Pedigrees demonstrate the familial relationships between the individual lanes of the autoradiograph. A demonstration of maternal and paternal concordance scoring is provided.



pUC 18
ladder



1 2 3 4

A C G T

Cm
Cp

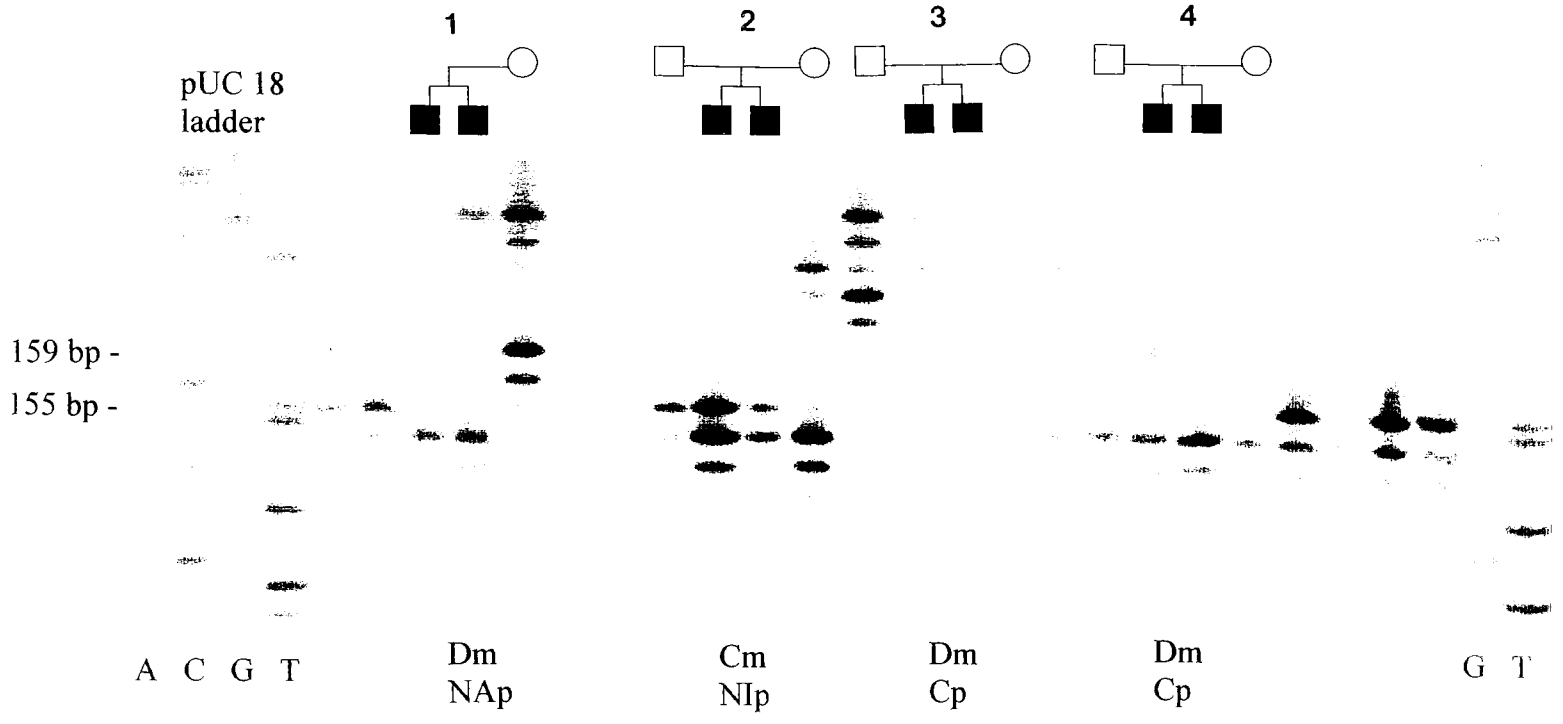
NIp

Dm
NAp

NI – NON-INFORMATIVE
NA – NOT AVAILABLE
C – CONCORDANT
D – DISCORDANT
m – MATERNAL
p – PATERNAL

Figure 2: Amplification of Alleles of Microsatellite Locus D17S250 (linked to SERT) from Genomic DNA of Autistic Sib Pairs and Their Parents.

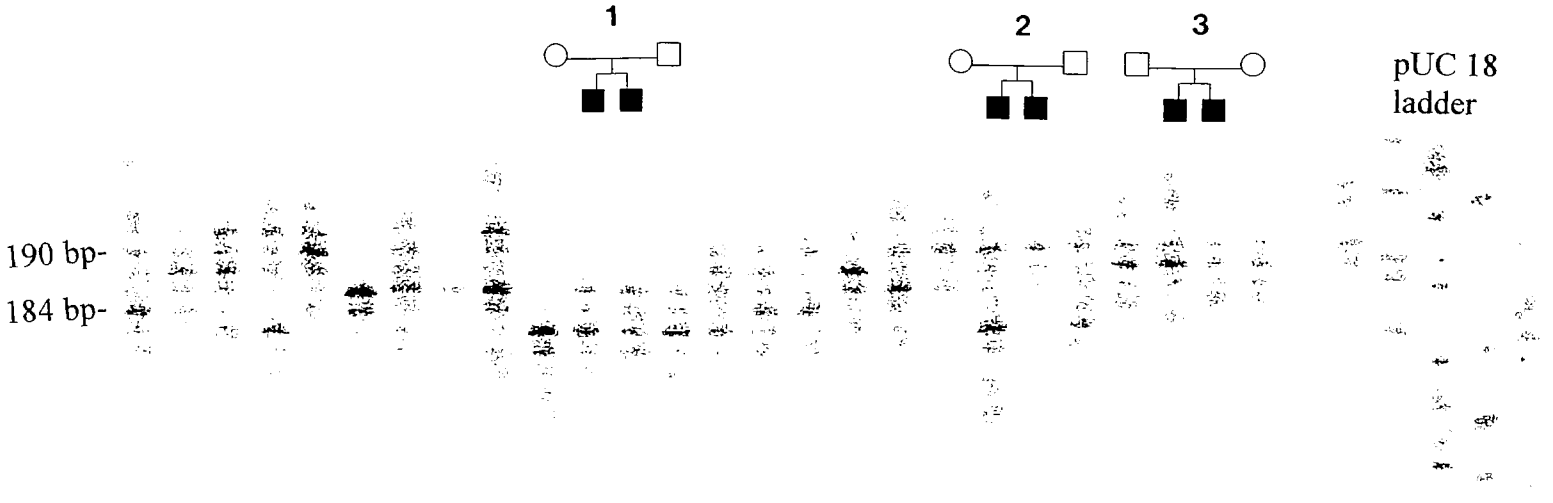
Allele sizes were determined by comparison to the pUC 18 sequence ladder. Pedigrees demonstrate the familial relationships between the individual lanes of the autoradiograph. A demonstration of maternal and paternal concordance scoring is provided.



NI – NON-INFORMATIVE
NA – NOT AVAILABLE
C – CONCORDANT
D – DISCORDANT
m – MATERNAL
p – PATERNAL

Figure 3: Amplification of Alleles of Microsatellite Locus D16S398 (linked to NET) from Genomic DNA of Autistic Sib Pairs and Their Parents.

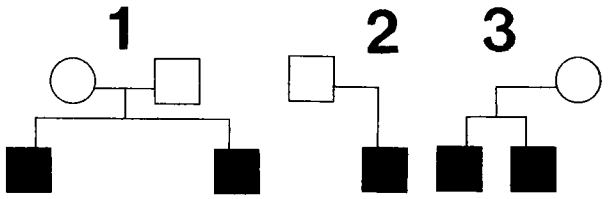
Allele sizes were determined by comparison to the pUC 18 sequence ladder. Pedigrees demonstrate the familial relationships between the individual lanes of the autoradiograph. A demonstration of maternal and paternal concordance scoring is provided.



NI – NON-INFORMATIVE
NA – NOT AVAILABLE
C – CONCORDANT
D – DISCORDANT
m – MATERNAL
p – PATERNAL

Figure 4: Amplification of Alleles of Microsatellite Locus D17S932 (linked to SERT) from Genomic DNA of Autistic Sib Pairs and Their Parents.

Allele sizes were determined by comparison to the pUC 18 sequence ladder. Pedigrees demonstrate the familial relationships between the individual lanes of the autoradiograph. A demonstration of maternal and paternal concordance scoring is provided.



pUC 18
ladder

- 205 bp

- 202 bp

201 bp allele -
shadow bands -

- 194 bp

191 bp allele -
shadow bands -

- 191 bp

Cm
Cp

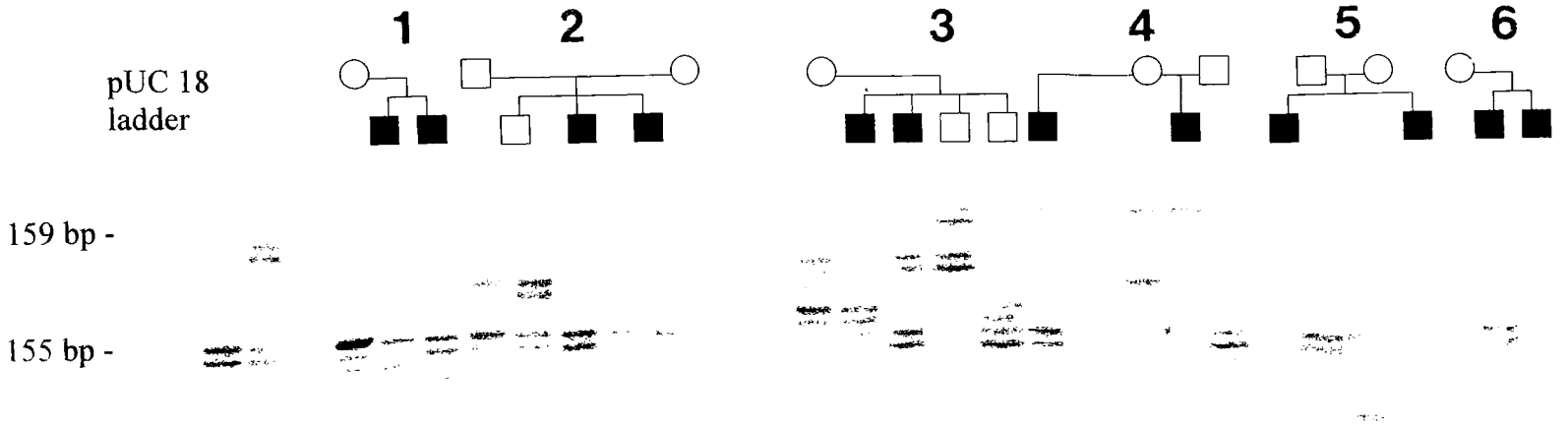
Dm
NAp

A C G T

NI – NON-INFORMATIVE
NA – NOT AVAILABLE
C – CONCORDANT
D – DISCORDANT
m – MATERNAL
p – PATERNAL

Figure 5: Amplification of Alleles of Microsatellite Locus D5S117 (linked to DAT1) from Genomic DNA of Autistic Sib Pairs and Their Parents.

Allele sizes were determined by comparison to the pUC 18 sequence ladder. Pedigrees demonstrate the familial relationships between the individual lanes of the autoradiograph. A demonstration of maternal and paternal concordance scoring is provided.



G	A	NIm	Cm Cp	Dm Dp	Cm DIFFERENT FATHERS	Cm NIp	Cm NAp

NI – NON-INFORMATIVE
NA – NOT AVAILABLE
C – CONCORDANT
D – DISCORDANT
m – MATERNAL
p – PATERNAL

Figure 6: Amplification of Alleles of 5HTTLPR, Functional Promoter Variant of the Serotonin Transporter Locus from Genomic DNA of Autistic Sib Pairs and Their Parents.

Figure depicts alleles separated on 2% agarose. The two 406 bp and 450 bp alleles observed here correspond to low and high transcriptional activity of the gene, respectively. Heterozygotes demonstrate the presence of a larger band presumably due to intra- or inter-molecular DNA structuring. Due to the consistency of this observation, the presence of the third band aided in the identification and genotyping of individuals.

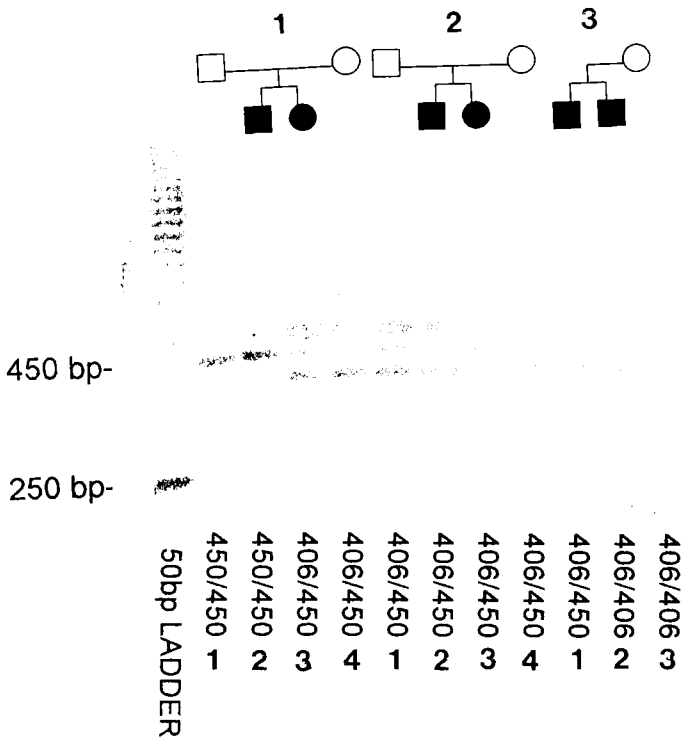

1
2
3
450 bp-
250 bp-
50bp LADDER
450/450 1
450/450 2
406/450 3
406/450 4
406/450 1
406/450 2
406/450 3
406/450 4
406/450 1
406/406 2
406/406 3
NEGATIVE
50bp LADDER

Figure 7: Amplification of Alleles of DAT1 VNTR, Located in the 3' UTR of the Dopamine Transporter Locus from Genomic DNA of Autistic Sib Pairs and Their Parents.

Figure depicts alleles separated on 2% agarose. Allele sizes were determined by comparison to the 50 bp ladder (bands represent 50 bp increments with double intensity bands at 250 bp and 500 bp).

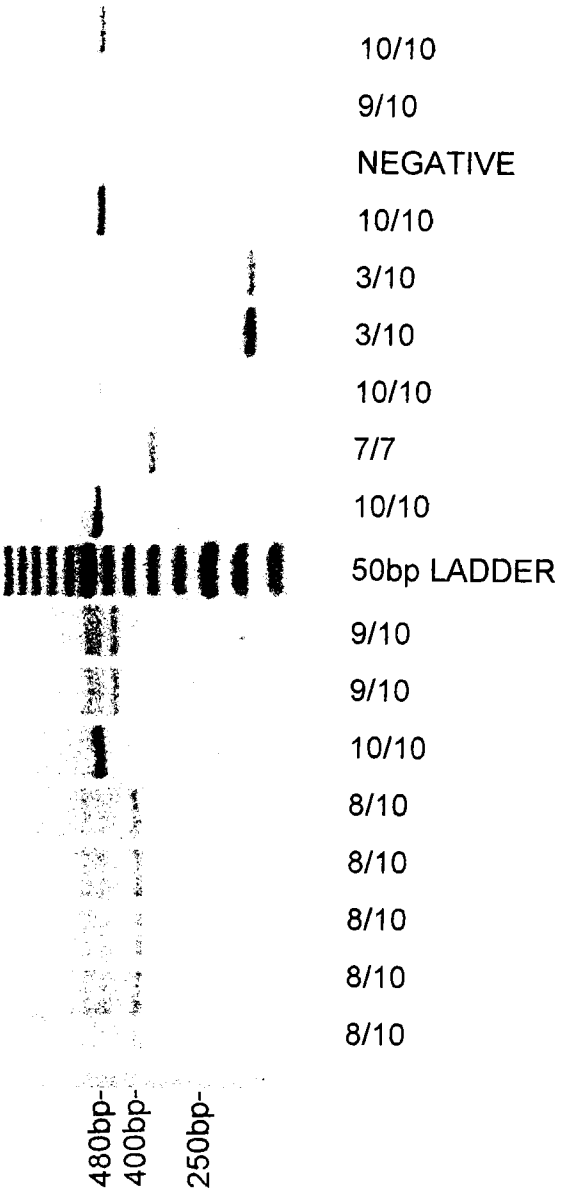

10/10
9/10
NEGATIVE
10/10
3/10
3/10
10/10
7/7
10/10
50bp LADDER
9/10
9/10
10/10
8/10
8/10
8/10
8/10
8/10
480bp-
400bp-
250bp-

Figure 8: Amplification of Alleles of STin2, Located in the Second Intron of the Serotonin Transporter Locus from Genomic DNA of Autistic Sib Pairs and Their Parents.

Allele sizes were determined by comparison to the pUC 18 sequence ladder. Pedigrees demonstrate the familial relationships between the individual lanes of the autoradiograph.

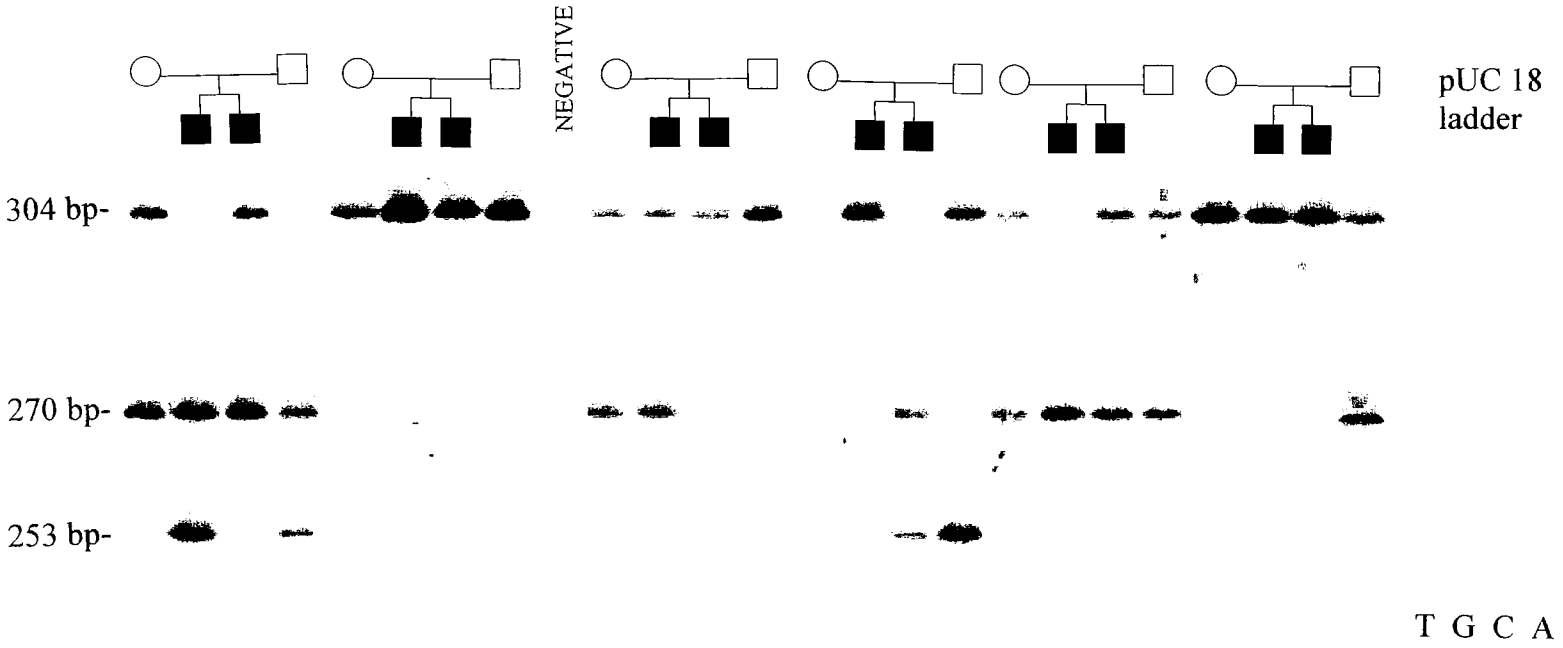

NEGATIVE
pUC 18
ladder
304 bp-
270 bp-
253 bp-
T G C A

Table 1: Heterozygosity Level, Allele Size Range and Primer Sequences for Intragenic and Linked Polymorphisms of SERT, NET and DAT1.

Candidate Gene	Chromosomal Location	Test locus	% HET	Allele Size Range(bp)	Primer Sequence (5'-3')	Reference
NET	16q12.2	D16S398	85	178-200	(446) CTT GCT CTT TCT AAA CTC CA (672) GAA ACC AAG TGG GTT AGG TC	Weber et al. (1992) pers. commun. (gdb)
		D16S389	77	96-122	16AC10B3F TGG TCC TGA TGT TTT AAT TTA GCA ATC CAG 16AC10B3R CTA ATT TCC ATC AGG AAT GTT AT	Shen et al. (1993) HMG 2:1505
		D16S451	84	83-113	16AC69F12F ATA TCC ATC ACC ATT CAT GGT TAC 16AC69F12R CTA AGG AAA GGT AGG ACC TTA AT	Shen et al. (1993) HMG 2:1505
SERT	17q11.2-q21	D17S250	83	133-173	(AC) GGA AGA ATC AAA TAG ACA AT (GT) GCT GGC CAT ATA TAT ATT TAA ACC	Weber et al. (1990) Nuc.Acid Res. 18:4640
		THRA-1	81	160-196	THRA-1(AC) CTG CGC TTT GCA CTA TTG GG THRA-1(TG) CGG GCA GCA TAG CAT TGC CT	Futreal et al.(1992) HMG 1:66
		D17S933	82	186-206	AFM254vg5a ACT CAC TGG GGT CCT GG AFM254vg5m TGT GGT TTC CTT ATA GAC TGT AGA	Gyapay et al. (1994) Nat Gen 7:246-339
		D17S932	83	189-205	AFM248yg9a GCT AAA AAT ACA CGG ATG G AFM248yg9m TGC AAG ACT GCG TCT C	Gyapay et al. (1994) Nat Gen 7:246-339
		STin2	41	253-304	8223 TGT TCC TAG TCT TAC GCC AGT G 8224 GTC AGT ATC ACA GGC TGC GAG	Lesch et al. (1994) J. Neural Transm. 95:157-164
		5HTTLPR	52	406-450	HTTp2A TGA ATG CCA GCA CCT AAC CC HTTp2B TTC TGG TGC CAC CTA GAC GC	Heils et al (1996) J. NeuroChem. 66: 2621-2624 Cook et al. (1996) Mol Psychiatry 2:247-250
DAT1	5p15.3	DAT1 VNTR	45	200-520	T3-5LONG TGT GGT GTA GGG AAC GGC CTG AG T7-3aLONG CTT CCT GGA GGT CAC GGC TCA AGG	Vanderbergh et al. (1992) Genomics 14:1104
		D5S117	62	145-201	(CA) TGT CTC CTG CTG AGA ATA G (GT) TAA TAT CCA AAC CAC AAA GGT	Weber et al. (1990) Nuc. Acid Res. 18:4035
		D5S417	72	92-110	(CA) TGG AAA CTA TGT ATC TTG GAG G (GT) GCN GGC TTT AGG GTG G	Gyapay et al. (1994) Nat Gen 7: 246-339

Table 2: Affected Sib Pair Concordance For Loci Linked to Biogenic Amine Transporter Genes NET, SERT and DAT1.

CANDIDATE GENE	TEST LOCUS	MATERNAL CONCORDANCE				PATERNAL CONCORDANCE			
		C	D	NI	%C	C	D	NI	%C
NET	D16S398	21	16	12	56.8 $\chi^2 = 0.68$ p = 0.41	17	18	7	48.6 $\chi^2 = 0.03$ p = 0.86
	D16S389	16	13	14	55.2 $\chi^2 = 0.31$ p = 0.53	18	11	10	62.1 $\chi^2 = 1.7$ p=0.19
	D16S451	13	4	17	76.5 $\chi^2 = 4.76$ p = 0.03	10	17	9	31 $\chi^2 = 1.81$ p = 0 18
SERT	D17S250	17	16	14	51.5 $\chi^2 = 0.03$ p = 0.86	14	12	15	53.8 $\chi^2 = 0.15$ p = 0.70
	THRA-1	25	14	10	64.1 $\chi^2 = 3.10$ p = 0.08	19	14	10	57.6 $\chi^2 = 0.76$ p = 0.38
	D17S932	15	17	13	46.9 $\chi^2 = 0.12$ p = 0.72	13	13	12	50 $\chi^2 = 0$
	D17S933	19	18	11	51.4 $\chi^2 = 0.03$ p = 0.86	15	16	9	48.4 $\chi^2 = 0.03$ p = 0.86
DAT1	DAT1 VNTR	10	10	25	50 $\chi^2 = 0$	7	5	29	58.3 $\chi^2 = 0.33$ p = 0.57
	D5S117	24	11	12	68.6 $\chi^2 = 4.83$ p = 0.03	14	12	15	53.8 $\chi^2 = 0.15$ p = 0.70
	D5S417	24	11	8	68.6 $\chi^2 = 4.83$ p = 0.03	16	15	8	51.6 $\chi^2 = 0.03$ p = 0.86

C= CONCORDANT
D= DISCORDANT
NI= NON-INFORMATIVE

Table 3: Identity by Descent (IBD) Allele Sharing Among Affected Sib Pairs for Pooled Data of SERT, NET and DAT.

	CANDIDATE GENE		
	DAT1	NET	SERT
r_{20} =	9	4	5
r_{21} =	14	22	21
r_{22} =	14	11	15
n_2 =	37	37	41
p_{20} =	0.24	0.11	0.12
p_{21} =	0.38	0.59	0.51
p_{22} =	0.38	0.30	0.37
s_2^2 =	0.0135	0.0135	0.0122
s_2 =	0.1162	0.116	0.110
t_2 =	1.20	1.64	2.27

where;

r_{20} = observed number of sib pairs with 0 marker alleles IBD and 2 aff'd members

r_{21} = observed number of sib pairs with 1 marker alleles IBD and 2 aff'd members

r_{22} = observed number of sib pairs with 2 marker alleles IBD and 2 aff'd members

n_2 = $r_{20}+r_{21}+r_{22}$

p_{20} = proportion of 0 marker alleles IBD geiven 2 aff'd sibs; p=r/n

p_{21} = proportion of 1 marker alleles IBD geiven 2 aff'd sibs; p=r/n

p_{22} = proportion of 2 marker alleles IBD geiven 2 aff'd sibs; p=r/n

s_2^2 = $1/2n_2$

If $n_1 = 0$

$t_2 = (p_{21} + 2p_{22} - 1)/s_2$

reject Ho (no linkage) if $t_2 > 0$ ($p \leq 0.05$)

Table 4: Transmission Disequilibrium Test for Symmetry Between Transmitted and Non-transmitted Alleles at Polymorphic Loci Linked to Candidate Genes SERT, NET and DAT1

CANDIDATE GENE	POLYMORPHIC LOCUS	T_{mh}* BOTH PARENTS	AUT MOTHERS	AUT FATHERS
SERT	STin2	2.14 df=2 p=0.4089	4 df=1 p=0.0318	3.6 df=1 p=0.0077
	THRA-1	10.92 df=8 p=0.1947	5.18 df=7 p=0.6914	8.77 df=8 p=0.3646
	D17S250	13.13 df=12 p=0.3541	11.49 df=10 0.309	6.68 df=9 p=0.7687
	D17S932	7.43 df=8 p=0.5278	6.5 df=7 p=0.4720	5 53 df=6 0.4804
	D17S933	13.54 df=9 p=0.1098	7.53 df=8 p=0.5506	7.82 df=8 p=0.5037
NET	D16S451	12.83 df=7 p=0.0614	6.6 df=5 0.2819	8 df=7 p=0.3838
	D16S389	16.16 df=10 p=0.0618	19.39 df=9 p=0.0011	14.56 df=10 0.0689
	D16S398	5.27 df=7 p=0.6620	7.37 df=7 p=0.4303	3.27 df=5 p=0.7096
DAT1	DAT1 VNTR	3.25 df=2 p=0.1099	1.51 df=2 p=0.3685	3.47 df=2 p=0.0823
	D5S117	7.25 df=9 p=0.6874	9.58 df=8 p=0.2657	6 df=7 p=0.6374
	D5S417	3.46 df=7 p=0.8859	2.49 df=7 p=0.9469	4.68 df=6 p=0.6853

* Tmh (TDT for marginal heterogeneity/homogeneity)
** two-tailed p values calculated using Monte Carlo Simulation (Reap Software)(Roff and Bentzen, 1989)

Table 5: Comparison of MPX Parental, Canadian Control and Published Population Allele Frequencies

		χ^2 *			
		MPX MOTHERS	MPX FATHERS	MPX MOTHERS	MPX FATHERS
CANDIDATE GENE	POLYMORPHIC LOCUS	vs. COMBINED CONTROLS		vs. FEMALE CONTROLS	vs. MALE CONTROLS
SERT	THRA-1	17.09 df=13 p=0.2112	35.44 df=14 p=0.0089	14.68 df=10 p=0.1362	22.23 df=14 p=0.0768
	D17S250	21.07 df=13 p=0.0663	10.93 df=11 p=0.4406	15.42 df=12 p=0.2068	7.09 df=11 p=0.8124
	D17S932[a]		2.33 df=7 p=0.9743	9.44 df=7 p=0.2120	
	D17S933	9.37 df=9 p=0.4046	11.9 df=10 p=0.2766	11.84 df=8 p=0.1426	11.9 df=10 p=0.2655
NET	D16S451[b]	14.25 df=10 p=0.1307	18.85 df=12 p=0.0641		
	D16S389[c]	22.99 df=12 p=0.0162	30.63 df=12 p=0.0008		
	D16S398	17 df=10 p=0.0727	12.21 df=10 p=0.2646	17.85 df=10 p=0.0404	12.76 df=9 p=0.1647
DAT1	DAT1 VNTR	3.16 df=6 p=0.8292		4.37 df=6 p=0.6236	
	D5S117[c]	13.19 df=9 p=0.1151		11.12 df=8 p=0.1783	
	D5S417	24.14 df=9 p=0.0068	12.05 df=9 p=0.2179	14.33 df=8 p=0.0573	17.44 df=9 p=0.0591

*χ^2 and p values (two-tailed) calculated using Monte Carlo simulation (Reap Software) (Roff and Bentzen, 1989)

[a] - only female controls available for comparison

[b] - published controls used for comparison

[c] - published controls used for comparison (shifting of distribution of allele frequencies required to more accurately reflect that of tested population)

Table 6: Comparison of Allele, Genotype and Haplotype Frequencies of MPX Parents and Canadian Control Populations at STin2 and 5HTTLPR.

		χ^2 *			
CANDIDATE GENE	POLYMORPHIC LOCUS	MPX MOTHERS vs. COMBINED CONTROLS	MPX FATHERS vs. COMBINED CONTROLS	MPX MOTHERS vs. FEMALE CONTROLS	MPX FATHERS vs. MALE CONTROLS
SERT	STin2 Allele	2.44 df=3 p=0.4889	3.00 df=3 p=0.3993	1.62 df=2 p=0.4323	6.60 df=3 p=0.0642
	STin2 Genotype	8.50 df=5 p=0.1189	3.25 df=5 p=0.6304	16.07 df=4 p=0.0005	7.63 df=5 p=0.1493
	5HTTLPR Allele	1.08 df=1 p=0.2767	1.44 df=1 p=0.2115	2.04 df=1 p=0.1371	0.66 df=1 p=0.3493
	5HTTLPR Genotype	1.60 df=2 p=0.4480	1.69 df=2 p=0.4489	2.66 df=2 p=0.2668	0.75 df=2 p=0.6452
	STin2/ 5HTTLPR Haplotype	12.91 df=12 p=0.3824	15.26 df=13 p=0.2920	20.97 df=11 p=0.0099	16.61 df=12 p=0.1316

*χ^2 and p values (two-tailed) calculated using Monte Carlo simulation (Reap Software) (Roff and Bentzen, 1989)

Table 7: TDT for Biallelic Polymorphic Locus, 5HTTLPR

	Number of Alleles Shared by Both Affected Children		
Transmitted Allele	0	1	2
S (406bp)	8	24	4
L (450bp)	4	24	8

$TDT\chi^2_{TOTAL}$ = 4.89 df=2, n.s.

Table 8: TDT of STin2 / 5HTTLPR Haplotype Transmission

STin2 5HTTLPR Haplotype	Transmitted Haplotype	Non-Transmitted Haplotype
9/S	0	1
10/S	2	1
12/S	2	4
9/L	0	1
10/L	1	5
12/L	9	2

$TDT\chi^2(T_{mh})$ = 10.12 df =5, p=0.0019

DISCUSSION

These results suggest a possible role for the DAT1, NET and SERT proteins in the etiology of autism/PDD. The polymorphic microsatellite markers were selected for study because of their proximity to the biogenic amine transporter genes, respectively. Elevated allele sharing at SERT, DAT1 and NET implies the presence of linkage to autism/PDD. These results also suggest that allelic variations or mutations in these genes contribute to the autistic/PDD phenotype in these sibships.

Although no evidence of association with the functional variant of SERT was observed, this study did not possess sufficient power to detect the presence of linkage or association due to the low PIC value of the SERT promoter variant locus and, therefore, decreased number of informative families. The recent report by Cook et al. (1997) demonstrating increased transmission of the short SERT allele to SPX autistic individuals using the transmission disequilibrium test (TDT) ($TDT\chi^2$=4.69, df=1 p=0.030), suggested evidence that the short allelic variant of this functional polymorphism, associated with decreased serotonin transporter transcriptional activity [Heils et al., 1996], may play an important role in the etiology of autism/PDD. Increased 12 copy/ long allele STin2 / 5HTTLPR haplotype ($TDT\chi^2$=10.12, df=5 p=0.0019) transmission in this study is in contrast to the increased transmission of short variant containing haplotypes ($TDT\chi^2$= 11.85, df=4, p=0.018) observed in Cook's study [Cook et al., 1997]. The increased 12/12 STin2 maternal genotype frequency in the present study is likely a factor in this result. Considerable linkage disequilibrium of these markers in ethnically diverse populations

[Gelernter, Kranzler and Cubells, 1997] could explain some of the observed inconsistencies between studies, as there may be ethnic disparities between the American and Canadian populations used in these studies. However, a more recent replication of Cook's study, in a German population, revealed significant evidence for association with haplotypes containing the long variant of 5HTTLPR (TDT p= 0.049), primarily due to the increased transmission of the long variant allele to affected individuals (TDT p=0.032) [Klauck et al., 1997].

Previous studies linking these polymorphisms to neuropsychiatric conditions have been reported. An increased frequency of the STin2.9 allele (seen very rarely in the present study) has been observed among individuals with affective disorder [Oglivie et al., 1996] and short 5HTTLPR variant has been linked to anxiety-related traits [Lesch et al., 1996]. Increased frequency of the12 copy allele of STin2 has also been found in subjects with bipolar affective disorder (p=0.00048) [Collier et al., 1996]. The potential transcriptional regulatory effect of the STin2 locus has been suggested due to the similarity to the IDDM2 locus (conferring susceptibility to Insulin Dependent Diabetes Mellitus) [Ogilvie et al., 1996; Heils et al., 1996]. Complex interaction with other regulatory elements of this gene as a potential mechanism of differential transcriptional activity have yet to be investigated, although, proximally located transcription factor binding sites could mediate the relationship between the STin2 VNTR and SERT expression [Lesch et al., 1994].

The lack of a significant increase in concordance at the VNTR locus in DAT1, failure to observe deviation from expected population allele frequencies reported by

Vandenbergh et al. [1992] and non-skewed transmission of parental alleles to autistic offspring reflects the low level of heterozygosity and thus small number of informative families at this locus. However, significant allele sharing between affected sib pairs at microsatellite loci in this region suggests a role for DAT1 in autism/PDD (Table 2).

No intragenic polymorphisms have been identified for the norepinephrine transporter; therefore, only linked microsatellites could be analyzed for this candidate gene. Increased transmission of particular maternal alleles at D16S389 did not coincide with those found in increased frequency upon comparison to published population-based controls [Shen et al., 1993] possibly due to population stratification (Tables 4 and 5). Similar to findings at SERT and DAT1, increased allele sharing among autistic siblings for NET markers (Table 2) suggests the potential involvement of this gene in the etiology of autism/PDD.

Parental allele frequency differences for THRA-1, D16S398 and D5S417 when compared with well-matched, sufficiently sized Canadian control populations ($N \approx 200$) did not reveal any one particular allele that seemed to be in excess. However, the distribution of alleles was altered signifying evidence for generalized genetic differences between first degree relatives of autistic probands and the general population. Under the multifactorial / threshold model of inheritance this phenomenon would be expected.

Linkage and allelic association for these markers was not supported when considering TDT findings. However, the power and validity of this test, in the context of this study, has to be considered. The aim of the TDT is to determine whether there is evidence for marker allele linkage disequilibrium with disease by looking at whether there

is asymmetry between transmitted and non-transmitted alleles. The TDT effectively uses the non-transmitted parental alleles as a control, avoiding the possibility of spurious association, which can occur using poorly matched or non-representative population-based controls. When applying this analysis to related affected individuals, alleles that are considered are only those that are mutually inherited from informative or heterozygous parents. The TDT, therefore, considers linkage and linkage disequilibrium simultaneously. Consequently, the TDT can only detect linkage in the presence of association [Speilman and Ewens, 1996]. A number of factors could contribute to failure to observe association, and therefore, linkage. In considering only those affected sib pairs demonstrating allelic concordance, the number of informative families decreases, leading to a decline in the ability or power to detect the presence of linkage or association. Ethnic diversity of the Canadian population could obscure the ability to detect the presence of association. Ancestral establishment of linkage disequilibrium between marker alleles and disease alleles could vary among those of different ethnic origin. If the disease susceptibility allele in question was of weak to moderate genetic effect, linkage equilibrium with nearby markers would be regained at a faster rate than would be expected under a single gene mode of inheritance. Furthermore, genetic loading or sub-threshold susceptibility in first-degree relatives of autistic individuals expected under the multifactorial / threshold model of inheritance invalidates the use of internal family-based controls. Therefore, failure to observe altered parental transmission of alleles to affected offspring does not invalidate the presence of linkage supported by analysis of IBD allele sharing among sib pairs.

Increasing the power of linkage detection under the affected sib pair (ASP) design was

made possible by pooling concordance information for polymorphic loci linked to each candidate gene. IBD determination for each individual marker lacked sufficient power due to the elimination of non-informative families. Therefore, to maximize the number of informative families, maintain power and to derive complete segregation information of the region of DNA containing the candidate gene, markers were analyzed by order of proximity to the candidate gene. This haplotype analysis is therefore, exempt from Bonferroni correction, which is applied when multiple loci have been tested.

Considering the estimates of population prevalence of autism and relative risk to siblings of affected individuals exclusively, sufficient power to detect linkage under LOD score based methods of linkage analysis is achieved by this study. However, under a multifactorial / threshold model of inheritance postulating a number of genes of weak to moderate genetic effect and the realistic probability of recombination between marker and candidate gene one has to consider means by which the method of study increases the probability of detecting linkage. An estimated 1.8% probability of linkage detection is attained by this study assuming a mid-range estimate of 0.02% incidence of autism/PDD in the general population, 3% risk to siblings of affected individuals, a locus accounting for 10% of the total genetic contribution (compensated for in calculation under additive model), 2% recombination between marker and candidate gene, 100% penetrance and effective sample size of 35 fully informative families (accounting for PIC<1) (Calculation in Appendix G). It is considered impossible to detect linkage at this level of power. The assumption of LOD score analysis with statistical significance attained at the 1000:1 odds ratio level is partially responsible for the low probability to detect linkage. The mean

statistic (reviewed by Blackwelder and Elston, 1985), basically a less stringent method of statistical analysis with a significance level of $p \leq 0.05$, boosts the estimate of power to 56.1%. Correction for the number of loci tested is a valid consideration for genome-scans to guard against the possibility of Type 1 error. However, the ASP method in combination with a candidate gene approach was employed here to avoid spurious associations and to improve the probability of linkage detection. Considering that biogenic amine transporter genes have been only recently mapped, and definitive physical location with respect to markers unavailable, several markers were used for each gene to assess affected sib pair concordance within the gene region. Application of the standard Bonferroni correction extinguishes attempts to increase the probability of detecting linkage when considering the need for multiple loci for testing each candidate gene. Suppose five loci were tested at a hypothetical candidate gene, and all showed concordance at p=0.012, 0.013, 0.017, 0.03 and 0.05, none would be deemed statistically significant because the standard Bonferroni correction requires that the p value be adjusted to p=0.01. The sequential Bonferroni method requires that p values be ranked, from greatest to least significant, then compared to significance levels which are divided by the number of non-independent tests minus one for each comparison. Applying the sequential Bonferroni correction to the previous example would require comparison of the observed ranked p values to significance levels of p=0.01, 0.0125, 0.0166, 0.025 and 0.05 (corresponding to $\alpha/5$, $\alpha/4$, $\alpha/3$, $\alpha/2$, and, $\alpha/1$). Only the lowest ranked p value, most likely representing the marker farthest from the gene, would be considered statistically

significant. To have to state that those markers closest to the gene were not statistically significant and that which is farthest away does qualify for statistical significance is inappropriate. Examples of such scenarios reveal the over-compensatory nature of this method as protection against chance significance in 1 of every 20 trials and signal the need to evaluate each data set unobstructed, taking into consideration the complex genetic environment from which they are extracted. The haplotype analysis used in this study combined information from the non-independent markers to create a more concise representation of regional chromosomal segregation. When testing IBD allele sharing among affected sib pairs for each of the candidate genes in this manner, they represent three separate, independent tests, thus eluding correction for non-independent, multiple testing. Continual efforts to increase the power of research design in the context of complex disease dissection are futile if this correction was to be persistently applied in such a blanketed fashion. In addition, the purpose of studies, such as the one presented here, is to provide an indication of whether further molecular research into a candidate gene is warranted. Taking into account the likelihood of finding significance, due to the constraints of reduced power, and, the substantial biological relevance of the candidate genes studied, it may be necessary to consider less highly significant results to prevent rejection of important leads [Lander and Kruglyuk, 1995]. Therefore, it is evident that haplotype analysis is the most powerful method for examination of this type of data.

We have recently reported evidence for the involvement of the dopamine beta hydroxylase (DβH) and monoamine oxidase A (MAOA) genes in the etiology of autism/PDD in these same families [Robinson et al, in preparation; Schutz et al (a), in

preparation]. Both enzymes are involved in the biosynthetic or degradative pathways of biogenic amines. On the basis of altered allele frequencies at the DBH and MAOA loci in the mothers of male multiplex sibships, but no increased concordance in the sib pairs themselves, we have proposed that abnormal biogenic amine levels in maternal serum expose the developing fetus to a potentially harmful uterine environment resulting in autism/PDD in genetically susceptible fetuses [Schutz et al (b), in preparation]. To explain discordance for autism in siblings and especially in twins in this model, it is necessary to hypothesize that fetus-specific factors modulate fetal susceptibility to this maternal effect of abnormal neurotransmitter levels. Variations in neurotransmitter uptake in the fetus, as determined by genetic variation at the transporter loci, could account for much of this fetal susceptibility. This would be reflected in increased allelic concordance at these loci in pairs of affected sibs. It is interesting to note that the SERT and NET proteins, but not DAT1, are expressed on the placenta and are directly exposed to maternal blood [Ramamoorthy et al., 1992; Balkovetz et al., 1989]. Presence on the placenta implies a role for these proteins in transplacental transport of biogenic amine neurotransmitters. The importance to normal development of biogenic amine transport across the placenta has been suggested by transgenic mouse studies, in which some DBH -/- fetuses of DBH +/- mothers survived, whereas DBH -/- offspring of DBH -/- mothers all died *in utero*, implicating NE transfer across the placenta [Thomas et al., 1995]. Furthermore, studies demonstrating that synergistic interactions between growth factors and catecholamine neurotransmitters, possibly of maternal origin, are required for the differentiation of catecholaminergic neurons [Du and Iacovitti, 1995]. Altered transport, either elevated or

reduced, could lead to the exposure of the fetus to inappropriate neurotransmitter levels, resulting in abnormal development and autism/PDD. Given the cross-affinities of these transporters, it is difficult to determine which specific biogenic amine(s) would be involved in this process. The absence of DAT1 expression on the placenta does not necessarily rule out a role for DAT1 in this process, as it could be involved in the response to maternal monoamines at the neuronal level in the developing fetus, rather than in transport across the placenta.

The parent-of-origin effects suggested by the data for THRA-1 and DAT1 are intriguing, in that they point to a role for genomic imprinting in the genetic mechanisms underlying autism/PDD. While this is an interesting possibility, these results need to be evaluated with caution. Although there appears to be no trend toward increased concordance for alleles from fathers, due to the small samples sizes, increased paternal concordances cannot be excluded, and a larger number of families need to be examined at each of these loci.

While it is formally possible that other genes in chromosomal regions represented by the polymorphic markers studied here are responsible for the increased concordance rates observed, SERT, NET and DAT1 are the most likely candidate genes in these regions based on their involvement in biogenic aminergic systems.

Future studies should investigate these regions in a larger sample of multiplex sibships, and with a more extensive set of closely linked polymorphic markers. Nevertheless, these results represent the first findings of significantly increased allelic concordance in affected sib pairs with autism/PDD and, taken together with our findings

at DBH and MAOA, suggest a novel model for the etiology of the autistic spectrum disorders in these families, and possibly for other complex neuropsychiatric disorders as well.

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CHAPTER 2:

Association between Maternal Genotype at the Dopamine beta Hydroxylase Locus and Fetal Genotype at the Serotonin Transporter Supports a Fetal Susceptibility / Maternal Effect Model for Autism/PDD

INTRODUCTION

Autism/PDD is a neurological disorder characterized by developmental delay, encompassing motoric abnormalities, delay or absence in language acquisition and deviant development of social relationships, as well as, emotional disturbances. There is a strong indication for the involvement of genetic factors in the etiology of the disorder, however, single gene models of inheritance fail to account for some of the key features of the disorder. Population incidence estimates for the disorder indicate 4-10 individuals are affected for every 10000 people. Relative risk to siblings of affected individuals is 2-6%, representing a frequency 60-100 times that of the general population incidence [Smalley et al., 1988]. Autism affects males predominantly, with four males affected for every one female [Ciaranello and Ciaranello, 1995]. These characteristics, in addition to considerable clinical and etiological heterogeneity [Ciaranello and Ciaranello, 1995], are better accounted for by a multifactorial / threshold model of inheritance.

Recent studies have demonstrated the existence of a possible maternal effect contributing to susceptibility to autism/PDD. In the process of screening multiple

incidence families for genetic factors predisposing individuals to autism, the presence of significantly different allele frequencies at the DβH (dopamine-beta hydroxylase) locus was observed among the mothers of autistic children when compared to a sex-matched Canadian control population (L^2=4.51, df=1, $p<0.05$) [Robinson, 1996]. The majority of mothers were homozygous for deletion alleles of a dinucleotide repeat polymorphism with a 19 bp (base pair) deletion/insertion located in the 5' untranslated region of the gene which significantly correlated with low DβH enzyme activity when compared to controls (F=5.45, df=59, $p<0.01$) [Robinson, 1996]. The presence of low activity levels of this enzyme, which catalyzes the conversion of dopamine (DA) to norepinephrine (NE) (Appendix A), would result in increased levels of dopamine (DA) and decreased production of norepinephrine (NE) and epinephrine (E) [Gary and Robertson, 1994]. DβH enzyme blockage *in vivo* causes increases in serotonin (5-HT) levels [Melmon, 1981]. Although DβH does not directly influence the synthesis of 5-HT, integrated regulation of these systems causes modulation of 5-HT levels through direct effects on DA, NE and E levels. Levels of DA, NE and 5HT are also commonly modulated by degradative actions of monoamine oxidase A (MAOA) (Appendix A and B). Maternal allele distributions of the MAOA CA dinucleotide repeat polymorphism and MAOA VNTR compared to a random sample of the Canadian population (Guthrie spots from newborns) were significantly different (χ^2=24.14, df=10, $p<0.0014$; χ^2=16.27, df=14, $p<0.0398$, respectively). The B2 allele of the MAOA VNTR was found far more often in mothers of two children affected with autism/PDD than would be expected based on control

frequencies (0.437 vs. 0.261). A 122 bp allele of the MAOA CA dinucleotide, found in the first intron of the MAOA gene, was also more frequent compared to controls (0.375 vs. 0.158). In fact, the B2-122 bp haplotype occurred significantly more often than would be expected from the observed frequencies of the respective alleles in the mothers (χ^2=44.3, p<<<0.001) [Schutz, 1998]. This provides evidence for the presence of linkage disequilibrium of these markers in the mothers. Correlations of this haplotype with MAOA enzyme activity variants have not been performed. The potential susceptibility alleles of DβH and MAOA were not transmitted with increased frequency to the autistic sibships, leading to the proposition of a maternal effect mechanism, such that altered MAOA and DβH enzyme levels in conjunction with fetal susceptibility factors could lead to modulation of important morphogenetic signals in embryological brain development predisposing individuals to autism/PDD [Schutz et al., 1998].

Normal embryological central nervous system (CNS) development is dependent upon morphogenetic signals, which trigger and modify the execution of intrinsic genetic programs in a concentration-, temporal- and spatial-dependent manner. Substances acting as morphogens direct processes including cell proliferation, migration, differentiation and cell death, as well as the process of tissue formation and growth outside of the central nervous system. Many studies have indicated an important functional role for biogenic amine neurotransmitter substances and their modulators as morphogens [Lauder and Krebs, 1978; Lauder and Krebs, 1984; Fiszman et al., 1991; Todd, 1992]. As a consequence of the concentration-dependent manner in which these morphogens stimulate

development, the potential for teratogenic effects of these substances is clear [Lauder, 1988]. Embryological neural development represents a paradoxical situation in which the differentiation of biogenic aminergic neurons requires exposure to the same neurotransmitter they differentiate to produce. Biogenic amines, in cooperation with growth factors, trigger the genetic induction of enzyme expression that characterizes the different neurons. Initiation of differentiation, therefore, requires that biogenic amines be synthesized extraneuronally. An alternative, and primary source for many mammalian embryos is neurotransmitters in maternal circulation [Burnikov, Shmukler and Lauder, 1996; Du and Iacovitti, 1996; Thomas, Matsumato and Palmiter, 1995; Zhou, Quaife and Palmiter, 1995]. The presence of biogenic amine transport proteins for serotonin and norepinephrine on the maternal-facing syncytiotrophoblast supports the importance for the transplacental transfer of biogenic amines to the developing embryo [Cool et al., 1991; Ramamoorthy et al., 1992; Ramamoorthy et al., 1993(a); (b); Lesch et al., 1994]. Orientation of the transporters in the membrane, to allow for vectorial transport in the maternofetal direction, also allows SERT and NET to clear biogenic amine substrates from the intervillous space, aiding in the prevention of premature vasoconstriction which can cause obstetric complications [Schroeter and Blakely, 1996]. Although the dopamine transporter is not expressed in placental tissue, NET and SERT show considerable substrate specificity and affinity for dopamine as well as their own ligands [Gordon and Olverman, 1978; Ramamoorthy et al., 1992; Faraj et al., 1994]. Early expression of transport proteins in neural and non-neural tissue during development would also contribute to accumulation of biogenic amines necessary in embryogenesis. DA, NE and

5-HT transporters ultimately function in the CNS to alter the availability of their respective neurotransmitters to post-synaptic neurons through re-uptake into the pre-synaptic neuron [Amara and Kuhar, 1993].

Direct indications for the importance of biogenic amine transporters in relation to autism/PDD are exemplified by studies of prenatal cocaine exposure. Autistic behaviors, developmental delay and delayed language acquisition have been observed in two studies of the effects of drug and alcohol exposure *in utero* [Davis et al., 1992; Harris et al., 1995]. Although cocaine was usually accompanied by the use of alcohol and other drugs, the absence of a high rate of autistic disorders among children exposed to alcohol and other drugs alone would suggest that this effect is specific to cocaine [Davis et al., 1992]. Ninety-four percent of drug-exposed children studied (N=70) expressed gross, fine and visual- motor disturbances, severe communication difficulties (failure to vocalize and language acquisition delay) and abnormalities of social interaction with primary caregivers and peers. Eleven percent of the children qualified for a diagnosis of autism under DSM-IIIR criteria for the disorder [Davis et al., 1992]. Binding and blockage of biogenic amine transport proteins in the placenta and/or developing fetal brain represents one of the principal teratogenic mechanisms of cocaine [Olsen, 1995]. SERT, NET and DAT1 represent the primary sites of pharmacological action of cocaine, as well as other psychostimulant drugs such as amphetamines and antidepressants [Ramamoorthy et al., 1995]. Inhibition of transport activity and intervillous clearance due to cocaine binding can have many effects on the amount of serotonin (5-HT), dopamine (DA) and norepinephrine (NE) [Ramamoorthy et al., 1995]. The neurotoxic effects of cocaine

binding could be related to dysregulated transfer of morphogenic substances from maternal circulation to the developing fetus and insufficient clearance of vasoconstrictive neurotransmitter substances from the intervillous space [Meyer, Shearman and Collins, 1996]. Maternal cocaine use is analogous to the proposed maternal effect model, in the way that both models induce fetal exposure to altered levels of 5-HT, DA and NE. It is necessary, however, to invoke fetal susceptibility, acting in conjunction with the maternal effect model, due to the insufficiency of the maternal effect model to account for some of the characteristics and features of the segregation patterns observed for autism/PDD. Consequently, the biogenic amine transporter genes were considered prime candidates for fetal susceptibility loci, due to their importance in fetal development, and later regulation of behavior through synaptic neurotransmission.

Investigations of markers for biogenic amine transporter genes have revealed statistically significant evidence for linkage. Concordance for alleles among sib pairs affected with autism/PDD was significantly increased for the dopamine transporter, DAT1 (t_2=1.20, $p<0.05$), the serotonin transporter, SERT (t_2=2.27, $p<0.05$) and norepinephrine transporter, NET (t_2=1.64, $p<0.05$). Examination of intragenic polymorphisms of the serotonin transporter revealed increased transmission of the 12 copy / long variant haplotype of the intronic VNTR locus (STin2) and functional promoter variant (5HTTLPR) to affected sib pairs (TDTχ^2=10.12, df=5, p=0.0019). This finding is in accordance with an earlier study of families with a single affected autistic child (N=65), showing biased allelic transmission and increased frequency of the higher transcriptional activity or long variant of 5HTTLPR (p=0.032) [Klauck et al., 1997]. Conversely, another

study of family trios (N=86) has revealed increased transmission of the low transcriptional activity variant of the serotonin transporter promoter region (TDTχ^2=4.69, df=1, p=0.030) to autistic individuals [Cook et al., 1996]. Differences with respect to the classification of autistic subjects in these studies could be pertinent to the cause of these inconsistencies.

Investigations of underlying brain abnormalities through post-mortem examination of autistic individuals support an embryological origin for autism/PDD. Characteristics of the lesions and structural abnormalities observed such as; increased neuron packing density in the limbic system, reduced numbers of Purkinje neurons and abnormalities of cranial nerve motor nuclei were consistent with abnormalities in the early neurodevelopmental program [Courchesne, 1991; Courchesne, 1997; Bauman, 1991; Rodier et al., 1996]. Cases linked to embryological exposure thalidomide more specifically placed this vulnerability window around the time of neural tube closure [Rodier et al., 1996].

The purpose of this study is to evaluate whether an interaction exists between maternal genotype at proposed maternal effect loci, DβH and MAOA, and affected sib pair allele sharing at proposed fetal susceptibility loci, SERT, NET and DAT1. This is examined by determining of the degree of interdependence between sibships concordant for chromosomal regions encompassing the transporter loci and maternal genotype at the DβH locus and MAOA locus. Based on a multifactorial / threshold mode of inheritance one might expect that an interaction between maternal and fetal genotype could be

reflected by clustering of sib pair concordance at transporter loci under maternal genotypes which may confer increased liability to autism/PDD. However, it is also reasonable to expect that more genetic liability be carried by the fetus in the absence of the maternal effect. This would be reflected by clustering of sib pair concordance for transporter loci under non-threatening maternal genotypes. The distribution of sib pairs with respect to maternal genotype was examined to test if clustering of transporter concordance among affected sib pairs was evident. If there is no association between fetal susceptibility conferred by the transporter loci and maternal effect, the number of affected sibs sharing two alleles identical by descent (IBD) should be evenly distributed among maternal genotypes for DβH and MAOA, as predicted by independent segregation.

METHODS

DβH and MAOA genotyping was performed by and as previously described by Paula Robinson and Chris Schutz [Robinson, 1996; Schutz, 1998]. Genotyping of markers for SERT, NET and DAT1 were performed as described in Chapter 1. Statistical analyses of observed affected sib pair allele concordance distributions in relation to maternal genotypes at DβH and MAOA was performed using the chi-square (χ^2) statistic. Distributions were compared to expected values based on independent segregation. Expected values were also calculated to compensate for the unequal maternal genotype frequencies.

RESULTS

Tables 1, 2 and 3 show the relationship between sibships concordant for alleles at SERT, NET and DAT1 markers and maternal genotype for the DβH locus. As previously stated, the DβH del/del genotype is strongly associated with reduced DβH enzyme activity levels [Robinson, 1996]. The distribution of sibship concordance for two alleles was compared to values predicted by independent segregation using the chi-square (χ^2) statistic. A non-significant excess of two alleles shared identical by descent for SERT was suggested in the context of the maternal del/del DβH genotype (χ^2=5.88, df=2, p= 0.053) indicating a trend towards an association between the two susceptibility factors. Although, the presence of an association between NET, DAT1 and DβH (NETχ^2=2.3, df=2, p= 0.317; DAT1χ^2=1.1, df=2, p= 0.577) was not observed, it is possible that larger sample sizes would reveal a correlation. Determining the relationship with maternal MAOA genotype entailed analysis of distribution of sibship concordance for two alleles under conditions of B2-122 bp haplotype presence or absence (Tables 4, 5 and 6). Again, sibship concordance for two alleles at transporter markers was compared to expected values based on independent segregation. No significant association was observed for any of the transporter loci when compared to the presence of the B2-122 bp haplotype of MAOA, found in increased frequency in mothers of multiple autistic offspring (SERTχ^2=2.47, df=1, p= 0.116; NETχ^2=0.10, df=1, p= 0.752; DAT1χ^2=0.06, df=1, p= 0.806). Interestingly, a trend towards increased allele sharing at SERT and DAT1 is

apparent for affected offspring of B2-122 bp haplotype carrying mothers, whereas allele sharing seems to be opposite for NET. This is also evident when looking at distributions under maternal DβH genotype. This phenomenon does not seem to be the product of an association between DβH and MAOA genotype in the mothers.

Table 1: Distribution of SERT Sib Pair Allele Sharing with respect to Maternal Genotype at DβH

SERT Loci (pooled)

Number of alleles shared IBD	Maternal Genotype at DβH Locus		
	+/+	del/+	del/del
0	2	2	1
1	7	11	3
2	1	5	9
2(expected)*	3.6	6.4	4.8

χ^2= 5.88, df=2, p= 0.053 (for 2 IBD distribution under maternal DβH genotype)

* expected values based on independent segregation with correction for proportion of mothers in each DβH genotype category

Table 2: Distribution of NET Sib Pair Allele Sharing with respect to Maternal Genotype at DβH

NET Loci (pooled)

Number of alleles shared IBD	Maternal Genotype at DβH Locus		
	+/+	del/+	del/del
0	2	1	1
1	4	9	9
2	4	6	1
2(expected)*	3.0	4.7	3.3

χ^2= 2.3, df=2, p= 0.317 (for 2 IBD distribution under maternal DβH genotype)

* expected values based on independent segregation with correction for proportion of mothers in each DβH genotype category

Table 3: Distribution of DAT1 Sib Pair Allele Sharing with respect to Maternal Genotype at DβH

DAT1 Loci (pooled)

Number of alleles shared IBD	Maternal Genotype at DβH Locus		
	+/+	del/+	del/del
0	2	5	2
1	5	6	3
2	3	5	6
2(expected)*	3.8	6.0	4.2

χ^2= 1.1, df=2, p= 0.577 (for 2 IBD distribution under maternal DβH genotype)

* expected values based on independent segregation with correction for proportion of mothers in each DβH genotype category

Table 4: Distribution of SERT Sib Pair Allele Sharing with respect to Maternal Genotype at MAOA

SERT Loci (pooled)

Number of alleles shared IBD	Maternal Genotype at MAOA Locus	
	No B2-122bp haplotype	B2-122bp haplotype
0	1	2
1	5	6
2	4	7
2(expected)*	4.4	6.6

χ^2= 2.47, df=1, p= 0.116 (for 2 IBD distribution under maternal MAOA genotype)
* expected values based on independent segregation with correction for proportion of mothers with and without MAOA B2-122 bp haplotype

Table 5: Distribution of NET Sib Pair Allele Sharing with respect to Maternal Genotype at MAOA

NET Loci (pooled)

Number of alleles shared IBD	Maternal Genotype at MAOA Locus	
	No B2-122bp haplotype	B2-122bp haplotype
0	1	0
1	4	10
2	4	1
2(expected)*	2.3	2.8

χ^2= 0.10, df=1, p= 0.752 (for 2 IBD distribution under maternal MAOA genotype)
* expected values based on independent segregation with correction for proportion of mothers with and without MAOA B2-122 bp haplotype

Table 6: Distribution of DAT1 Sib Pair Allele Sharing with respect to Maternal Genotype at MAOA

DAT1 Loci (pooled)

Number of alleles shared IBD	Maternal Genotype at MAOA Locus	
	No B2-122bp haplotype	B2-122bp haplotype
0	1	3
1	4	3
2	4	7
2(expected)*	4.5	6.5

χ^2= 0.060, df=1, p=0.806 (for 2 IBD distribution under maternal MAOA genotype)
* expected values based on independent segregation with correction for proportion of mothers with and without MAOA B2-122 bp haplotype

DISCUSSION

The suggestion of correlation between siblings concordant for alleles at SERT markers and maternal genotype at the DβH locus implies that genetic liability at the SERT locus may contribute to fetal vulnerability to autism/PDD by acting in conjunction with altered maternal DβH enzyme levels that produce higher dopamine and serotonin levels and decreased norepinephrine. It is difficult however, to eliminate an interaction between these fetal susceptibility loci and a maternal effect, based on these data, due to the absence of particular fetal susceptibility alleles for each of the transporter genes, the presence of multiple pathways which can cause alterations in biogenic amine availability and the temporal aspect of the maternal effect. Although increased affected sib pair concordance for regions encompassing the transporter loci indicates the presence of linkage, failure to find specific associated susceptibility alleles, which would confer increased fetal vulnerability to the disorder, required that the degree of dependence of fetal vulnerability to maternal genotype at MAOA and DβH be estimated using affected sib pair concordance. This requires one to assume that affected sib pair concordance for two alleles IBD directly translates into sharing of fetal susceptibility alleles. While this may be possible, it is also likely that some affected sib pairs discordant for alleles of markers located some distance away from the gene, may be concordant for susceptibility alleles due to recombination between the marker and the gene. Therefore, without specific fetal susceptibility alleles, interpretation of the distribution of affected sib pair concordance at transporter loci in relation to maternal genotype at MAOA and DβH remains highly

speculative. It is also possible that a maternal effect is present but not evident through examination of maternal DβH and MAOA genotypes. A number of factors could cause the same proposed effect conferred by maternal genotypes at DβH and MAOA. Alterations of other biosynthetic and degradative enzymes in the serotonergic and dopaminergic pathways would also cause altered biochemical profiles creating the stressful intrauterine environment increasing susceptibility of the fetus to autism/PDD. Alternatively, external factors such as maternal stress, drug abuse, poor nutrition and compromised health could also lead to alteration in biogenic amine concentrations [Weinstock, Fride and Hertzberg, 1988; Meyer, Shearman and Collins, 1996; Chen et al., 1992]. The important factor to consider is the window of vulnerability which has been suggested by the developmental time period most consistent with neurological abnormalities in autistic individuals [Bauman, 1991; Rodier et al., 1996; Courchesne, 1997]. The presence and timing of alternative sources of maternal effect could not be accounted for in this investigation; therefore, the present study can not eliminate the possibility of interaction between the fetal susceptibility loci and a maternal effect. In addition, it is difficult to establish a relationship between maternal and fetal vulnerability factors without knowing the exact molecular defect and the magnitude of etiological effect conferred by all of the loci. For example, the magnitude of transporter defect or the presence of a number of transporter defects carried by a fetus could boost genetic liability to a level that does not necessitate maternal effect.

Altered biochemical profiles could be mimicked by abnormalities in the expression, specificity or activity of any, or a combination, of the biogenic amine transporters, all of

which are linked to autism/PDD. Evidence for the involvement of SERT, NET and DAT1 in the etiology of autism has been supported by increased allele sharing at regions encompassing the transporter loci among affected sib pairs.

The multifactorial / threshold model of inheritance predicts sub-threshold genetic loading in first-degree relatives of autistic probands, and could also contribute to the pathogenesis of the disorder. The existence of higher rates of related neuropsychiatric disorders among relatives of autistic individuals [Smalley, McCracken and Tanquay, 1995] and the presence of altered allele, genotype and haplotype distributions for SERT, NET and DAT1 loci observed in the parental population when compared to population-based controls would suggest an increased liability for the disorder among relatives of affected individuals. Most striking is the discovery of hyperserotonemia (to a lesser extent) among parents and unaffected siblings of autistic children [Leventhal et al., 1990], signifying the presence of genetic liability among relatives, as well as being another potential factor for maternal effect. In addition, another major determinant in the regulation of extracellular biogenic amine levels is oxidative metabolism by MAOA. MAOA has been a target locus of pharmacological treatments and many research efforts to dissect the pathogenesis of numerous neuropsychiatric disorders. Findings of altered haplotype frequencies among mothers of sibships affected with autism/PDD has indicated that MAOA also represents a maternal effect locus [Schutz, 1998]. Although unproven, it is likely that the B2-122 bp haplotype found with greater frequency among the mothers is in linkage disequilibrium with gene sequence variants, which affect enzyme activity levels. Due to the uncertainty of phenotypic consequences at the MAOA locus, we currently can not forecast high or

low activity levels into the maternal effect model; however, it is likely that the effects of MAOA would amplify those proposed for the low DβH activity levels, in either a multiplicative or additive fashion.

The effects of gestational cocaine exposure (also linked to the etiology of autism) examined in rats revealed up-regulation of SERT and NET placental expression [McGrath et al., 1997; Padbury et al., 1997] resulting in an increase in all intrauterine and fetal biogenic amines [Padbury et al., 1997], presumably to compensate for cocaine transport blockage. Conversely, prolonged drug exposure and/or chronic intrauterine stress results in decreased biogenic amine transporter expression, subsequent effects of which include decreased clearance of DA, 5-HT and NE from the intervillous space, critical to maintenance of placental blood flow. Therefore, the biochemical profile and pathophysiological effects of cocaine exposure are dependent on the dose and timing of exposure. Likewise, the effects of altered maternal serum biogenic amines are concentration- and temporal-dependent, and their effects highly dependent on the degree of fetal vulnerability.

The maternal effect and fetal susceptibility loci found in association with autism/PDD could precipitate many different teratogenic biochemical profiles. Altered biogenic amine levels from maternal serum, confounded by abnormal transport of DA, NE and 5-HT at the placenta or abnormal placental transfer of these factors alone, could directly lead to changes in the neurodevelopmental processes including cell differentiation, synaptogenesis and cell death resulting in adverse behavioral outcomes. One also needs to consider the indirect and cascade effects evoked by altered biogenic amine exposure.

Biogenic amine transport blockage, due to gestational cocaine exposure in rats, has revealed effects on neuroendocrine systems, such as increased release of corticosteroids [McGrath et al., 1997], increasing the risk of further intrauterine stress. Insufficient clearance of vasoconstrictive biogenic amines from the intervillous space, resulting in decreased placental blood flow and fetal hypoxia, could also be a major factor contributing to embryological brain damage [Padbury et al., 1997].

A number of studies have suggested that the increased frequency of obstetric complications (OCs) in relation to autism/PDD signify a possible contributing environmental factor in the etiology of autism/PDD [Folstein and Rutter, 1977; Steffenberg et al., 1989]. However, most autistic individuals examined lack the lesions characteristic of those caused by perinatal insult; instead most abnormalities suggest an earlier embryological origin for the disorder [Bailey et al., 1995; Rodier et al., 1996; Courchesne, 1997]. It is conceivable that the higher incidence of obstetric complications is a consequence of fetal abnormalities rather than the cause [Goodman, 1988; Weinberger, 1995], especially considering the cascading effects of genetic predisposition discussed above. Several studies have cited the importance of maintaining proper biogenic amine regulation in the placenta. MAOA inhibitor-induced and genetically-induced hyperserotonemia result in pregnancy complications [Gujrati et al., 1996]. In fact, perfusion of the uterus with MAOA inhibitors, such as parglyine, has been used as a method to induce abortion [Koren, Pfeifer and Sulman, 1965; 1966]. Parglyine is effective because of the loss of biogenic amine regulation and thus protective effect conferred by MAOA expressed in the placenta and amniotic fluid.

Abnormalities in the levels of biogenic amines could be produced in a number of ways. Gestational cocaine exposure, fetal susceptibility at the serotonin, dopamine and norepinephrine transporters alone or in combination with each other or maternal hyperserotonemia, maternal DβH enzyme levels, and possibly MAOA activity could all result in altered biochemical profile. Additionally, these fetal susceptibility loci could act in conjunction with environmentally-induced alteration of biogenic amines in maternal serum during the critical developmental period proposed for autism/PDD. Dysregulation of exogenous and endogenous biogenic amines can result in embryological developmental abnormalities consistent with the behavioral and neurological disturbances seen in autism/PDD. Variable expressivity with respect to clinical presentation could be related to the degree of vulnerability or degree of alteration in the concentration of morphogens and variability of subsequent indirect effects on interdependent systems. Etiological heterogeneity could be explained by the presence of several divergent pathways that give rise to a common outcome. The ability for environmental factors to mimic intrauterine conditions conferred by altered maternal serum levels and/or fetal transporter loci are consistent with this hypothesis. The differences with respect to the frequency with which the different genders are affected with the disorder could be the effect of an unidentified X-linked susceptibility gene or the presence of an association with the MAOA gene (encoded on the X chromosome). Although an association with MAOA was found in the mothers of affected male sibships, an association between the affected sibships and MAOA was not apparent [Schutz, 1998]. Efforts to uncover this association may have been restricted due to the reduced power of the study in which it was examined [Schutz, 1998].

Alternatively, the increased vulnerability of male fetuses to prenatal insult may be an important factor in the predominance of a male affected population [Knobloch and Pasamanick, 1974; Folstein and Rutter, 1977]. Abnormalities of biochemical markers seen in the autistic population (such as hyperserotonemia and low plasma norepinephrine, epinephrine, DβH and MAOA levels [reviewed by Anderson, 1987]) are certainly consistent with the proposed anomalies at the genetic level.

The need for further investigation into the potential interaction of fetal and maternal vulnerability factors is clear. Possible fetal susceptibility alleles, representing inherent abnormalities of transporter proteins in the placenta and fetal brain, have yet to be characterized at the molecular level. Determination of the relationship between the MAOA B2-122 bp haplotype and MAOA activity levels are required for defining the exact function and degree of its association to autism/PDD. Although several aspects of this fetal susceptibility / maternal effect model have yet to be proven, it represents the most comprehensive and consistent theory for the etiology of autism/PDD offered to date.

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GENERAL SUMMARY AND CONCLUSIONS

Studies aimed at dissecting the complex genetic etiology of autism/PDD have, in the past, been relatively unsuccessful. Efforts to increase the power of the research design were made in this study to increase the power of detecting linkage by using the affected sib pair approach in combination with a candidate gene strategy. Markers for pathophysiologically and biochemically relevant candidate genes were used to analyze allele sharing among sib pairs concordant for a broader autistic phenotype. Increased allele sharing among sib pairs affected with autism/PDD for regions encompassing the biogenic amine transporter loci, SERT, NET and DAT1, indicate the presence of linkage. Failure to observe increased parental transmission of particular marker alleles to affected sib pairs precluded identification of susceptibility alleles using analysis by the transmission disequilibrium test (TDT). However, familial genetic loading predicted by the multifactorial / threshold model of inheritance proposed for autism/PDD (discussed in the General Introduction) is likely a primary factor obscuring the ability to identify the potential susceptibility alleles. Significant differences observed when parental allele frequencies where compared to population-based controls indicate possible susceptibility alleles among first-degree relatives of autistic children, however, without proof that these alleles are increased in frequency among the affected population they can not be labelled as such. Examination of intragenic polymorphisms of the serotonin transporter gene (SERT)

revealed significant haplotype and genotype frequency differences among mothers of two autistic children and controls. Investigations of the functional polymorphism (5HTTLPR) and intronic VNTR (STin2) of the SERT gene have found conflicting results regarding potential susceptibility alleles for autism [Cook et al., 1997; Klauck et al., 1997]. Increased transmission of the haplotype containing the 12 copy allele of STin2 and increased transcriptional activity variant of SERT to affected sib pairs, in accordance with findings of Klauck et al. [1997], suggests possible susceptibility alleles for autism, however, these results need to be replicated with a larger sample size due to the low number of informative families.

The presence of fetal susceptibility genes for autism/PDD aid in explaining the characteristic features of the disorder unaccounted for by the maternal effect model proposed [Schutz et al., 1998]. Additionally, the prevalence of affected sib pairs concordant for markers for the serotonin transporter gene with mothers carrying low DβH activity alleles suggests an interaction between the fetal susceptibility gene and maternal effect locus. The presence of an association between maternal DβH genotype was not seen for NET and DAT1, nor was an association observed for any of the transporter loci and maternal MAOA haplotype. However, an interaction can not be excluded due to inability to directly assess particular fetal susceptibility alleles in relation to maternal genotype.

A number of follow-up studies are prompted by this research. Increased concordance among affected sib pairs for maternal alleles at markers linked to the transporter loci, suggests the possibility of an imprinting mechanism active within regions encompassing the transporter loci. Although biallelic serotonin transporter expression has

been observed in the brain [Austin et al., 1994] this does not preclude tissue-specific imprinting of SERT, DAT1 and NET. Closer analysis of the transporter genes is also required to establish susceptibility alleles for autism/PDD.

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Appendix A: The Catecholamine (DA, NE and E) Synthesis and Metabolic Pathway

Tyrosine
↓ Tyrosine hydroxylase (TH)
L-dopa
↓ Dopa decarboxylase (aromatic amino acid decarboxylase)
Dopamine (DA) →(via MAOA, MAOB, COMT) →DOPAC, HVA
↓ Dopamine-β-hydroxylase
Norepinephrine (NE) →(via MAOA, MAOB, COMT) →DHPG, MHPG, VMA
↓ Phenylethanolamine N-methyltransferase (PNMT)
Epinephrine (E) → (via MAOA, MAOB, COMT) →DHPG, MHPG, VMA

Metabolic Enzymes

MAOA- monoamine oxidase A
MAOB- monoamine oxidase B
COMT- catechol-O-methyltransferase

Major Metabolites

DOPAC- dihydrophenylacetic acid
HVA- homovanillic acid

DHPG- dihydroxyphenylglycol sulfate
MHPG- methoxyhydroxyphenylglycol sulfate
VMA- vanillylmandelic acid

(Adapted from Goldstein DS, Lenders JWM, Kaler SG, Eisenhofer G (1996): Catecholamine Phenotyping: Clues to the Diagnosis, Treatment, and Pathophysiology of Neuropsychiatric Disorders. J. Neurochem. 67:1781-1790.)

Appendix B: The Serotonin (5-HT) Synthesis and Metabolic Pathway

Tryptophan
↓ Tryptophan hydroxylase (TpH)
5-hydroxytryptophan (5HTP)
↓ Aromatic amino acid decarboxylase
5-hydroxytryptamine (5-HT) →(via MAOA) →5–HIAA
(Serotonin)

Metabolic Enzyme

MAOA- monoamine oxidase A

Major Metabolite

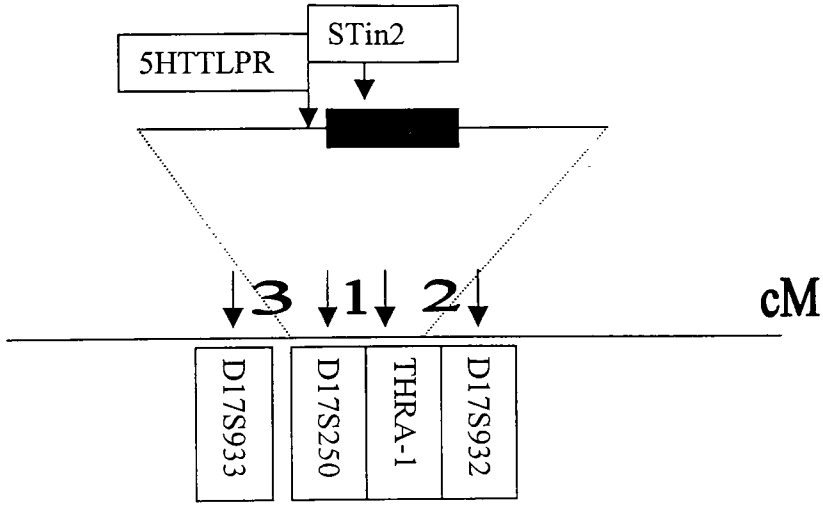
5-HIAA- 5-hydroxyindoleacetic acid

(Adapted from Gilman JT, Tuchman RF (1995): Autism and Associated Behavioral Disorders: Pharmacotheraputic Intervention. The Annals of Pharmacotherapy 29:47-56.)

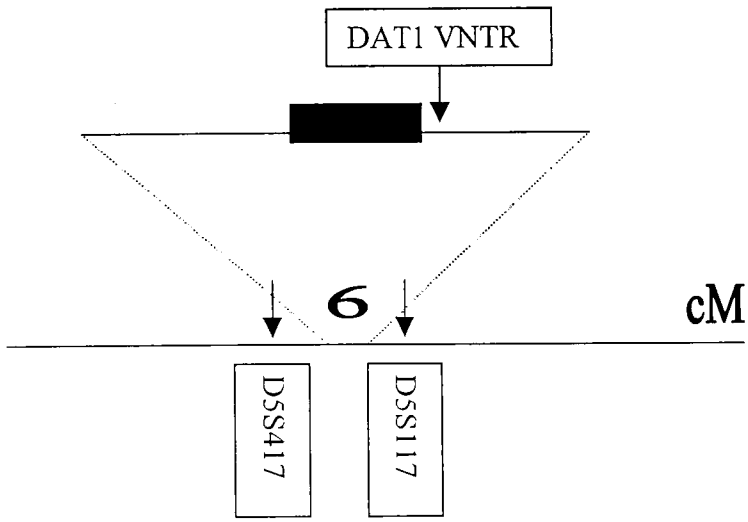
Appendix C: Order and Distance of Markers for Biogenic Amine Transporters; SERT, NET and DAT1.

Distances are given in centiMorgans (cM). Order and distance are estimated from several sources (see Chapter 1) including the Human Genome Database and NCBI Database. Some discrepancies with respect to the order of markers on chromosome 16 proximal to the norepinephrine transporter gene (NET) have been found in the literature. The order presented here is the most probable, based on recombination events observed between markers in the test population.

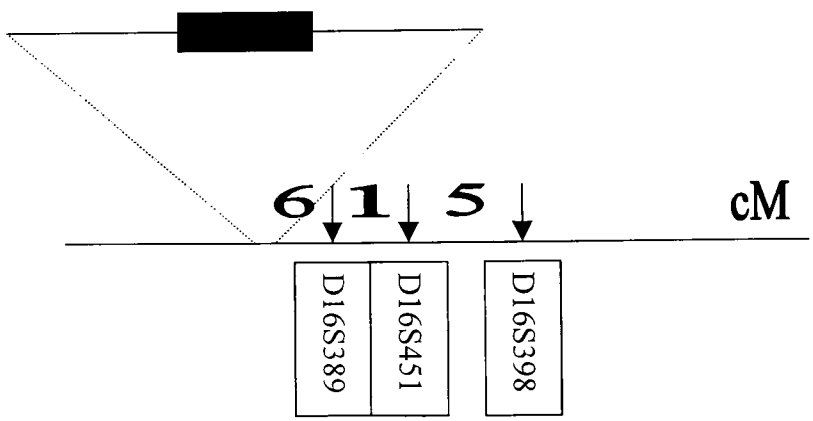
SERT – 17q11.2-q12



DAT1 – 5p15.3



NET – 16q12.2



Appendix D: Maternal and Paternal Concordance Relationships for Multiplex Sibships Affected with Autism/PDD.

C denotes identity by descent allele sharing (concordance) for affected sib pairs. D denotes discordance. NI indicates that genotype of the parent was non-informative for delineation of alleles. NA denotes cases in which a parent was not available for genotyping. Blanks indicate concordance could not be determined due to inability to genotype individuals critical for determination of concordance.

AUT FAMILY	D17S250		THRA-1		STin2		5HTTLPR		D17S932		D17S933		D16S389		D16S398		D16S451		DAT1 VNTR		D5S417		D5S117	
MALE MULTIPLEX	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL
1	NI	NA	NI	NA	D	NA	NI	NA	D	NA	D	NA	C	NA	C	NA	C	NA	NI	NA	C	NA	D	NA
2	NI	NA	C	NA	NI	NA	C	NA	C	NA	NI	NA		NA	C	NA	C	NA	NI	NA			C	NA
3	C	NI	C	D	NI	D	D	NI	NI	D	C	NI	NI	C	C	C	C	C	NI	C	C	C	C	NI
4	C	C	C	C	NI	C	D	NI	C	C	C	C	C	NI	C	D	C	D	NI	D	D	C	NI	D
5	NI	D	C	D	D	NI	D	NI	C	D	C	D	NI	C	NI	C	NI	C	D	NI	D	D	NI	NI
6	NI	NI	NI	NI	NI	NI	NI	NI	NI	D	NI	NI	C	C	C	C	C	C	NI	D	C	D	C	NI
7	D	NA	D	NA	NI	NA	NI	NA	NI	NA	NI	NA		NA	C	NA	NI	D	NI	NA	NI	NA	D	NA
8	C	NI	NI	NI	D	D	D	D	D	C	C	D	C	D	D	C	C	D	D	NI	NI	NI	NI	C
9	D	C	C	C	NI	C	NI	NI	D	NI	D	NI		NI	NI	D		NI	NI	C	C	C	C	NI
10	D	C	D	C	NI	C	NI	C	D	C	D	C	NI	NI	NI	NI	NI	NI	NI	D	C	C	C	C
11	NI	NI	C	C	C	NI	NI	NI	C	NI	C	NI	NI	NI	D	NI	NI	NI	NI	C	C	C	C	C
12	NI	D	C	NI	D	NI	D	D	NI	D	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	D	D	C	NI
13	C	NI	C	C	NI	C	C	NI	C	C	C	C	D	D	D	NI	D	NI	C	NI	C	C	C	NI
14(Different Fathers)	D		NI		NI		NI		D		D		NI		C		C						C	
15	C	D	C	D	NI	NA	NI	NI	NI	NA	C	NA	C	D	C	NA	NI	NA	NI	NA	D	D	D	D
16	NI	D	NI	C	NI	NI	NI	D	C	C	D	NA	C	D	C	D	NI	D	C	NI	NI	NI	C	C
17	D	NA	NI	NA	NI	NA	NI	NA	D	NA	D	NA	D	NA	D	NA	NI	NA	NI	NA	C	NA	NI	NA
18	D	C	NI	NI	C	NI	NI	C	D	C	C	C	NI	C	D	D	NI	C		C	C	NI	C	C
19	D	C	NI	C	NI	NI	NI	NI	D	C	D	C	C	NI	NI	D			NI	NI	C	C	D	C
20	C	C	C	C	NI	NI	NI	NI	C	C	C	C	D	C	D	C	D	NI	NI	NI	C	C	NI	C
21	C	D	D	NI	NI	NI	NI	NI	D	D	NI	D	D	C	D	D			C	NI	D	D	C	NI
22	C	C	C	C	NI	NI	C	NI	C	NI	C	C	D	C	D	C			D	D	NI	C	NI	NI
23	C	D	C	D	D	D	D	D	NI	NI	D	D	C	C	D	C	NI	C	C	NI	C	D	C	D
24	D	NI	D	NI	D	D	NI	D	D	C	NI	C	D	D	D	D	NI	NI	NI	NI	NI	D	C	D
25	C	C	C	C	NI	D	C	NI	C	C	C	D			NI	D	C	NI	NI	NI			NI	D
26	NI	NI	C	D	NI	NI	C	NI	C	D	C	D	C	C	NI	D	NI		NI	NI	C	D	C	C
27	D	D	D	D	D	NI			NI	NI	D	D	D	C	C	C			D	NI	C	D	D	NI
28	NI	NI	D	D					D	C	C	D			C	D			NI	C	C	NI	C	NI
29			C	D	NI	NI			D	D	C	NI	NI	C	D	NI			C	NI			C	C
30	C	NA	C	NA	NI	NA	NI	NA	NI	NA	NI	NA	NI	NA	NI	NA		NA	C	NI	NI	NA	C	NA
31	C	NI	C	C	NI	NI	C	NI	C	C	C	NI	NI	C	C	C	NI		NI	NI	D	D	C	D
32	C	C	C	C	NI	NI	NI	D			D	C	D	NI	C	NI	NI		NI	NI	C	NI	NI	C
33	D	D	D	D	C	NI	NI	D	D	D	C	D	C	D	C	C	C	NI	D	NI	D	D	D	D
34	D	NI	NI	NI	NI	NI	D	NI	NI	NI	NI	NI	C	C	C	D	NI	?	NI	D	D	D	D	D
35	C	D	NI	D	NI	NI	C	NI	C	D	D	D	NI	NI	NI	C	C	C	D	NI	D	NI	NI	C
36	D	NI	D	NI	NI	NI	NI	D	D	NI	D	C	NI	NI	C	D	NI	D	C	NI	C	C	D	NI
37	C	D	C	D	NI	D	NI	NI	C	D	C	D	D	D	NI	D	D	D	NI	C	C	C	D	C
	D17S250		THRA-1		STin2		5HTTLPR		D17S932		D17S933		D16S389		D16S398		D16S451		DAT1 VNTR		D5S417		D5S117	
	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL
38	NI	NI	C	D	NI	NI	NI	NI	NI	D	C	C	C	NI	NI	C			C	NI	C	C	NI	NI
39	NI	NI	C	C			NI	D			D	D	D	C	D	C	D	C	NI	NI	NI	NI		
40	D	C	C	NI	NI	NI	D	NI	NI	NI	D	D	NI		D	C			NI	C	NI	C	C	NI
41			D	C					NI	NI	NI	C	NI	C	C	NI	C	C			C	D	C	NI
42	NI	NI	D	C	NI	C	D	NI	D	NI	D	C	C	C	C	C			D	NI	C	C	D	D
43	D	D	D	D	NI	NI	NI	D	D	NI	NI	D	D	D	D	D			C	NI	C	C	C	C
44	C	C	C	NI	NI	NI	C	C	C	C	C	NI			C	D			C	NI			C	D
45	C	C	C	C	NI	NI	C	NI			NI	C	D	D	D	D			D	NI			D	D
46	NI	C	D	C	NI	NI							C	D	C	D					C	D		
47	D	D	D	C			NI	NI	D	D	D	D	D	C	D	C	NI	C	D	NI	D	D	NI	D
48	D	C	D	C	NI	NI	NI	NI	NI	NI	D	C	C	D	NI	D	C	D	D	NI	D	C	NI	C
49	NI	NI	C	D	NI	D	D	D	C	D	D	D	C	C	C	C	C	C	NI	NI	C	NI	C	NI

Appendix E: Transmission Disequilibrium Tables for all Multiallelic Markers Linked to and Within SERT, NET and DAT1.

For each locus maternal, paternal and combined parental transmitted and non-transmitted alleles were evaluated to detect the presence of allelic association. The basis of transmission disequilibrium testing (TDT) is the use of non-transmitted alleles (or in some cases haplotypes) as family-based controls, avoiding the potential of spurious associations which can occur if population-based controls are not well matched. This method of analysis requires that only alleles shared by affected siblings be counted, therefore, sample size is decreased for this reason. Summary of statistical evaluation is provided in Table 4.

MICROSATELLITE MARKER THRA-1

Transmitted Allele	Non-transmitted Allele															TOTAL
	160	162	164	168	170	172	174	176	178	180	182	184	186	188	196	
160																0
162			1		1	1	3									6
164																0
168																0
170							3	1	3							7
172		1			2											3
174						3		1	7	3						14
176					1		2									3
178					3		2	1								6
180					2		1		2				2			7
182																0
184																0
186																0
188																0
196																0
TOTAL	0	1	1	0	9	4	11	3	12	3	0	0	2	0	0	46

MICROSATELLITE MARKER THRA-1

Transmitted Allele	Non-transmitted Maternal Allele															TOTAL
	160	162	164	168	170	172	174	176	178	180	182	184	186	188	196	
160																0
162						1	3									4
164																0
168																0
170							2		2							4
172		1														1
174								1	4	2						7
176					1											1
178					2		2	1								5
180					1		1		2				1			5
182																0
184																0
186																0
188																0
196																0
TOTAL	0	1	0	0	4	1	8	2	8	2	0	0	1	0	0	27

MICROSATELLITE MARKER THRA-1

Transmitted Allele	Non-transmitted Paternal Allele															TOTAL
	160	162	164	168	170	172	174	176	178	180	182	184	186	188	196	
160																0
162			1		1											2
164																0
168																0
170							1	1	1							3
172					2											2
174						3			3	1						7
176							2									2
178					1											1
180					1								1			2
182																0
184																0
186																0
188																0
196																0
TOTAL	0	0	1	0	5	3	3	1	4	1	0	0	1	0	0	19

MICROSATELLITE MARKER D17S932

Transmitted Allele	Non-transmitted Allele									TOTAL
	189	191	193	195	197	199	201	203	205	
189										0
191			2		1	1				4
193		2			3	3				8
195						1				1
197	1		2				1*			4
199		1	1		2*		1	1		6
201					2	2				4
203						1				1
205						1				1
TOTAL	1	4	5	0	8	9	1	1	0	29

* greater than combined total of parents due to situation in which
both parents heterozygous for same alleles and pattern of transmission ambiguous
to which parent transmitted concordant/discordant allele

MICROSATELLITE MARKER D17S932

Transmitted Allele	Non-transmitted Maternal Allele									TOTAL
	189	191	193	195	197	199	201	203	205	
189										0
191			1			1				2
193		1			1	3				5
195						1				1
197	1		1							2
199		1	1							2
201					1					1
203						1				1
205										0
TOTAL	1	2	3	0	2	6	0	0	0	14

MICROSATELLITE MARKER D17S932

Transmitted Allele	Non-transmitted Paternal Allele									TOTAL
	189	191	193	195	197	199	201	203	205	
189										0
191			1		1					2
193		1			2					3
195										0
197			1							1
199							1	1		2
201					1	2				3
203										0
205						1				1
TOTAL	0	1	2	0	4	3	1	1	0	12

MICROSATELLITE MARKER D17S933

Transmitted Allele	Non-transmitted Allele										TOTAL
	188	190	192	194	196	198	200	202	204	206	
188											0
190			4*	5		2		1	2		14
192				1			1				2
194					1	1	1		1		4
196		1	1			1		1			4
198		1	2	2	1				1		7
200	1										1
202		1									1
204		1		1		1					3
206					1						1
TOTAL	1	4	7	9	3	5	2	2	4	0	37

* greater than combined total of parents due to situation in which both parents heterozygous for same alleles and pattern of transmission ambiguous to which parent transmitted concordant/discordant allele

MICROSATELLITE MARKER D17S933

Transmitted Allele	Non-transmitted Maternal Allele										TOTAL
	188	190	192	194	196	198	200	202	204	206	
188											0
190			1	1		1					3
192				1			1				2
194					1		1				2
196			1			1		1			3
198		1	2	2					1		6
200											0
202											0
204						1					1
206					1						1
TOTAL	0	1	4	4	2	3	2	1	1	0	18

MICROSATELLITE MARKER D17S933

Transmitted Allele	Non-transmitted Paternal Allele										TOTAL
	188	190	192	194	196	198	200	202	204	206	
188											0
190			1	4		1		1	2		9
192											0
194						1			1		2
196		1									1
198					1						1
200	1										1
202		1									1
204		1		1							2
206											0
TOTAL	1	3	1	5	1	2	0	1	3	0	17

MICROSATELLITE MARKER D17S250

Transmitted Allele	Non-transmitted Allele														TOTAL
	133	143	149	151	153	155	157	159	161	163	165	167	169	173	
133															0
143															0
149															0
151					1	2	1			2					6
153	1					4	1				1			1	8
155			1		2							1	1		5
157				1		2									3
159				2		1	1								4
161												1			1
163							1		1						2
165						1									1
167					1	1				1					3
169															0
173															0
TOTAL	1	0	1	3	4	11	4	0	1	3	1	2	1	1	33

MICROSATELLITE MARKER D17S250

Transmitted Allele	Non-transmitted Maternal Allele														TOTAL
	133	143	149	151	153	155	157	159	161	163	165	167	169	173	
133															0
143															0
149															0
151					1		1			1					3
153	1					2	1							1	5
155													1		1
157				1		1									2
159				1			1								2
161															0
163									1						1
165															0
167					1	1									2
169															0
173															0
TOTAL	1	0	0	2	2	4	3	0	1	1	0	0	1	1	16

MICROSATELLITE MARKER D17S250

Transmitted Allele	Non-transmitted Paternal Allele														TOTAL
	133	143	149	151	153	155	157	159	161	163	165	167	169	173	
133															0
143															0
149															0
151						2				1					3
153						2					1				3
155			1		2							1			4
157						1									1
159				1		1									2
161												1			1
163							1								1
165						1									1
167										1					1
169															0
173															0
TOTAL	0	0	1	1	2	7	1	0	0	2	1	2	0	0	17

MICROSATELLITE MARKER D16S451

Transmitted Allele	Non-transmitted Allele												TOTAL
	87	89	95	97	99	101	103	105	107	109	111	113	
87													0
89													0
95					1				1				2
97					1	1	2		1	2			7
99							1						1
101				4	1								5
103													0
105				2		1			2				5
107				3	1								4
109				1		1							2
111													0
113													0
TOTAL	0	0	0	10	4	3	3	0	4	2	0	0	26

* greater than combined total of moms and dads due to situation in which both parents heterozygous for same alleles and pattern of transmission ambiguous to which parent transmitted concordant/discordant allele

MICROSATELLITE MARKER D16S451

Transmitted Allele	Non-transmitted Maternal Allele												TOTAL
	87	89	95	97	99	101	103	105	107	109	111	113	
87													0
89													0
95													0
97						1	1			1			3
99													0
101				3									3
103													0
105				2					1				3
107				1									1
109				1									1
111													0
113													0
TOTAL	0	0	0	7	0	1	1	0	1	1	0	0	11

MICROSATELLITE MARKER D16S451

Transmitted Allele	Non-transmitted Paternal Allele												TOTAL
	87	89	95	97	99	101	103	105	107	109	111	113	
87													0
89													0
95					1				1				2
97					1		1			1			3
99							1						1
101				1	1								2
103													0
105						1			1				2
107				2	1								3
109						1							1
111													0
113													0
TOTAL	0	0	0	3	4	2	2	0	2	1	0	0	14

MICROSATELLITE MARKER D16S398

Transmitted Allele	Non-transmitted Allele												TOTAL
	178	180	182	184	186	188	190	192	194	196	198	200	
178													0
180													0
182					2	5	2						9
184					1								1
186			1			2		1					4
188			3				4	1					8
190			4	2		1		1		2			10
192			1	1		2	1						5
194			1	1									2
196							1						1
198													0
200													0
TOTAL	0	0	10	4	3	10	8	3	0	2	0	0	40

MICROSATELLITE MARKER D16S398

Transmitted Allele	Non-transmitted Maternal Allele												TOTAL
	178	180	182	184	186	188	190	192	194	196	198	200	
178													0
180													0
182						2	1						3
184					1								1
186			1			1		1					3
188			1				1						2
190			2			1		1		2			6
192				1		2							3
194			1	1									2
196							1						1
198													0
200													0
TOTAL	0	0	5	2	1	6	3	2	0	2	0	0	21

MICROSATELLITE MARKER D16S398

Transmitted Allele	Non-transmitted Paternal Allele												TOTAL
	178	180	182	184	186	188	190	192	194	196	198	200	
178													0
180													0
182					2	3	1						6
184													0
186						1							1
188			2				3	1					6
190			2	2									4
192			1				1						2
194													0
196													0
198													0
200													0
TOTAL	0	0	5	2	2	4	5	1	0	0	0	0	19

MICROSATELLITE MARKER D16S389

Transmitted Allele	Non-transmitted Allele														TOTAL
	96	98	100	102	104	106	108	110	112	114	116	118	120	122	
96															0
98															0
100				1											1
102			1		2	1						1	1		6
104				5		8		1		1		1			16
106					3*					1					4
108						1									1
110					1										1
112															0
114															0
116						2		1							3
118				1			1			1					3
120									1						1
122															0
TOTAL	0	0	1	7	6	12	1	2	1	3	0	2	1	0	36

* greater than combined total of moms and dads due to situation in which both parents heterozygous for same alleles and pattern of transmission ambiguous to which parent transmitted concordant/discordant allele

MICROSATELLITE MARKER D16S389

Transmitted Allele	Non-transmitted Maternal Allele														TOTAL
	96	98	100	102	104	106	108	110	112	114	116	118	120	122	
96															0
98															0
100															0
102			1		1							1	1		4
104				1		5		1		1					8
106										1					0
108						1									1
110					1										1
112															0
114															0
116						1									1
118															0
120															0
122															0
TOTAL	0	0	1	1	2	7	0	1	0	2	0	1	1	0	16

MICROSATELLITE MARKER D16S389

Transmitted Allele	Non-transmitted Paternal Allele														TOTAL
	96	98	100	102	104	106	108	110	112	114	116	118	120	122	
96															0
98															0
100				1											1
102					1	1									2
104				4		3						1			8
106					2										2
108															0
110															0
112															0
114															0
116						1		1							2
118				1			1			1					3
120									1						1
122															0
TOTAL	0	0	0	6	3	5	1	1	1	1	0	1	0	0	19

MICROSATELLITE MARKER D5S117

Transmitted Allele	Non-transmitted Allele												TOTAL
	145	147	149	151	153	155	157	159	161	163	165	201	
145				1									1
147													0
149				1				1					2
151	1		1		1	7	1	1	1		1		14
153				1		1							2
155				7	2								9
157				1	1	3							5
159				1									1
161								1					1
163													0
165													0
201						1							1
TOTAL	1	0	1	12	4	12	1	3	1	0	1	0	36

MICROSATELLITE MARKER D5S117

Transmitted Allele	Non-transmitted Maternal Allele												TOTAL
	145	147	149	151	153	155	157	159	161	163	165	201	
145				1									1
147													0
149				1									1
151					1	6		1	1				9
153				1		1							2
155				3	2								5
157					1	2							3
159				1									1
161													0
163													0
165													0
201						1							1
TOTAL	0	0	0	7	4	10	0	1	1	0	0	0	23

MICROSATELLITE MARKER D5S117

Transmitted Allele	Non-transmitted Paternal Allele												TOTAL
	145	147	149	151	153	155	157	159	161	163	165	201	
145													0
147													0
149								1					1
151	1		1			1	1				1		5
153													0
155				4									4
157				1		1							2
159													0
161								1					1
163													0
165													0
201													0
TOTAL	1	0	1	5	0	2	1	2	0	0	1	0	13

MICROSATELLITE MARKER D5S417

Transmitted Allele	Non-transmitted Allele										TOTAL
	92	94	96	98	100	102	104	106	108	110	
92											0
94							1				1
96											0
98		2	1		2	4	2		2		13
100				3			2	1			6
102					3		2				5
104				4	1	2		1			8
106				1		1					2
108								1			1
110											0
TOTAL	0	2	1	8	6	7	7	3	2	0	36

MICROSATELLITE MARKER D5S417

Transmitted Allele	Non-transmitted Maternal Allele										TOTAL
	92	94	96	98	100	102	104	106	108	110	
92											0
94							1				1
96											0
98		1	1			2	1		1		6
100				2			2	1			5
102					2		1				3
104				1	1	2					4
106				1							1
108								1			1
110											0
TOTAL	0	1	1	4	3	4	5	2	1	0	21

MICROSATELLITE MARKER D5S417

Transmitted Allele	Non-transmitted Paternal Allele										TOTAL
	92	94	96	98	100	102	104	106	108	110	
92											0
94											0
96											0
98		1			2	2	1		1		7
100				1							1
102					1		1				2
104				3				1			4
106						1					1
108											0
110											0
TOTAL	0	1	0	4	3	3	2	1	1	0	15

VNTR LOCUS STin2 (SERT INTRONIC VNTR)

Transmitted Allele	Non-transmitted Allele			TOTAL
	9	10	12	
9				0
10			7*	7
12	2	6*		8
TOTALS	2	6	7	15

* greater than combined total of parents due to situation in which both parents heterozygous for same alleles and pattern of transmission ambiguous to which parent transmitted concordant/discordant allele

VNTR LOCUS = STin2 (SERT INTRONIC VNTR)

Transmitted Allele	Non-transmitted Maternal Allele			TOTAL
	9	10	12	
9				0
10			2	2
12	2			2
TOTALS	2	0	2	4

VNTR LOCUS = STin2 (SERT INTRONIC VNTR)

Transmitted Allele	Non-transmitted Paternal Allele			TOTAL
	9	10	12	
9				0
10			1	1
12		4		4
TOTALS	0	4	1	5

VNTR LOCUS = DAT1 VNTR

Transmitted Allele	Non-transmitted Allele			TOTAL
	8	9	10	
8		1	1	2
9			6*	6
10		9		9
TOTAL	0	10	7	17

* greater than combined total of parents due to situation in which both parents heterozygous for same alleles and pattern of transmission ambiguous to which parent transmitted concordant/discordant allele

VNTR LOCUS = DAT1 VNTR

Transmitted Allele	Non-transmitted Maternal Allele			TOTAL
	8	9	10	
8		1		1
9			4	4
10		5		5
TOTAL	0	6	4	10

VNTR LOCUS = DAT1 VNTR

Transmitted Allele	Non-transmitted Paternal Allele			TOTAL
	8	9	10	
8			1	1
9			1	1
10		4		4
TOTAL	0	4	2	6

Appendix F: Parental, Canadian Control and Published Population Allele, Genotype and Haplotype Frequencies for Polymorphic Loci Linked To and Within SERT, DAT1 and NET.

5HTTLPR Genotype Frequencies

5HTTLPR Genotype	Female Controls	Male Controls	Male and Female Controls	Male and Female MPX Mothers	Male and Female MPX Fathers
s/s	0.22	0.20	0.20	0.13	0.15
l/s	0.63	0.53	0.56	0.60	0.54
l/l	0.16	0.26	0.23	0.27	0.32

5HTTLPR Allele Frequencies

5HTTLPR Allele	Female Controls	Male Controls	Male and Female Controls	Male and Female MPX Mothers	Male and Female MPX Fathers
s(406bp)	0.53	0.47	0.49	0.43	0.41
l(450bp)	0.47	0.53	0.51	0.57	0.59

STin2 Genotype Frequencies

STin2 Genotype	Female Controls	Male Controls	Male and Female Controls	Male and Female MPX Mothers	Male and Female MPX Fathers
9/10	0.02		0.001		0.02
9/12		0.01	0.001	0.04	0.02
10/10	0.08	0.07	0.08	0.18	0.12
10/12	0.78	0.41	0.54	0.43	0.55
12/12	0.12	0.49	0.36	0.35	0.29
11/12		0.01	0.001		

STin2 Allele Frequencies

STin2 Allele	Female Controls	Male Controls	Male and Female Controls	Male and Female MPX Mothers	Male and Female MPX Fathers
9(253bp)	0.01	0.01	0.01	0.02	0.02
10(270bp)	0.48	0.28	0.35	0.40	0.40
11(287bp)		0.01	0.00		
12(304bp)	0.51	0.71	0.64	0.58	0.57

STin2 / 5HTTLPR Haplotype Frequencies

STin2 / 5HTTLPR Haplotype	Female Controls	Male Controls	Male and Female Controls	Male and Female MPX Mothers	Male and Female MPX Fathers
9/10 s/s	0.02		0.01		
9/12 s/s				0.02	
10/10 s/s	0.02		0.01		0.03
10/12 s/s	0.12	0.09	0.10	0.06	0.03
12/12 s/s	0.06	0.12	0.10	0.06	0.10
9/10 l/s					0.03
9/12 l/s					0.03
10/10 l/s	0.04	0.03	0.03	0.13	0.03
10/12 l/s	0.53	0.20	0.32	0.26	0.30
12/12 l/s	0.06	0.29	0.21	0.17	0.15
11/12 l/s		0.01	0.01		
9/12 l/l		0.01	0.01	0.02	
10/10 l/l	0.02	0.04	0.03	0.06	0.08
10/12 l/l	0.14	0.13	0.13	0.11	0.23
12/12 l/l		0.09	0.06	0.09	0.03

D17S250 Allele Frequencies

D17S250 Allele	Female Controls	Male Controls	Male and Female Controls	Male and Female MPX Mothers	Male and Female MPX Fathers
133				0.01	
143	0.02	0.005	0.01		
149	0.03	0.01	0.02		0.01
151	0.12	0.11	0.12	0.11	0.07
153	0.27	0.28	0.28	0.38	0.38
155	0.13	0.19	0.16	0.09	0.23
157	0.15	0 14	0.14	0.18	0.12
159	0.07	0.03	0.05	0.03	0.03
161	0.06	0.06	0.06	0.04	0.05
163	0.07	0.06	0 06	0.07	0.05
165	0.04	0.02	0.03	0.01	0.02
167	0.05	0.07	0.06	0.04	0.03
169	0.01	0.01	0.01	0.02	
173				0.01	

THRA-1 Allele Frequencies

THRA-1 Allele	Female Controls	Male Controls	Male and Female Controls	Male and Female MPX Mothers	Male and Female MPX Fathers
160		0.003	0.002		0.01
162	0.03	0.04	0.03	0.08	0.06
164	0.01	0.01	0.01		
166	0.01		0.003		
168	0.01	0.01	0.01	0.01	
170	0.28	0.31	0.30	0.17	0.20
172	0.09	0.06	0.07	0.04	0.08
174	0.32	0.29	0.30	0.36	0.35
176	0.03	0.05	0.04	0.03	0.05
178	0.14	0.14	0.14	0.15	0.13
180	0.08	0.07	0.07	0.12	0.04
182	0.01	0.01	0.01	0.02	0.02
184		0.01	0.003	0.01	0.01
186		0.003	0.002		0.02
188					0.01
196					0.01

D17S932 Allele Frequencies

D17S932 Allele	Female Controls	Male and Female MPX Mothers	Male and Female MPX Fathers
189		0.01	
191	0.06	0.09	0.09
193	0.15	0.24	0.15
195	0.11	0.04	0.09
197	0.23	0.23	0.22
199	0.24	0.28	0.23
201	0.17	0.10	0.18
203	0.05	0.01	0.03
205			0.01

D17S933 Allele Frequencies

D17S933 Allele	Female Controls	Male Controls	Male and Female Controls	Male and Female MPX Mothers	Male and Female MPX Fathers
186		0.01	0.005		
188					0.01
190	0.25	0.26	0.25	0.19	0.36
192	0.12	0.11	0.12	0.16	0.07
194	0.29	0.33	0.31	0.27	0.26
196	0.05	0.04	0.04	0.07	0.02
198	0.11	0.13	0.12	0.17	0.11
200		0.05	0.03	0.04	0.01
202	0.07	0.02	0.04	0.02	0.02
204	0.12	0.06	0.08	0.06	0.12
206				0.01	0 01

D16S398 Allele Frequencies

D16S398 Allele	Female Controls	Male Controls	Male and Female Controls	Male and Female MPX Mothers	Male and Female MPX Fathers
178	0.01		0.003		
180	0.05	0.06	0.05		0.01
182	0.16	0.17	0.17	0.23	0.20
184	0.09	0.11	0.10	0.09	0.06
186	0.16	0.17	0.16	0.09	0.10
188	0.28	0.23	0.25	0.19	0.30
190	0.19	0.15	0.17	0.23	0.24
192	0.03	0.06	0.05	0.08	0.06
194	0.02	0.02	0.02	0.03	
196	0.02	0.03	0.02	0.03	0.02
198					
200		0.01	0.003	0.01	0.01

D16S451 Allele Frequencies

D16S451 Allele	Published Controls *	Male and Female MPX Mothers	Male and Female MPX Fathers
83	0.01		
87			0.03
89		0.01	0.03
95			0.04
97	0.21	0.33	0.23
99	0.09	0.15	0.14
101	0.13	0.08	0.13
103	0.03	0.08	0.06
105	0.24	0.13	0.14
107	0.16	0.14	0.16
109	0.08	0.09	0.04
111	0.03	0.01	
113	0.02		

*(Shen, Y. et al 1993)

D16S389 Allele Frequencies

D16S389 Allele	Published Controls *	Shifted Published Controls (+6bp)	Male and Female MPX Mothers	Male and Female MPX Fathers
90	0.02			
92	0.01			
96	0.22		0.01	
98	0.39	0.02	0.01	
100	0.14	0.01	0.01	0.01
102	0.03	0.22	0 13	0.20
104	0.01	0.39	0.39	0 38
106	0.08	0.14	0.25	0.10
108	0.06	0.03	0.02	0.01
110	0.04	0.01	0.03	0.04
112		0.08		0.01
114	0.01	0.06	0.07	0.06
116		0.04	0.03	0.04
118			0.03	0.11
120		0.01	0.01	0.03
122				0.01

* (Shen, Y.et al 1993)

D5S417 Allele Frequencies

D5S417 Allele	Female Controls	Male Controls	Male and Female Controls	Male and Female MPX Mothers	Male and Female MPX Fathers
92	0.01	0.01	0.01		
94	0.01	0.03	0.02	0.02	0.05
96	0.02	0.01	0.01	0.03	
98	0.53	0.45	0.48	0.36	0.45
100	0.11	0.15	0.14	0.20	0.14
102	0.07	0.09	0.08	0.10	0.08
104	0.20	0.18	0.19	0.20	0.19
106	0.05	0.08	0.07	0.06	0.07
108				0.03	0.03
110		0.003	0.002		

D5S117 Allele Frequencies

D5S117 Allele	Published Controls *	Shifted Published Controls (-2bp)	Male and Female MPX Mothers	Male and Female MPX Fathers
145		0.01	0.01	0.02
147	0.01		0.01	
149		0.02	0.01	0.02
151	0.02	0.33	0.43	0.48
153	0.33	0.05	0.13	0.08
155	0.05	0.45	0.29	0.26
157	0.45	0.08	0.06	0.06
159	0.08	0.01	0.03	0.02
161	0.01	0.05	0.02	0.04
163	0.05			
165				0.01
201			0.01	

*(Weber, J. et al 1990)

DAT1 VNTR Allele	Published Controls *	Male and Female MPX Mothers	Male and Female MPX Fathers
3(200bp)	0.01	0.01	
5(280bp)	0.01		
7(360bp)	0.01	0.02	
8(400bp)	0.02	0.01	0.01
9(440bp)	0.24	0.25	0.28
10(480bp)	0.70	0.72	0.71
11(520bp)	0.02		

*(Vandenbergh et al., 1992)

Appendix G: Calculation of Power, the Probability of Linkage Detection.

Power of Affected Sib Pair Analysis for Detecting Linkage of Disease Susceptibility Loci with Polymorphic Markers
Adapted from N. Risch (1991): Am J Hum Genet 46:229-241.
Power is defined as the probability of detecting linkage, if it exists, given parameters defined (ie. relative risk to sibs, PIC value of marker, recombination between marker and candidate gene, amount of genetic variance accounted for by candidate gene and sample size) assuming 100% penetrance.

Parameters to define:
λ_R = relative risk = recurrence risk to

$$\frac{\text{relative of affected sib}}{\text{risk to general population}}$$

λ_S = sib risk = 40-600 (depending on which epidemiological data is used)
α_{S0} = prior probability of zero maternal OR paternal alleles shared IBD (null expectation) = 0.5
Z_{S0} = probability of zero alleles shared IBD by two affected sibs = α_{S0} / λ_S
n_0 = number of 0 IBD pairs in sample size of N
Formulae used:
LOD score test for significance
$T = n_0 \log10 (n_0 / N\alpha_{S0}) + (N- n_0) \log10 [N- n_0 / (N- n_0) \alpha_{S0}]$
Where $T \geq 3$ (odds ratio 1000:1) is the threshold for significance.
Calculate W, the threshold value of n_0 for significance (1000:1 odds ratio), if $n_0 \leq W$, then $T \geq 3$.

Calculate P (power) probability of observing $n_0 \leq W$
$P = \Sigma^{W}_{no=0} [N!/n_0! (N- n_0)! (Z_{S0})^{no}(1 \quad Z_{S0})^{N-no}]$

(eg.) DAT1 data
N = 35, n_0 = 11, N- n_0 = 24

T = 11 log10 [11/35(0.5)] + 24log10 [24/35-35(0.5)]
T = -2.218+3.292
T = 1.074 (not significant)
Calculate W
N = 35, n_0 = 6, N- n_0 = 29
T = 6 log10 (6/17.5) + 29log10(29/17.5)
T = -2.789+6.361
T = 3.57
(for n_0 = 7, N- n_0 = 28, T=2.929)
Therefore, W = 6

Need to define parameters for calculation of P.
Under the assumptions of:
Zero recombination
Sib risk = 50 (very conservative)
Locus accounts for 100% genetic variance
100% penetrance
100% marker polymorphism
P= 100% if N=35

Under more realistic parameters:
Recombination fraction = $\theta = 0.02$
Locus accounts for 10% of genetic variance
[under additive model (approximates genetic heterogeneity, no mathematical model for multifactorial / threshold model)]
K = K1+K2....Kn
Where K = (prevalence), n = number loci involved in genetic effect

Calculate GENE SPECIFIC sib risk (compensates for reduced genetic effect).
Conservative gene specific sib risk = $\lambda_{GS(con)} = 50\ (0.01) = 0.5$
(based on 2% sib risk, 0.04% population prevalence)
Less conservative gene specific sib risk = $\lambda_{GS(lesscon)} = 150\ (0.01) = 1.5$
(based on 3% sib risk, 0.02% population prevalence)
Optimal gene specific sib risk = $\lambda_{GS(opt)} = 600\ (0.01) = 6$
(based on 6% sib risk, 0.01% population prevalence)

Compensate for recombination:

$$\lambda_{GS(con)'} = \lambda_{GS(con)} / 1\text{-}4\theta\ (1-\theta)\ (\lambda_{GS(con)}\text{-}1)$$
$$= 0.5/1\text{-}0.08(0.98)(-0.5) = 0.48$$

$$\lambda_{GS(lesscon)'} = \lambda_{GS(lesscon)} / 1\text{-}4\theta\ (1-\theta)\ (\lambda_{GS(lesscon)}\text{-}1)$$
$$= 1.44$$

$$\lambda_{GS(opt)'} = \lambda_{GS(opt)} / 1\text{-}4\theta\ (1-\theta)\ (\lambda_{GS(opt)}\text{-}1)$$
$$= 4.31$$

Calculate $Z_{S0} = \alpha_{S0} / \lambda_S$:
$Z_{S0} = \alpha_{S0} / \lambda_{GS(con)'} = 0.5/0.48 = 1.0$ (considered impossible to detect linkage)
$Z_{S0} = \alpha_{S0} / \lambda_{GS(lesscon)'} = 0.5/1.44 = 0.347$
$Z_{S0} = \alpha_{S0} / \lambda_{GS(opt)'} = 0.5/4.31 = 0.12$

$$P_{(lesscon)} = [35!/0!35!\ (0.347)^0\ (0.653)^{35}] + \ldots [35!/6!29!\ (0.347)^6\ (0.653)^{29}] = 0.018$$

Therefore, the study achieves1.8% power to detect linkage if it exists, given population size of 35 under the assumptions of 2% recombination, sib risk =150, locus accounting for 10% of total genetic variance, 100% penetrance.
Would require approx. 100 fully informative families (100% marker polymorphism) to achieve 80% power to exclude locus.

$P_{(opt)} = [35!/0!35!\ (0.12)^0\ (0.88)^{35}]+\ldots[35!/6!29!\ (0.12)^6\ (0.88)^{29}] = 0.881$
Therefore, the study achieves 88.1% power to detect linkage if it exists given population size of 35, under the assumptions of 2% recombination, sib risk = 600, locus accounting for 10% of genetic variance, 100% penetrance, 100% marker polymorphism.

In actuality, power to detect linkage exists somewhere in this range. Where depends on the most accurate estimate of sib risk.
Using less stringent statistical analyses provides the advantage of increased power to detect linkage because you are effectively decreasing the level of significance required.

To illustrate this point I have applied less stringent statistical analyses using parameters defined under the less conservative sib risk value.
Using a chi-square test or mean statistic, the nominal significance level is $p \leq 0.05$.
For N=35:
W= 11, the threshold value of n_0 for significance (if $n_0 \leq W$, then $p \leq 0.05$).
$P\ \chi^2 = [35!/0!35!\ (0.347)^0\ (0.653)^{35}]+\ldots[35!/6!29!\ (0.347)^{11}\ (0.653)^{24}]$
$P\ \chi^2 = 0.561$
A considerable increase in the probability that one will detect linkage if it exists.
And, the test is still within accepted levels of stringency.

Because a number of inferred parameter values were used in the calculation of power, it is likely that power values calculated here are inaccurate. This is unavoidable due to the inability to define and, thus, represent the mode of inheritance mathematically and/or quantify the total genetic variance accounted for by candidate genes studied. A value 10% genetic variance was adopted from estimates for quantitative trait loci (QTL) of weak to moderate effect (Plomin R, Owen M, McGuffin P (1994): The Genetic Basis of Complex Human Behaviors. Science 264:17331739).