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GENETIC ANALYSIS OF COMPLEX

NEURODEVELOPMENTAL DISORDERS:

A MODEL FOR THE GENETIC ETIOLOGY OF AUTISM AND THE RELATED PERVERSIVE DEVELOPMENTAL DISORDERS

AND

MAPPING OF A GENE RESPONSIBLE FOR X-LINKED MENTAL RETARDATION

By

CHRISTOPHER KEVIN SCHUTZ, B.Sc.

A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree

Doctor of Philosophy

McMaster University

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Abstract

The genetic factors underlying many of the major neuropsychiatric disorders have so far escaped elucidation, due to their complex segregation patterns and confounding influences such as heterogeneity and non-genetic influences. Approaches which are specifically suited to these types of problems have been developed, and are currently being applied to the genetic analysis of complex disorders. Two examples of such approaches are described here: the identification of genes involved in the etiology of autism and the pervasive developmental disorders (autism/PDD) using affected sib pair and allelic association methods, and the linkage analysis of X-linked mental retardation in an extended pedigree.

The excess of males in the autistic/PDD population suggests the involvement of a locus on the X chromosome in the etiology of this group of disorders. In thirty-two pairs of affected brothers (male multiplex sibships), no evidence of increased sharing of maternal alleles was found at any of 16 highly polymorphic loci distributed along the X chromosome. However, at loci in the monoamine oxidase A gene in Xp11.23-11.4, the mothers of these affected sib pairs showed decreased heterozygosity. A haplotype consisting of the B2 and 122 bp alleles at two polymorphisms in the gene was found to be significantly more frequent in mothers of male multiplex sibships than in controls ($\chi^2=16.22$, 14 df, $p<0.05$). As the affected siblings were not concordant for this haplotype more often than expected by chance, these data suggest that maternal genotype plays a role in the etiology of autism/PDD, i.e. that altered maternal MAOA activity results in the exposure of the fetus to abnormal monoamine neurotransmitter levels at a critical point in development, leading to autism/PDD.

In the same set of male multiplex sibships, evidence was found for increased concordance
near the serotonin transporter in 17q11.2-q21 ($\chi^2=7.11$, 1 df, p<0.01) and the dopamine transporter in 5p13.3 ($\chi^2=5.24$, 1 df, p<0.025). Concordance was significantly increased for maternal, but not paternal, alleles at these loci. No significant increase in concordance was found for a locus linked to the norepinephrine transporter gene in 16q12.2. These findings suggest roles for the serotonin and dopamine transporters in the etiology of autism/PDD in these families.

On the basis of these results in male multiplex families and other evidence from the literature, a model for the etiology of autism/PDD is proposed, in which the disorder arises from exposure of the fetus at a critical stage of development to abnormal monoamine neurotransmitter levels from maternal serum, due to a combination of maternal and fetal genotypes, and influenced by environmental factors. This model explains most of the unusual genetic features of autism/PDD.

Two-point linkage analysis was used to map the locus responsible for X-linked mental retardation with macrocephaly and seizures (MRX38) in a single family with five affected males, to Xp21.1-p22.13, with a peak lod score of 2.71 with no recombination. The region of localization is bounded by recombination events with DXS1226 distally and DXS1238 proximally, defining a genetic interval of approximately 14 cM. The MRX38 locus maps to a region overlapping several other MRX localizations, but appears to represent a novel X-linked mental retardation entity.
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in me have always been constants; and to my parents Herbert and Alice, whose trust and patience have been stretched to their limits but could not be broken, and whose love and support know no limits: to them I give my love in return, and dedicate this thesis.
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General Introduction

The advent of molecular genetic technologies and progress in the Human Genome Project have facilitated rapid advancements in our understanding of the genetic bases of many human disorders. Many disease genes have been identified in the absence of any knowledge regarding their biochemical functions, by virtue of positional cloning approaches which permit the mapping and eventual cloning of such genes on the basis of genetic linkage to polymorphic markers. Such approaches have been successful with a large number of disorders exhibiting monogenic inheritance patterns, including cystic fibrosis, Duchenne muscular dystrophy, Huntington disease, and Fragile X mental retardation. Identification of these genes and characterization of the molecular defects underlying their associated disease phenotypes has led to more accurate carrier identification and prenatal testing, and may well yet lead to more effective treatments.

As important as these advances in understanding monogenic disorders are, the majority of disorders affecting large proportions of the population are less amenable to simple linkage mapping methods. These include the major psychiatric disorders such as autism, schizophrenia and bipolar affective disorders as well as very common conditions such as mental retardation, hypertension, diabetes, cancer susceptibility and heart disease. As these disorders are much more prevalent and take a much higher toll with respect to public health than do the relatively rare monogenic diseases, understanding their etiologies represents a
major priority.

Although there is clear evidence for strong genetic influences in many complex disorders, these represent special problems for human geneticists. The complexity arises from a number of possible sources. These include: etiologic heterogeneity, in which the same disorder may arise from mutations in different genes in different families (genetic heterogeneity) or from non-genetic or environmental causes (phenocopies); clinical heterogeneity, in which phenotypic variation within and/or between families can complicate the establishment of clear and consistent inclusion criteria, possibly leading to mistyping of individuals and making comparisons across studies difficult; polygenic inheritance, in which interactions between genetic loci make it difficult to assess the significance of a single gene without consideration of the genetic background; and interactions between genetics and the environment, i.e. genes contributing to a susceptibility to a particular disorder, but which are insufficient to produce the disease phenotype in the absence of some environmental factor. For many common disorders, several of these sources appear to coexist, leading to very complex etiologies.

In the face of such complexity, traditional lod-score-based linkage analysis methods have generally not been successful. These methods rely on following the segregation pattern of alleles at a single genetic locus through large multigenerational families, to detect cosegregation with the disease phenotype; linkage information from different pedigrees in which the same disorder segregates may be combined to increase the power of the analysis. In most
complex disorders, such large multigenerational pedigrees are rare, and the
possibility of etiologic heterogeneity makes the combination of data from different families unwise in many cases. Thus, linkage analyses of complex disorders often lack the power required to detect linkage. Furthermore, the possibility of phenocopies, incomplete penetrance and mistyping of individuals can provide incorrect or contradictory information, resulting in false inclusion or exclusion of a genetic region in linkage studies, or in inconsistencies across studies. Given the failure of traditional lod-score-based linkage methods in mapping genes underlying or contributing to common complex disorders, other approaches have been designed which are generally more robust and therefore better suited to this type of gene mapping. These include affected relative pair methods and association studies. In affected relative pair methods, only affected individuals in a pedigree are considered, reducing the problem of mistyping relatives and eliminating the need for extensive pedigrees. Allelic association or linkage disequilibrium studies are population-based analyses of unrelated affected individuals; these reduce both the need for large families and the problem of misclassification. These methods have their own limitations, most notably the requirement for large sample sizes for adequate statistical power, but are generally more promising approaches for elucidating the genetic underpinnings of common complex disorders.

The methodological considerations required for linkage analysis of complex disorders are illustrated in this thesis through two examples of
neurodevelopmental disorders: firstly, the identification of genes involved in the etiology of autism and the related pervasive developmental disorders (PDD); and secondly, the mapping of X chromosome-linked mental retardation (XLMR) genes. Each of these studies presents its own problems with respect to experimental design. In the former case, while genetic factors clearly exert a strong influence on the etiology of autism/PDD, there is wide clinical variation, no clear inheritance pattern is apparent, and environmental influences appear to play a role in at least some families. Thus, efforts to map the genes contributing to the prevalence of autism/PDD must be able to deal with the potential presence of etiologic, genetic and clinical heterogeneity as well as polygenic inheritance. In this study, affected sib pair and allelic association methods are used to examine the roles of X-linked and autosomal candidate loci in autism/PDD, in order to develop a model for the etiology of this group of disorders. In the second example, an XLMR locus, segregating in a large family and characterized by macrocephaly and seizures, is mapped to Xp21.1-p22.13. While the segregation pattern of XLMR syndromes is usually clear, clinical heterogeneity within families as well as phenotypic overlap between distinct XLMR entities makes it difficult to combine linkage information from different families. Thus XLMR loci can be mapped only in single families large enough to be sufficiently informative. In both cases, however, appropriate study design makes it possible to increase our understanding of the genetic basis of complex disorders.
Chapter I:

Autism/PDD - Literature Review

Autism, first described by Kanner in 1943, is a developmental disability characterized by impairments in reciprocal social interaction, in imagination and in verbal and non-verbal communication, and by a pattern of repetitive, stereotypic and sometimes self-injurious activities. In addition to these core features, autistic individuals also commonly show specific deficits or delays in language, motor coordination and responses to sensory stimuli, as well as difficult behaviour and disturbances in eating, drinking and sleeping. Intelligence may range from severely retarded to above average, often with specific cognitive deficits. It is now recognized that classical autism as described by Kanner represents one end of a spectrum or continuum of conditions called the pervasive developmental disorders (PDDs), which share these clinical features but differ from each other in the number and severity of symptoms, patterns of behaviour and natural history (Szatmari 1992). While autism is considered the most severe form of PDD, the most recent edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV; APA 1994) also describes several other subtypes, including Asperger syndrome, atypical autism, autism not otherwise specified, Rett syndrome and disintegrative disorder of childhood. These are typically classifications of individuals with some autistic behaviours, but not enough to qualify for a diagnosis of classical autism.

Autism as defined by Kanner occurs in the population at a frequency of approximately one in 2500 live births. As the less severe forms of PDD are more common, the overall prevalence of autistic spectrum disorders is estimated at one in 200, making this group one of the
most frequent disorders of childhood (Ciaranello and Ciaranello 1995). While the sex ratio among affected individuals varies greatly among studies, more males than females are diagnosed with a form of PDD, with an average sex ratio of around four males to every female (Ciaranello and Ciaranello 1995).

Etiology of autism/PDD

The etiology of autism and the related pervasive developmental disorders (autism/PDD) is not understood. Early notions that autism arose due to abnormal child-rearing or lack of affection from parents have long been discounted, as siblings of autistic children have a relatively low risk of being affected themselves, and autism/PDD occurs with the same frequency in all social strata. Instead, research into the etiology of autism/PDD has focused on biological causes for the disorder.

Autism is known to be associated with a number of pre- and perinatal conditions causing brain damage. These include maternal rubella, untreated phenylketonuria, anoxia at birth, viral infection and trauma of the brain (Garrow et al. 1984). In addition, autistic-like symptoms are frequently found in children exposed prenatally to drugs such as alcohol and cocaine (Harris et al. 1995). Thus it seems likely that the disorder is organic in nature, resulting from altered or impaired brain development.

These pre- and perinatal environmental influences do not appear to be the only causes of autism/PDD, as such factors cannot be identified as causative in most patients. Rather, there is considerable evidence for familial aggregation in autism. The risk to siblings of autistic probands is estimated to be between 2% and 8%, or between 50 and 200 times higher than the
general population rate (Smalley et al. 1988, Ritvo et al. 1989), indicating high familiality. It has also been shown that other forms of PDD run in the families of autistic children. For example, the co-twins of MZ autistic probands can have atypical autism or Asperger syndrome (Folstein & Rutter 1977, Le Couteur et al. 1992), and the risk of Asperger syndrome and atypical autism in the non-twin siblings of autistic probands is 2.8% (Bolton et al. 1994). Thus, the overall risk of both autism and other PDD types to siblings of PDD probands is 5%-6% (Szatmari and Mahoney 1993). Another type of impairment that has recently been reported to occur in first degree relatives is a "minor variant" of PDD characterized by similar impairments in reciprocal social interaction, communication and interests but not enough to merit a diagnosis of PDD. For example, Bolton et al. (1994) found that roughly 20% of siblings of autistic probands had social or communication impairments or a restricted pattern of interests compared to 3% of siblings of Down syndrome controls (p<0.001). These studies taken as a whole indicate that what is heritable is not only autism, but a susceptibility to some form of PDD. Other, more distant relatives are also more likely to be affected, particularly when milder forms of PDD are included. While some of this familiality may be attributable to common environmental influences, there is considerable evidence for the contribution of genetic factors to the incidence of autism/PDD. Four twin studies have reported that the concordance rate for autism is much higher among monozygotic (MZ) twins than among dizygotic (DZ) twins (Folstein and Rutter 1977, Smalley et al. 1988, Silliman et al. 1989, Rutter et al. 1990). As such differences in concordance between MZ and DZ twins are taken as evidence of genetic factors in a disorder, these studies argue for a genetic influence in autism. Furthermore, autistic-like characteristics have been shown to be associated with a number of known genetic disorders, including tuberous sclerosis,
neurofibromatosis, phenylketonuria, fragile X syndrome, and others (Ritvo et al. 1989). It is unclear whether these associations reflect a role for these genes or for closely linked loci in the normal etiology of autism, or whether autistic-like behaviours are common sequelae of these other disorders. In any case, genetic influences clearly play a role in the etiology of autism/PDD in at least some families.

Genetic analysis of autism/PDD

Given the high heritability of autism/PDD, one would expect it to be possible to isolate major gene effects. However, the low risk to sibs, the low concordance rates in MZ twins and the unusual sex ratio are incompatible with any simple Mendelian pattern of inheritance, i.e. autosomal recessive, dominant, or X-linked modes. Furthermore, variable expressivity within families generally argues against a monogenic pattern of inheritance, although exceptions do exist, e.g. tuberous sclerosis and neurofibromatosis type 1. Thus autism and PDD are probably not the results of defects in a single gene, but rather the etiology of the autistic spectrum disorders is likely complex. Such complexity could arise in several ways, and so a number of general models for the etiology of autism/PDD need to be considered, including mixed, oligogenic, and polygenic models (Smalley et al. 1988, Jorde et al. 1991).

Mixed models suggest that a number of autism/PDD genes are segregating in the population, each of which is sufficient to cause a form of autism/PDD. That is to say genetic heterogeneity exists, with different autism/PDD genes segregating in different families. Simple mixed models involving a small number of autism/PDD genes are inadequate, as they could explain the unusual sex ratio, for example, but cannot explain the segregation patterns or clinical
variability observed in many individual families, or the low concordance rate in MZ twins. In contrast, oligogenic and polygenic models invoke the interaction of several genes to contribute to an autistic/PDD phenotype, through the additive or multiplicative effects of alleles at each locus. The cumulative effect may be required to exceed some level in order to result in an affected individual (threshold model); alternatively autism/PDD can be viewed as a quantitative trait or continuum on which different disease classifications are essentially arbitrary definitions (continuous trait model). The latter model allows for phenotypic variation among relatives within a pedigree, and for milder manifestations of PDD in some individuals which do not meet diagnostic criteria. It should be noted that mixed and polygenic models are not mutually exclusive: one could imagine autism segregating as a single-gene disorder in some families, and as a continuous trait in others. Clearly, given the association of autism with a number of disorders of known genetic etiology as noted above, some genetic heterogeneity must exist within the autistic population. What is unclear is whether genetic heterogeneity exists among the idiopathic cases of autism/PDD, i.e. those cases in which no associated disorder is evident.

Given this complexity, it is perhaps not surprising that the linkage studies carried out to date have proven unsuccessful at identifying genes or genetic regions linked to autism (Spence et al. 1985, Jorde et al. 1991). Lod-score-based linkage methods are generally ill-suited for mapping genes in the presence of significant heterogeneity or low penetrance. Furthermore, the inclusion criteria used were narrow definitions of autism: the possibility exists that relatives with very mild PDD might have gone undiagnosed and therefore been typed as unaffected, leading to false results in these studies.

More recent investigations have therefore concentrated on three ways of improving the
power to detect genes of major effect: broader inclusion criteria, in order to obtain as much
information from families as possible; definition of relevant subgroups, as suggested above; and
the use of more suitable and robust analytical tools and experimental methods.

Association studies, in which allele frequencies at a particular locus are examined in a
large group of affected individuals, are one alternative. As association studies only use
individuals with a diagnosis of autism/PDD, the risks of incorrect classification are minimized;
however, the choice of inclusion criteria remains an issue with regard to heterogeneity. In fact,
etiologic heterogeneity is likely to be increased in case-control association studies compared to
linkage studies, as sporadic cases are more likely to be included. Another problem posed by
association studies is the need for a well-matched control sample for comparison of allele
frequencies. Differences in ethnic composition and background between cases and controls can
account for variations in allele frequencies between the two populations. It is therefore important
to test several different control groups, in order to determine whether such stratification exists.
The haplotype relative risk method, in which the parental alleles not transmitted to the affected
individual are used to determine control frequencies, has been proposed as an alternative (Falk
and Rubinstein 1987, Terwilliger and Ott 1992). This method, however, requires analysis of the
parental genotypes as well as those of patients, and may not be applicable to all study designs.
The most important shortcoming of association studies is their inefficiency in genomic scans.
Association studies are essentially tests of linkage disequilibrium between the test locus and the
disorder (Hodge 1994). As linkage disequilibrium falls off very rapidly with increasing genetic
distance, association studies are typically very poor at detecting loci linked at some distance from
the test locus, i.e. greater than 1-2 cM. Scans of whole chromosomes, or of the entire human genome, using tests of association would require very dense genetic maps with an extremely high number of polymorphic test loci. While such maps and markers are becoming available as a result of efforts to map the human genome, such scans remain highly inefficient, especially given the number of individuals required for such studies. Thus, association studies are primarily warranted for the analysis of specific candidate loci, i.e. genes suspected to have some involvement in the disorder on the basis of their biochemical roles, proximities to chromosome abnormalities associated with the disorder or demonstrated roles in other, similar disorders.

Another method for detecting loci contributing to complex disorders is the affected sib pair (ASP) method (Risch 1990). In this method, the genotypes of pairs of affected sibs are compared to determine concordance for the inheritance of parental alleles. Assuming a completely heterozygous test locus, a sib pair may be concordant for both a paternal and a maternal allele, concordant for one parental allele, or discordant for both parental alleles. Decreased marker heterozygosity results in some parents being homozygous, or in parents sharing alleles, and the mating being uninformative for that test locus. In the absence of linkage between the test locus and the disorder, we expect a ratio of 1:2:1 of the above concordance states from Mendelian independent segregation, whereas a significant increase in concordance in pairs of affected sibs is indicative of linkage. Two distinct measures of concordance can be used, depending on the availability of parental genotypes: if the parents' genotypes are known, the identity-by-descent (IBD) of shared alleles in the affected sib pair may be established; in the absence of parental genotype information, identity-by-state (IBS) is used, in which instance the
observation of shared alleles in the affected sibs is weighted by the probability of the parents being heterozygous for those alleles, i.e. by the allele frequencies. Thus IBS methods, in addition to being vulnerable to errors in specification of allele frequencies, tend to be less powerful than corresponding IBD approaches, and it is preferable to obtain genotypes from parents as well as affected sib pairs where possible.

Similar methods using more distantly related affected individuals, so-called affected pedigree member or APM methods, have also been used in the analysis of complex disorders (Lange 1986, Weeks and Lange 1988). In theory, these are more powerful than ASP approaches, since more distant relatives are less likely to share alleles by chance; however, such relative pairs may be rare, and genotyping of intervening relatives in the pedigree is necessary in order to establish identity-by-descent. If these genotypes are unavailable, the researcher is forced to use IBS statistics, and the power of the analysis is compromised to some degree. The ASP method has therefore proven to be the most popular of these approaches in mapping genes involved in complex disorders. Affected relative pair methods, and particularly ASP methods, have been used successfully in recent years to identify susceptibility loci in diabetes (Field et al. 1994, Luo et al. 1995) and multiple sclerosis (Ebers et al. 1996, Haines et al. 1996, Sawcer et al. 1996), as well as in the somewhat controversial mapping of a locus influencing male sexual orientation (Hamer et al. 1993).

Several considerations are important in the design of ASP studies, in order to maximize the statistical power of the approach. The first, clearly, is sample size: the greater the number of sib pairs examined, the greater is the theoretical power which can be achieved. The second issue is marker heterozygosity, or the polymorphic information content (PIC) of the markers used.
Low heterozygosity of a locus results in more homozygous parents and uninformative matings, lowering the effective sample size and therefore the statistical power to detect linkage at that locus. Markers with high heterozygosities (>50%), most commonly microsatellite or short tandem repeat (STR) based loci, are therefore preferred in sib pair studies. These are tracts of repeats of simple sequences, generally di-, tri- or tetranucleotides, which are highly polymorphic, ubiquitous throughout the genome and readily scored using the polymerase chain reaction (PCR) with primers designed from unique flanking sequences (Weber and May 1989).

The third major consideration in the design of ASP studies is the number of test loci used. Concordance rates for a test locus fall off toward expected values with increasing genetic distance between the test locus and a disease gene, as a result of recombination. Thus a test locus too far away from a disease gene may lack the power to detect the effect of that gene in a sample of a given size. Using a series of linked test loci evenly spaced along the genetic map of the chromosome increases the likelihood of one or more test markers showing increased concordance due to linkage to the disease gene. The number of markers required therefore depends on the number of chromosomes examined, the genetic length of each chromosome, and the desired spacing between markers, or map density. It is generally considered that a map density of 5-10cM intervals between test loci is adequate for most studies (Brown et al. 1994). The continuing progress in the mapping of the human genome, has led to map densities approaching 1cM becoming available for many chromosome regions. The advantages in power offered by using such a dense map are however offset both by logistical considerations of time and cost and by the statistical issue raised by multiple testing of the same sample. Any level of significance selected for an analysis predicts a type I error rate (e.g. 5% significance level
suggests one false positive result in twenty trials). Testing of a large number of loci will therefore result in some proportion of these loci demonstrating increased concordance by chance, rather than due to linkage to a disease gene. This problem is typically dealt with by dividing the significance level by the number of tests performed (Bonferroni correction; Holm 1979). The result of this correction is that a much larger deviation from expected concordance rates must be observed at any locus in order to be deemed significant. In most cases, this loss of power more than offsets any advantage of a more dense genetic screen. The problem of multiple testing may also be circumvented through the use of multipoint ASP methods, which examine the entire data set at once (Goldgar 1990), but in general it is preferable to perform an initial scan using a coarser map; if a locus of interest is detected, its position can be subsequently refined using a more dense map of the candidate region.

The final issue affecting the power of an ASP study design is the degree to which a putative disease locus contributes to the overall prevalence of the disorder in the sample population, also referred to as the effect size of the disease locus. Risch (1990) has demonstrated that this effect size can be expressed in terms of the sibling relative risk ($\lambda_s$), the proportion of the increased risk to a sibling of an affected individual which can be attributed to that locus. Effect sizes are not quantities whose real values can be determined in the absence of a complete etiological model, however, but rather theoretical variables which can be used as experimental parameters in statistical tests. It is also important to note that these quantities, effect size and $\lambda_s$, are not necessarily identical to their corresponding values in the general population, as pairs of affected sibs are a subset of the affected population which has been selected with bias.

Effect size can be viewed as an indication of the complexity of a genetic model, as it may
reflect heterogeneity in the sample (i.e. the number of families in which a specific disease locus is involved) or polygenic inheritance (i.e. the degree to which a specific disease locus contributes toward a threshold for diagnosis), or both. Thus, the real effect size in a sample of sib pairs is highly dependent on the composition of that sample, which is why it is difficult to compare across ASP studies or to extend results to the general population. Nevertheless, the smaller the effect size of a specific disease locus in a sample, the greater is the number of families required in that sample in order to achieve sufficient power to detect that locus. By the same argument, a much larger sample is required in order to statistically exclude a genetic region, since a contributing locus could exist in that region whose effect size is too small to be detected with a smaller sample. For this reason, exclusions are more properly expressed in the context of a minimum effect size.

Once all these concerns regarding statistical power are addressed, the ASP method is the most effective method for detecting loci contributing to complex disorders, as it offers several advantages. It is more robust to misclassification than linkage methods in extended pedigrees, since only affected individuals are included in the analysis, although inclusion criteria remain an issue; it requires only nuclear families; and most importantly, unlike lod-score based segregation analysis, which is very vulnerable to model misspecification, the ASP method does not require specification of a genetic model (Risch 1990). The ASP approach simply tests for linkage of the test locus to a disease gene, without assumptions regarding other factors. Further, it is less vulnerable to recombination between the test marker and the disease locus, and is therefore far better suited to chromosome and genome scans than association studies. The major weaknesses of the ASP method are the need for a large number of sibships, the reliance on highly
polymorphic test loci, and the vulnerability to etiologic heterogeneity and sampling strategies. In addition, it is important to bear in mind that affected sib pairs represent a selected subset of the affected population, and that in the face of heterogeneity results obtained from these sibships may not be transferable to the whole.

Any approach used to identify genes involved in the etiology of autism/PDD would benefit from reducing heterogeneity in the population under study, as mixed etiologies in the sample reduce the power of any type of analysis. The problem of heterogeneity may be addressed in two different ways. The first is to reduce etiologic heterogeneity by limiting inclusion criteria for families, for example by excluding apparent sporadic cases associated with pre- or perinatal factors or chromosome abnormalities, or by selecting only multiply affected families for study. Similarly, sampling only families consistent with a particular mode of transmission, e.g. X-linked recessive transmission, may enrich the study population for a particular etiology. The sampling bias in these types of strategies means care must be taken in the interpretation of any findings with respect to the overall prevalence of the disorder. The second approach is to reduce clinical heterogeneity by defining subgroups in the affected population on the basis of severity, clinical features or biochemical observations. The underlying assumption here is that differences in clinical presentation would reflect distinct etiological mechanisms. Given the reduced clinical concordance observed in siblings and in dizygotic and monozygotic twins in autism/PDD (Ciaranello and Ciaranello 1995), however, it does not seem as though subgroups defined on the basis of severity or the presence or absence of specific behaviours are relevant. A strategy which included a severely affected individual with classical
autism but did not include his sibling with a milder form of PDD would not seem to represent the most effective use of the genetic information available in that family.

Neurophysiological and Neurochemical findings in autism/PDD

Biochemical and neurophysiological observations in autistic/PDD subjects might be expected to prove more consistent within sibships and across families and studies, if they reflect the underlying causal mechanisms, but in fact few consistent findings in these areas have been made. Studies of the neuropathophysiology of autism have focused on the brainstem, limbic and forebrain structures, cerebellum and cerebral cortex, as these regions are involved in the normal behaviours which are impaired in autistic individuals (Courchesne 1997). Little evidence has been found to implicate the brainstem, limbic or forebrain regions. Autopsy studies on six autistic subjects found the loss of granule and Purkinje cells from the posterolateral neocerebellum and archicerebellar cortex, accompanied by abnormally large neurons in the deep cerebellar and inferior olivary nuclei, suggesting an immature neuronal phenotype (Bauman and Kemper 1985). The authors proposed that the development of cerebellar circuitry was disrupted by the early loss of Purkinje cells, resulting in the persistence of a nonfunctional fetal neuronal pattern which is not replaced by the normal adult circuitry pattern of the cerebellum (Bauman and Kemper 1989). No consistent findings of cerebellar size abnormalities have been made (Rumsey et al. 1988, Courchesne et al. 1988, Piven et al. 1990). The cerebral cortex has been examined in autism due to its role in information processing, cognition, language and learning, but few consistent differences have been found between brains of autistic subjects and controls. Piven et al. (1990) found malformations of the gyrus in 7 of 13 autistic subjects. These are thought to
arise from defects in neuronal migration during the first six months of gestation. Hashimoto et al. (1988) found evidence for a reversed cortical asymmetry in autistic and mentally retarded subjects compared to controls. Consistent abnormalities in cortical metabolism have not been observed (Rumsey et al. 1985, DeVolder et al. 1987, Siegel et al. 1992).

It is possible that heterogeneity exists even at the neuropathophysiological level, or that structural differences underlying the autistic phenotype are too subtle to have been detected as yet; whatever the reason, few consistent structural changes have been identified. Given the complexities and number of genes and trophic factors involved in neural development, it is unlikely that the observation of specific structural abnormalities would lead to the identification of causal genetic mechanisms in any case. Rather, dissection of the genetic pathways contributing to the neuropathology of autism/PDD is more likely to result from the identification of specific biochemical defects. Thus, subgrouping of patients on the basis of biochemical abnormalities may be a more fruitful approach. Most such studies have focused on neurotransmitters, their metabolites, and hormones, particularly the monoamine transmitters serotonin (5-HT), dopamine (DA) and norepinephrine (NE). Platelet 5-HT levels have consistently been found to be elevated in autistic individuals vs. controls (Schain and Freedman 1961, Anderson et al. 1990, Cook and Leventhal 1996). In a comparison of autistic individuals with and without affected relatives, 5-HT levels were significantly higher in those with affected relatives; both groups were significantly higher than normal controls. These results suggest that hyperserotonemia may represent a genetic liability for autism (Piven et al. 1991). Similarly, DA levels have been reported to be increased in the serum of autistic individuals and their parents (Martineau et al. 1988). No consistent evidence exists for altered NE levels (Anderson 1987).
The significance of altered monoamine levels in autistic individuals is unclear, especially since serum levels do not necessarily correlate with brain and cerebrospinal fluid levels. While these alterations could be secondary consequences of the basic defect in autistic individuals, they are the only consistent biochemical markers for the disorder.

Based on these observations, the genes encoding proteins involved in the monoamine metabolic pathways and in monoaminergic synaptic transmission have been the targets of several genetic studies (Comings et al. 1996, Robinson 1996). Various strategies have been applied, including association and sib pair studies, to examine the potential roles of these enzymes and receptors in autism, but no clear evidence for the involvement of any of these candidates has been found.

In the course of testing sib pair concordance rates at autosomal candidate genes for autism, our laboratory has recently found an allelic association at the dopamine beta hydroxylase (DBH) gene in 9q34 (Robinson 1996). The enzyme DBH converts DA to NE, and is present in noradrenergic and adrenergic neurons as well as in serum (Friedman and Kaufman 1965). It was found that the mothers of autistic/PDD sib pairs carried a 19 base pair (bp) deletion allele at the DBH locus significantly more frequently than normal controls. This increase was not, however, reflected in increased concordance for the deletion allele in the affected sib pairs, nor was a similar increase in frequency detectable in the fathers of these sib pairs. The latter two observations argue against an interpretation of the allelic association data as reflecting a role for the DBH gene in the sib pairs themselves; rather we have suggested that an abnormality in maternal DBH expression or activity levels contributes to the occurrence of autism/PDD in the child. In other words, the fetus could be subjected to a maternal effect which may cause
abnormal neural development leading to autism/PDD in the child. Consistent with this model is the finding that DBH enzymatic activity levels correlated with DBH genotype, with deletion homozygotes having low activity, heterozygotes having intermediate activity and individuals without the deletion having the highest activity (Robinson 1996). Thus a fetus could be exposed to high DA levels (or decreased NE levels) as a result of low DBH activity in the mother, and therefore be at risk for an autistic or PDD phenotype.

Such a model would explain the failure of linkage and sib pair studies to identify genes of major effect in affected individuals, but fails, on its own, to explain the skewed sex ratio in the autistic population, the low risk to sibs of affected individuals, and the presence of DZ and MZ twins discordant for autism/PDD. It also cannot explain all cases of autism/PDD, since not all mothers carried the DBH deletion allele or had low DBH activity. It does, however, represent the first plausible model for the etiology of at least some autism/PDD in the population, if these other issues can be addressed.

Given the complex genetics of the autistic spectrum disorders, it seems likely that other genes in the monoaminergic pathways are also involved. If the maternal effect mechanism proposed for DBH is essentially correct, it would follow that any factors, genetic or environmental, which affect the exposure of the developing fetus to abnormal monoamine levels would modulate the severity or risk of an autistic/PDD phenotype. It is reasonable, therefore, to apply the methods developed for the analysis of complex disorders to further examine some of the genes involved in monoaminergic pathways in the autistic population, with the goal of developing a model for the genetic etiology of autism and the pervasive developmental disorders.
Chapter II:

Autism and the X chromosome: No linkage to microsatellite loci

detected using the affected sib pair method

Abstract

The etiology of autism and the related pervasive developmental disorders (autism/PDD) is poorly understood, although it is clear that genetic factors play a major role. Autism/PDD appears to be a heterogeneous group of disorders, making genetic analysis difficult in the absence of etiologically definable subgroups. The excess of males in the affected population has led to suggestions that an X-linked locus could play a role in the causation of autism/PDD. To examine this, we have investigated the genotypes of 32 families with two or more affected boys, using a broad classification of autism/PDD, at a series of 16 highly polymorphic loci (DXS237, 5'DMD, DXS 538, MAOA VNTR/CA, MAOA CA, MAOB, DXS453, DXS3, DXS458, DXS454, DXS424, DXS294, DXS102, DXS548, FMR-1 and DXS1113) distributed along the entire X chromosome with an average interlocus distance of 12 cM, in order to identify regions of significantly increased concordance among pairs of brothers. No locus tested showed a significant increase in concordance, suggesting that there are no genes of major effect located on the X chromosome which contribute to increased susceptibility to autism/PDD.
Introduction

An involvement of the X chromosome in the genetic etiology of autism/PDD has long been postulated, on the basis of two sets of observations. While the sex ratio among autistic individuals varies between studies, most show a large excess of affected males, with an average across studies of around 4 males to every female (Ciaramello and Ciaramello 1995). An X-linked recessive allele expressed in hemizygous males has been suggested to account for this excess. The second line of evidence stems from the apparent association of autism with the fragile X syndrome. Estimates of the degree of association vary widely, ranging from 0 to 53% (Payton et al. 1989, Bolton and Rutter 1990). This variation is largely due to differences in ascertainment and diagnostic criteria for both autism and fragile X syndrome. Piven et al. (1991) used the Autism Diagnostic Interview (ADI; LeCouteur et al. 1989), a standardized instrument for diagnosing autism, and found a frequency of fragile X syndrome of 2.7% in autistic subjects, as determined by cytogenetic detection of the fragile site in Xq27.3. Autistic behaviour, particularly impaired communication and social interactions and perseverative behaviour, have also been described in the fragile X population (Brown et al. 1982). This apparent association between autism and fragile X syndrome is controversial: it is unclear whether autistic behaviour with fragile X represents a phenotypic variant of the syndrome, a distinct clinical entity resulting from a different mutation in the FMR1 gene, or the comorbidity of the two disorders. The issue was further complicated by the discovery of two additional fragile sites in Xq27-q28, FRAXE (Knight et al. 1993) and FRAXF (Hirst et al. 1993). Expansion of the trinucleotide repeat in FRAXE is associated with mental retardation (Knight et al. 1993), whereas no disorder has yet been found to be associated with FRAXF. It is conceivable that the apparent association of
autism with fragile X results at least in part from misclassification of FRAXE or FRAXF fragility as FRAXA, and that one of these fragile sites, rather than the FMR1 gene, is associated with autism. Characterization of the molecular events underlying these fragile sites, namely trinucleotide repeat expansion, has permitted direct molecular analysis of these repeats in autistic individuals. Hallmayer et al. (1994) found no evidence of trinucleotide repeat expansion in FMR1 in 79 autistic children of 35 families, and were able to exclude linkage of autism to markers in the Xq27.3 region. Holden et al. (1996a) found no trinucleotide repeat expansion at FMR1, FRAXE or FRAXF, and no significant increase in allelic concordance at these loci among pairs of brothers diagnosed with autism or a related PDD. Thus it would appear that the genes associated with these three fragile sites are not major contributors to the frequency of autism in these families, and presumably not in the general population. The observation of autistic-like behaviours in some fragile X individuals remains unexplained, however. It may be that autistic behaviours are common sequelae to both fragile X syndrome and the autistic spectrum disorders, i.e. that the biochemical or developmental pathways of the two disorders overlap in some manner (Fisch 1993). This seems plausible, given the apparent etiologic heterogeneity of the autistic spectrum disorders.

While the possibility of X-linkage in autism/PDD remains intriguing, an X-linked mode of inheritance is clearly not sufficient to account for all autism/PDD in the population, for several reasons. First, an X-linked mode of inheritance predicts a much larger excess of affected males. Second, the recurrence risk to male siblings, the probability of a brother of an affected boy being affected himself, is about 3% (Smalley et al. 1988), whereas a strictly X-linked model would predict a recurrence risk of 50%. Similarly, X-linkage fails to account for the reduced
phenotypic concordance in monozygotic twins (64%) or for the clinical differences between affected monozygotic twins. Finally, the segregation pattern of autism in some extended pedigrees is clearly inconsistent with an X-linked mode of inheritance, exhibiting transmission of the disorder to sons from the paternal line (Hallmayer et al. 1996). Thus, if an X-linked autism gene exists, it cannot be the only genetic factor in operation; other non-sex-linked loci must contribute to the genetic etiology of the disorder as well.

Two basic models involving X-linked and autosomal loci can be envisioned. The first is a mixed model, in which an X-linked locus causes autism/PDD in some families, whereas in other families an autosomal mode of inheritance is present. The second is a multifactorial or polygenic model, in which several loci contribute increased susceptibility toward a threshold level for a diagnosis of "affected". In this scenario an X-linked locus would confer greater susceptibility in males than in females, resulting in a sex ratio biased toward males. It should be noted that these models are not mutually exclusive; one could for instance envision a mixed model involving X-linked recessive inheritance in some families, with an autosomal, polygenic pattern in others. Furthermore, the effects of environmental influences can be factored in as well, allowing for an etiologic model of considerable complexity.

Regardless of the model applied, the involvement of several genes and environmental factors implies etiologic and genetic heterogeneity. In any sample of autistic/PDD families, a mixture of mechanisms may be operating. Genes having causative roles in some pedigrees may not be involved in others. Heterogeneity has a serious confounding effect on methods of linkage analysis which rely on multiple families, since positive and negative measures of linkage such as lod scores are additive across families and tend to cancel each other out. Linkage analysis in
single extended pedigrees with multiple affected individuals is an alternative, but such families are rare. Furthermore, in addition to the usual difficulties in obtaining diagnoses on and genetic material from extended relatives, the choice of diagnostic criteria becomes a serious issue for a complex disorder such as autism/PDD. Findings of mild manifestations of autistic behaviours in the parents and relatives of autistic individuals are controversial (Wolff et al. 1988, Delong and Dwyer 1988, Folstein and Piven 1991) and misclassification of the affected status of individuals in a pedigree can severely diminish the ability to detect linkage. In the face of these difficulties, it is not surprising that early attempts at linkage analysis of autism were unsuccessful (Spence et al. 1985).

Association studies are an alternative to linkage-based approaches, in which large numbers of affected individuals are genotyped at a candidate locus to test for linkage disequilibrium between the test locus and the disorder, as detected by deviations in allele frequencies between cases and controls. These types of studies are most effective when used to examine candidate loci selected on the basis of their known functions and suspected involvements in the etiology of the disorder. To date, while the possibility of association of several autosomal loci with autism has been examined (eg. Spence et al. 1985, Comings et al. 1991, Hérault et al. 1993, Daniels et al. 1995, Robinson 1996, Cook et al. 1997a), only one association study of autism with X-linked loci has been performed. Petit et al. (1996) found no evidence for a statistically significant association of autism with any of 8 X-linked RFLP markers in a sample of 90 autistic children. A more thorough examination using a larger number of more highly polymorphic markers may, however, yet reveal an association between an X-linked locus
and autism.

Another alternative to linkage analysis in large families is the affected sib pair (ASP) method, which determines the degree of allelic concordance among pairs of affected siblings as a measure of linkage with a disease locus (Risch 1990). Analysis of allelic concordance at X-linked loci in pairs of affected boys represents a special case of the ASP method. In considering only affected brother pairs, genotype information from the father is not required, since boys receive no paternal X chromosome contribution (with the exception of the two pseudoautosomal regions). Thus only the maternal genotype is required, which is advantageous in two respects. First, blood samples from fathers tend to be more difficult to obtain, for a variety of reasons including availability and reluctance to give blood. Second, and more importantly, there is only one relevant transmitting parent required to be heterozygous at a test locus, and so a sibship is more likely to be informative.

In the absence of linkage between a test locus and the disorder, Mendelian segregation predicts a 1:1 ratio of concordance to discordance among informative sib pairs. A statistically significant increase in concordance is therefore suggestive of linkage. Thus for detection of an X-linked susceptibility gene, a series of polymorphic loci distributed evenly along the length of the chromosome may be tested for their concordance rates by genotyping pairs of affected brothers. Loci exhibiting significantly greater than 50% concordance in informative families could therefore be located in chromosome regions containing the susceptibility gene.

Three studies to date have applied the ASP method to detecting linkage of autism to the X chromosome. Hallmayer et al. (1994), in addition to their lod score based linkage analysis of the
Xq27.3 region, examined concordance rates for four loci in that region, and found no evidence for increased concordance. In a second study, the same group (Hallmayer et al. 1996) recently extended this sib pair analysis to a scan of the X chromosome using 35 markers in 38 pairs of autistic siblings. They were able to exclude the presence of an X-linked gene of major effect contributing to autism in their sample, but could only exclude 17% of the X chromosome from containing a gene of small effect ($\lambda_s<1.5$), and in fact found weak evidence (lod score of 1.24) for such a gene linked to the DXS424 locus in Xq24 (Hallmayer et al. 1996). Both these studies included affected female as well as affected male siblings. We suggest that if an X-linked autism susceptibility locus exists, then male multiplex sib pairs are likely to represent a more homogeneous subgroup among the autistic population, and this subgroup would thus be enriched for the putative X-linked locus. In other words, inclusion of male-female and female-female affected pairs may have introduced heterogeneity in those studies, reducing the power to detect an X-linked locus. Hallmayer et al. (1994) found no evidence of heterogeneity in their sample, but this related specifically to linkage to the fragile X syndrome locus in Xq27.3 (i.e. none of the families were linked to this region), and has no bearing on the homogeneity of their sample with respect to other loci. Furthermore, it has been established that there is an increased risk for some form of PDD, not necessarily classical autism, in families with an autistic individual (Szatmari 1992). An X-linked susceptibility locus may play a role in these types of families, and might be underrepresented in sib pairs with affected females relative to male-male pairs. Thus we have focused our studies on male multiplex sib pairs with autism or PDD as a more homogeneous sample for X chromosome studies. As cited earlier, we found no evidence for increased concordance in these male multiplex autism/PDD sib pairs at the FMR1, FRAXE or FRAXF loci.
(Holden et al. 1996a). We report here the results in those and additional male MPX families of an ASP scan of the X chromosome.

Materials and Methods

Most of the families used in this study, as well as the inclusion criteria used, have been described previously (Szatmari et al. 1996; Holden et al. 1996a). All parents were phenotypically normal with respect to the autistic spectrum. Genomic DNA was extracted from peripheral lymphocytes of affected individuals and all available parents. In some cases, blood spots were used as a source of target DNA (Holden et al. 1996b).

The microsatellite repeat loci listed in Table I were examined in the study, using the oligonucleotide primers described in the associated references cited (primers obtained from Research Genetics, Huntsville, AL or synthesized by Mobix, McMaster University). Polymerase chain reactions (PCRs) were carried out using 50 ng template DNA, 1X PCR buffer, 1.0 mM MgCl₂, 200 μM each dNTP, 10 μM each primer, 0.5 U Taq DNA polymerase (BRL) in 10 μl volumes in thin-walled amplification tubes in a Perkin-Elmer Cetus 400 or 480 thermal cycling apparatus. Initial PCR conditions typically involved an incubation at 94°C for four min, followed by 28 cycles of 94°C for 30sec, 55°C for 1min, 72°C for 1min. Reaction conditions were optimized for each locus by varying the annealing temperature. An additional five cycles were used when amplifying unpurified DNA from blood spots. Reactions incorporated α³²P-labelled dCTP, and were subsequently electrophoresed on 6% denaturing polyacrylamide gels

28
<table>
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and visualized overnight by autoradiography.

Families were scored for each locus on the basis of their genotypes. Siblings were scored as concordant if both brothers inherited the same maternal allele identical by descent, discordant if they received different alleles from their mother, or non-informative if their mother was homozygous at that locus. Allele assignments were confirmed by a second, independent scorer. Concordances were calculated as the proportion of concordant sibships among total informative families. A \( \chi^2 \) test was employed to compare the observed degree of concordance against 50% concordance expected in the absence of linkage. Analyses were corrected for multiple non-independent testing by dividing the significance level \( \alpha \) by the number of tests performed (Bonferroni correction; Holm 1979).

Results

Scoring of concordance for X-linked loci

Genotyping of all 32 affected sib pairs and their mothers was performed for all loci, with the exception of FMR-1, for which genotyping was completed for 30 of 32 families. Allele sizes were assigned by comparison with sequence ladders, or according to corresponding reported allele frequency distributions. Complete genotype data for each individual is included in Appendix I. Examples of autoradiograms showing concordance results for 10 different loci are given in Figure I.

The seven alleles at the DXS458 locus (Figure 1a) range in size from 178 to 190 bp (Weber et al. 1990). Six of the seven alleles are present in the nine females’ genotypes in Figure 1a). Alleles appear as groups of three bands, with the middle band most intense. The most
Figure I. Scoring of concordance in male multiplex autism/PDD sibships

Examples of autoradiograms provided to demonstrate the scoring of PCR-based markers for 10 X-linked polymorphic loci. Each set of three lanes represents one pedigree, in the order mother - affected son - affected son, as indicated by the pedigrees above each panel. The concordance state is indicated below each sibship: C denotes a concordant sibship, D a discordant sibship and N a non-informative sibship resulting from the homozygosity of the mother at this locus. Where concordance could not be determined due to failure of an allele to be amplified, no concordance state is indicated. Alleles segregate as a group of bands, due to stuttering as a result of Taq polymerase slippage during PCR extension. The most intense band is considered the true allele size for scoring purposes.

a) DXS458, b) 5'DMD, c) DXS237, d) DXS454, e) DXS102, f) DXS294, g) MAOA VNTR, h) MAOA microsatellite.
a) DXS 458

b) 5' DMD

c) DXS 237
d) DXS 454

N C NCDND

e) DXS 102

NNNNDDCCDN

f) DXS 294

CNCDNCDC
g) MAOA VNTR

h) MAOA CA
The 5'DMD locus exhibits nine alleles ranging in size from 88 to 108 bp (Hugnot et al. 1991). Five of the nine alleles are represented in the genotypes of the nine mothers of male multiplex sibships depicted in Figure 1b). Alleles appear as an intense band (used to obtain allele sizes), flanked by a 2 bp larger faint band and several progressively more faint bands.

Genotypes of nine families at the DXS237 locus are shown in Figure 1c). The DXS237 locus has eight alleles, ranging in size from 153 to 167 bp (Gedeon et al. 1992), of which only four are present in these families. Alleles appear as two approximately equally intense bands, flanked by a larger, faint band and a series of smaller, progressively more faint stutter bands. The relatively low degree of heterozygosity at this locus is evident from the high frequency of one allele (161 bp) in these females.

Each of the four alleles reported at the DXS454 locus (Weber et al. 1990) is represented in the genotypes of the nine families depicted in Figure 1d). The amplification products of each allele appear as a group of four bands, with a most intense band flanked by two less intense bands 2 bp larger and smaller, and a fourth, faint band 4 bp smaller than the most intense band. The concordance states of the second and fourth sib pairs could not be determined from this autoradiogram due to the lack of detectable amplification product from the mother (sib pair 2) or from both affected boys (sib pair 4). Concordance in these families was determined in a subsequent amplification reaction (not shown).

Five of the eight alleles described at the DXS102 locus (Gedeon et al. 1992) are present in Figure 1e). Each allele appears as a doublet of equal intensity separated by 2 bp, flanked by somewhat weaker bands 2 bp larger and 2 bp smaller, and a series of progressively weaker smaller bands in 2 bp intervals. The larger band of each doublet was used to determine allele
sizes. In the cases of the second and ninth families in Figure 1e), no amplification product was visible from one sib in each pair. The faint bands present in those lanes resulted from leakage of samples from neighbouring lanes during loading. From the homozygous genotypes of their mothers, however, their alleles at this locus could be inferred. Similarly, the maternal genotypes for the third and fifth families were not visible. Based on the discordance at this locus in the sib pair of the fifth family, the mother could be inferred to be heterozygous. The genotype of the mother in the third family, as well as all other genotypes not visible on this autoradiogram, were confirmed by subsequent reanalysis.

The DXS294 locus has 10 reported alleles, ranging from 122 to 148 bp (Gedeon et al. 1991). Eight alleles are visible in Figure 1f). Alleles appear as a doublet with the larger band more intense, flanked by a weaker band 2 bp larger and another weaker band 4 bp smaller than the most intense band. The most intense band was used for sizing. The second affected sibling in the seventh family apparently shows a mutation from 144 bp in the mother to a novel, 130 bp allele. This result was replicated in two different amplifications from original DNA stocks, and may be the result of either a novel mutation in the affected boy, or of confusion of a sample during the DNA extraction or handling process.

The MAOA polymorphism represented in Figure 1g) consists of a 23 bp VNTR motif directly adjacent to a dinucleotide repeat, and is characterized by 15 alleles ranging in size from 285 to 388 bp (Hinds et al. 1992). Only four of these 15 alleles are represented in the eight families depicted in Figure 1g). Alleles appear as doublets of equal intensity, flanked by weaker bands 2 bp larger and smaller. Two weaker doublets 12 bp and 24 bp below the top band of the first doublet are also present, presumably representing slippage by one and two VNTR repeat
units, respectively. The concordance states of two families could not be determined from this autoradiogram due to the lack of visible amplification product in one affected sib each. These were determined by subsequent reanalysis.

A second polymorphism in the MAOA gene, a simple dinucleotide repeat, is shown in Figure 1h). The seven alleles at this locus range in size from 112 to 126 bp (Black et al. 1991). Five of the seven alleles are represented in the genotypes of the seven mothers of male sib pairs depicted. The alleles follow the basic pattern of an intense band flanked by two weaker bands 2 bp larger and smaller, with another still weaker band 4 bp smaller, but is complicated somewhat by some non-specific amplification products in the same size range, as well as fainter shadow bands of slightly lower electrophoretic mobility, presumably due to some incorporation of radiolabel into the (dG.dT)-rich strand.

Allelic concordance for X-linked loci

The observed states of allelic concordance for each locus in each of the male MPX sibships are shown in Table II, along with the total numbers of concordant, discordant and noninformative families at each locus. Family AUT 063 contains three affected boys, and therefore represents three non-independent sib pairs. For a given locus, this family could be concordant for all three sibs, concordant for two pairs and discordant for one, or non-informative. As the prior probability of concordance of 50% does not hold true for this sib trio, family AUT 063 was not included when calculating the degree of concordance at each locus, but is shown in Table II for completeness. Concordance was thus determined from the remaining 31 families typed.
| Locus      | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | # of C families | % C |
|------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----------------|-----|
| DXS 237    | D  | N  | N  | D  | C  | N  | N  | N  | C  | N  | D  | C  | N  | N  | N  | D  | C  | N  | N  | N  | N  | D  | C  | C  | N  | 8  | 7  | 10  | 53.3 |
| S'DMD      | D  | C  | D  | D  | C  | D  | D  | C  | D  | C  | D  | D  | C  | D  | C  | D  | C  | D  | C  | D  | N  | D  | C  | C  | N  | 11 | 13 | 7   | 45.8 |
| DXS 538    | D  | C  | N  | D  | D  | C  | N  | D  | C  | N  | D  | C  | N  | D  | D  | N  | D  | D  | C  | C  | N  | D  | D  | C  | N  | 10 | 11 | 10  | 47.6 |
| MAOA VNTR  | N  | N  | N  | D  | N  | N  | N  | D  | C  | N  | D  | C  | N  | D  | C  | N  | D  | C  | N  | D  | C  | N  | D  | C  | N  | 10 | 11 | 10  | 47.6 |
| MAOA CA    | D  | N  | N  | D  | N  | N  | N  | D  | C  | D  | N  | N  | C  | D  | N  | C  | N  | D  | C  | N  | D  | C  | N  | N  | 10 | 9  | 14  | 47.8 |
| MAOB       | D  | N  | D  | N  | D  | C  | D  | D  | D  | D  | N  | D  | D  | C  | D  | C  | D  | D  | C  | D  | I  | C  | N  | N  | 8  | 14 | 8   | 39.1 |
| DXS 483    | C  | N  | C  | C  | D  | C  | D  | D  | D  | D  | D  | N  | D  | N  | D  | C  | D  | N  | D  | D  | C  | N  | C  | N  | 8  | 15 | 8   | 34.8 |
| DXS 3      | D  | D  | C  | C  | N  | D  | C  | D  | N  | C  | N  | D  | N  | C  | N  | N  | N  | N  | N  | N  | N  | C  | C  | N  | 10 | 9  | 12  | 52.6 |
| DXS 488    | D  | C  | N  | N  | D  | C  | D  | D  | C  | N  | N  | N  | C  | N  | C  | N  | N  | C  | N  | D  | N  | N  | C  | C  | N  | 7  | 10 | 14  | 41.2 |
| DXS 454    | N  | N  | C  | C  | N  | C  | D  | N  | D  | N  | N  | C  | D  | C  | N  | C  | D  | N  | C  | D  | C  | D  | D  | N  | 11 | 10 | 10  | 52.4 |
| DXS 424    | C  | N  | C  | D  | N  | C  | D  | D  | N  | D  | C  | N  | D  | C  | D  | N  | D  | C  | D  | C  | D  | D  | N  | 10 | 9  | 8   | 60.0 |
| DXS 284    | N  | N  | N  | D  | D  | C  | D  | N  | D  | N  | N  | N  | N  | D  | D  | C  | D  | C  | D  | C  | D  | C  | D  | 8  | 11 | 12  | 42.1 |
| DXS 102    | N  | N  | N  | D  | D  | C  | D  | N  | D  | N  | N  | N  | N  | D  | D  | C  | D  | C  | D  | C  | D  | C  | D  | 8  | 15 | 15  | 50.0 |
| DXS 548    | C  | N  | D  | N  | C  | N  | D  | N  | N  | N  | N  | N  | N  | N  | N  | N  | N  | N  | N  | N  | N  | D  | N  | 6  | 13 | 10  | 31.8 |
| FMR-1      | N  | C  | D  | N  | C  | N  | D  | N  | C  | D  | D  | D  | D  | D  | D  | C  | D  | N  | D  | C  | N  | D  | D  | 11 | 11 | 10  | 50.0 |

Table II. Concordance states for male multiplex autism/PDD siblings
No locus showed significantly greater than 50% concordance in this sample of male multiplex families. In most regions of the chromosome, concordance among informative families was below or around 50%. While concordance was greater than 50% among informative families at four loci (53.3% at DXS237, 52.6% at DXS3, 52.4% at DXS454 and 60.9% at DXS424), these increases in concordance were not statistically significant, especially after correcting for the number of statistical tests performed.

Heterozygosity at X-linked loci

The degree of heterozygosity observed at each locus was determined using the genotypes of all 32 mothers of male multiplex sibships. For most of the loci tested, the number of families which were informative was consistent with the heterozygosities or polymorphism informational content (PIC) values reported in the literature (Table III). At the two MAOA loci in Xp11.23-11.4, however, it was noted that heterozygosity in the mothers of these male multiplex sibships was reduced. As a result, fewer sibships were informative at this locus than was expected (p<0.05 for each locus).

Discussion

The lack of a significant increase in concordance at any of the 15 X chromosome loci tested suggests that there is no evidence for an autism/PDD susceptibility gene linked to any of these markers segregating in these male multiplex sibships. In particular, our results do not support the suggestion by Hallmayer et al. (1996) of linkage in the Xq23 region, as we did not observe a significant increase in concordance at the DXS424 locus. While it must be noted that
Table III. Heterozygosity at X-linked test loci in multiplex autism/PDD sibships

<table>
<thead>
<tr>
<th>Locus</th>
<th>Observed HET</th>
<th>Expected HET</th>
</tr>
</thead>
<tbody>
<tr>
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<td>15/31</td>
<td>14/31</td>
</tr>
<tr>
<td>5‘DMD</td>
<td>24/31</td>
<td>24/31</td>
</tr>
<tr>
<td>DXS 538</td>
<td>21/31</td>
<td>24/31</td>
</tr>
<tr>
<td>MAOA VNTR</td>
<td>16/31*</td>
<td>23/31</td>
</tr>
<tr>
<td>MAOA CA</td>
<td>17/31*</td>
<td>22/31</td>
</tr>
<tr>
<td>MAOB</td>
<td>23/31</td>
<td>20/31</td>
</tr>
<tr>
<td>DXS 453</td>
<td>23/31</td>
<td>22/31</td>
</tr>
<tr>
<td>DXS 3</td>
<td>19/31</td>
<td>20/31</td>
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<td>23/31</td>
<td>26/31</td>
</tr>
<tr>
<td>DXS 294</td>
<td>23/31</td>
<td>23/31</td>
</tr>
<tr>
<td>DXS 102</td>
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<td>22/31</td>
</tr>
<tr>
<td>DXS 548</td>
<td>16/31</td>
<td>19/31</td>
</tr>
<tr>
<td>FMR-1</td>
<td>19/29</td>
<td>18/29</td>
</tr>
<tr>
<td>DXS 1113</td>
<td>22/31</td>
<td>23/31</td>
</tr>
</tbody>
</table>

* p<0.05
the composition of the two samples is different, as Hallmayer et al. included sibships with affected females, a sample such as ours containing only male affected sibs should have been expected to be more powerful in detecting an X-linked locus. Nevertheless, other differences in the nature of the two sets of families, such as the inclusion criteria used, may exist to account for our failure to replicate the DXS424 results.

While we can exclude the possibility of a major X-linked gene causing autism/PDD in all these families, these results should not be taken as an exclusion of the X chromosome from involvement in the etiology of autism/PDD. As this study represented an initial screen of the chromosome, the map density used was relatively coarse, with an average distance between test loci of 12 cM; a gene of smaller effect size could be situated in one of these intervals, and the combined implications of reduced effect size and recombination could result in the failure to detect linkage in a relatively small sample size. A much larger number of families and markers must be screened in order to exclude a chromosome from containing a susceptibility gene of reduced effect size, particularly given the possibilities of complex modes of inheritance and etiologic and genetic heterogeneity.

The finding of reduced heterozygosity at the MAOA locus is intriguing. Monoamine oxidase A is involved in the degradation of the biogenic amines, a class that includes the neurotransmitters serotonin, dopamine and norepinephrine. As abnormalities in serum levels of these biogenic amines and their metabolites have been reported in autistic individuals and their parents (Anderson 1987, Martineau et al. 1994), the monoaminergic systems have been implicated in the etiology of the disorder. Alterations in MAOA function could potentially account for these abnormal levels and thereby contribute to the disorder. The lack of
against the inheritance of a predisposing maternal MAOA allele as a major effect in the expression of the autistic/PDD phenotype. The possible role of the MAOA gene in the etiology of autism/PDD will be examined further.
Chapter III:

Autism and the X chromosome: Evidence in male multiplex sibships for the involvement of the monoamine oxidase A gene

Abstract

The genetics of autism and the related pervasive developmental disorders (autism/PDD) are poorly understood, although it is clear that genetic factors play a major role in the etiology of these disorders. The excess of males in the affected population has led to suggestions that an X-linked locus could contribute to the causation of autism/PDD. In a previous study, we examined the genotypes of 32 families with two or more affected boys (male multiplex sibships) at a series of 16 highly polymorphic loci distributed along the entire X chromosome, in order to identify regions of significantly increased concordance among pairs of affected brothers using the affected sib-pair method. While none of these loci showed evidence for increased concordance, significant allelic association was detected with two polymorphisms located within the monoamine oxidase A (MAOA) gene located in Xp11.23-11.4. At each locus, a particular allele was found to be significantly more frequent in the mothers of these male multiplex sibships than in a random sample of normal controls (p<0.01 in both cases). The two alleles are in strong linkage disequilibrium in these families. Affected siblings were, however, not necessarily concordant for the maternal MAOA haplotype. We interpret these results to suggest that a maternal effect mechanism may contribute to the causation of autism/PDD in these families, wherein a mutation in MAOA
associated with this haplotype causes abnormal biogenic amine levels to be present in the maternal blood. Exposure of the fetus to these abnormal levels may result in defects in neural development leading to autism/PDD.

Introduction

On the basis of the excess proportion of males to females in the Autism/PDD population, it has been suggested that a locus on the X chromosome could contribute to the etiology of autistic spectrum disorders (Hallmayer et al. 1994). At least two types of models incorporating an X-linked locus could account for the complex inheritance pattern: firstly, a mixed model in which autism segregates as an X-linked disorder in some families and as an autosomal recessive disorder in others; or secondly, polygenic or threshold models involving an X-linked recessive autism susceptibility locus, which would be much more likely to contribute to the phenotype in males than in females. Combinations of these two types of models are also conceivable, of course.

The suggestion of an X-linked locus involved in the etiology of autism has led investigators to scan the X chromosome for linkage. Hallmayer et al. (1996) recently reported a small positive multipoint lod score (1.24) at the DXS424 locus in Xq24 in affected sib pairs, possibly suggesting the presence of a gene of small effect in this region. In our own study of 32 male autistic/PDD sib pairs, we found no evidence for linkage at DXS424, nor at any other of the 15 highly polymorphic loci tested (see Chapter II). Thus linkage and affected sib pair...
approaches to date have failed to identify an X-linked locus of major effect to account for the male-biased sex ratio in autism/PDD. Nevertheless the X chromosome remains a focus of study.

An alternative to linkage-based approaches to identifying genes involved in complex disorders is the allelic association approach, which relies on the comparison of allele frequencies at a given locus between unrelated patients and controls. A significant difference in the frequency of an allele at the test locus between the two groups may arise as a result either of a causal role of that allele in the disorder, or of linkage disequilibrium between that allele and a tightly linked causal mutation. The detection of linkage disequilibrium therefore argues for the very close proximity of the test locus to the gene contributing to the disorder, and can be a very significant finding. As noted previously, association studies have the advantage of examining only affected individuals, thereby avoiding the problems of misclassification of relatives and allowing for larger sample sizes. Association studies pose a number of strategic difficulties, however. The identification of an appropriate control group is a major issue, since differences in allele frequencies between patients and controls could result from differences in ethnic composition and geographic origin. Heterogeneity in the affected population is also a concern, as sampling unrelated affected individuals may result in a patient group of mixed etiology in which it is more difficult to detect a locus of smaller effect. Furthermore, allelic association studies are tests of linkage disequilibrium between the test locus and the disorder gene. As linkage
disequilibrium falls off very rapidly with increasing genetic distance, a large number of very closely linked test loci would have to be screened in order to scan a large genetic region. Tests of association are therefore more useful for examining the potential roles of specific genes which have been identified as candidates on the basis of other biochemical, pharmacological or genetic evidence, and are not generally an efficient method for scanning whole chromosomes or of an entire genome.

Nevertheless, Petit et al. (1996) carried out an allelic association study for eight X-linked restriction fragment length polymorphism (RFLP) markers in autistic individuals and found a difference in allele distribution between patients and controls at the DXS287 locus in Xq23, which was however non-significant when a Bonferroni correction was used to adjust for the number of statistical tests.

This is the only study of allelic association of X-linked loci with autism described to date.

In the course of our own concordance scan of the X chromosome in male MPX sibships, while we reported no findings of significantly increased concordance rates (see Chapter II), we noted an unusual result at the monoamine oxidase A (MAOA) locus in Xp11.23-11.4. Far fewer affected sib pairs were informative for a polymorphism in the MAOA gene than was predicted from the allele frequencies reported in the literature (Hinds et al. 1991), i.e. more mothers of male multiplex autistic/PDD sibships than expected were homozygous at this locus.
Monoamine oxidase (MAO) catalyses the degradative oxidation of the monoamine neurotransmitters, including serotonin (5-HT) and the catecholamines dopamine (DA) and norepinephrine (NE). It is expressed in catecholaminergic and serotonergic synapses, where its role is the clearing of excess transmitter, as well as in a variety of other tissues. Two forms of MAO exist, denoted A and B, which are highly similar in amino acid sequence and structure but differ in their substrate specificities and pharmacological properties. MAOA and MAOB are encoded by two adjacent genes located in Xp11.23-11.4 which are thought to have arisen from a gene duplication event (Grimsby et al. 1991).

As several studies have demonstrated abnormalities in serum monoamine levels in autistic individuals and their parents (Anderson 1987, Martineau et al. 1994, Cook and Leventhal 1996), and genetic variation in MAO activity could account for these abnormal levels, the MAOA and MAOB genes are prominent candidates for roles in the etiology of autism/PDD in their own right. Their location on the X chromosome is intriguing, as it suggests that one or both of these genes could account for the sex bias among affected individuals. The possibility of altered allele frequencies at the MAOA locus suggested by the reduced heterozygosity observed in the mothers of male multiplex sibships was therefore examined further in this study. The results suggest the possible involvement of the monoamine oxidase A (MAOA) gene in the etiology of autism/PDD, based not on increased concordance in affected brothers at the MAOA locus, but on the increased frequency of a particular MAOA haplotype in
Materials and Methods

The families used in this study were collected on the basis of the inclusion criteria described previously (Szatmari et al. 1996). Genomic DNA was extracted from peripheral lymphocytes of affected individuals and all available parents. In some cases genomic DNA was obtained from dried blood spots (Holden et al. 1996b).

Dried blood spots were also obtained from a series of sequentially born, unrelated Canadian males, taken in the course of postnatal screening for phenylketonuria (Ontario Ministry of Health). These served as a presumably normal representative control sample of alleles from the Ontario population.

The polymorphic loci listed in Table I were examined, using the oligonucleotide primers and reaction conditions described in the associated references cited (primers obtained from Research Genetics, Huntsville, AL, or synthesized by MOBIX, McMaster University, Hamilton, ON). Polymerase chain reactions (PCRs) were carried out using 50 ng template DNA in 10 µl volumes in thin-walled amplification tubes in a Perkin-Elmer Cetus 400 or 480 thermal cycling apparatus. To amplify from blood spots, five additional cycles of amplification were used. Reactions incorporated α-32P-labelled dCTP, and were subsequently electrophoresed on 6% denaturing polyacrylamide gels and
<table>
<thead>
<tr>
<th>Locus</th>
<th>Chromosomal Localization</th>
<th>%HET</th>
<th>Reference</th>
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<tbody>
<tr>
<td>MAOA VNTR/CA</td>
<td>Xp11.23-11.4</td>
<td>75</td>
<td>Hinds et al. (1992) Genomics 13:896</td>
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<td>0.0cM</td>
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<td>64</td>
<td>Grimsby et al. (1992) NAR 20:924</td>
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visualized overnight by autoradiography. Allele assignments were confirmed by a second, independent scorer.

Allele frequencies at the two polymorphisms in the MAOA gene and the MAOB microsatellite polymorphism in mothers of male multiplex sibships were compared to those in normal control males, as well as to those reported in the literature. Differences in allele frequencies were tested using the MONTE program of the REAP statistical package (McElroy et al. 1992), which calculates a $\chi^2$ from the observed data, then performs a Monte Carlo simulation to determine the number of simulated data sets which exceed the observed $\chi^2$ and obtain an estimated probability of falsely rejecting the null hypothesis (no significant difference between allele distributions). The maximum number of simulations permitted by the program (10000) were performed for each comparison.

Results

Allele frequencies at MAOA locus

While examining the MAOA VNTR/CA repeat polymorphism (Hinds et al. 1991) for concordance rates in the male MPX sibships, it became clear that far fewer sibships were informative than was expected from literature values, i.e. there appeared to be an excess of mothers in the sample who were homozygous at this locus. To investigate this, we examined allele frequencies at the MAOA VNTR/CA polymorphism and compared them to values reported in the literature and to allele frequencies observed in a random sample of the general population
obtained from Guthrie spots of newborn males (Table II). The allele distribution was significantly different from both the control group ($\chi^2=16.27$, 14 df, $p=0.0398$) and the previously reported study ($\chi^2=57.65$, 14 df, $p=0.0011$; Hinds et al. 1991). The allele distributions in the control group and the reported study also differed significantly ($\chi^2=26.70$, 14 df, $p=0.0051$; Hinds et al. 1991).

One particular allele, denoted B2, was found to be far more frequent in the mothers of male MPX sibships than in the control group or in either previously reported study. The B2 allele was present at a frequency of 43.7%, much higher than the reported frequency of 16.1% and the observed frequency in controls of 26.1%. Of 32 mothers of male MPX sibships, 6 were homozygous for the B2 allele, 14 were heterozygous for B2, and 12 had two other alleles. In other words, 20 of 32 females (62.5%) carried at least one copy of the B2 allele. As the control allele frequencies were obtained from males, genotype frequencies could not be directly compared between mothers of MPX sibships and controls. From the allele frequencies observed in the controls, assuming Hardy-Weinberg equilibrium, we would expect only 2 B2 homozygous individuals, 12 heterozygous, and 18 with two other alleles. Thus genotypes containing B2 alleles appear more common in these mothers than in the general population, although not significant at the level of $\alpha=0.05$ ($\chi^2=3.35$, 2 df, $p=0.1861$).

In order to further investigate the possible involvement of MAOA in the etiology of autism/PDD, we studied another highly polymorphic marker, a
Table II. Allele frequencies at Monoamine Oxidase A VNTR/CA polymorphism

Reported allele frequencies are taken from Hinds et al. (1992).

<table>
<thead>
<tr>
<th>Allele</th>
<th>Reported</th>
<th>Observed in controls</th>
<th>Observed in mothers of male MPX sibships</th>
<th>Observed in mothers of female MPX sibships</th>
<th>Observed in mothers of male SPX</th>
<th>Observed in mothers of female SPX</th>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
A dinucleotide repeat located in the first intron of the MAOA gene (Black et al. 1992). Again, no evidence of increased concordance in the sibs was detected, but the allele distributions were significantly different between the mothers of male MPX sibships and the newborn male controls ($\chi^2=24.14$, 10 df, $p=0.0014$) as well as one of two reported frequency distributions (Black et al. 1991: $\chi^2=54.44$, 7 df, $p<0.0001$). The allele distribution in the male controls and in the controls of Black et al. (1991) also differed significantly ($\chi^2=25.99$, 10 df, $p=0.0001$) (Table III). In contrast, a second reported allele distribution (Hotamisligil et al. 1994) did not differ significantly from those observed either in mothers of male MPX sibships ($\chi^2=9.96$, 10 df, $p=0.2417$) or in the Guthrie spot controls ($\chi^2=16.53$, 10 df, $p=0.0524$). The two previously reported distributions differed significantly ($\chi^2=45.48$, 10 df, $p<0.0001$). Thus the interpretation of allele frequencies at the MAOA microsatellite polymorphism in the mothers of male MPX sibships is unclear, and depends on the choice of control group used for comparison.

As with the VNTR/CA polymorphism in MAOA, a particular allele, sized at 122 bp, was more frequent in the mothers of male MPX sibships (24/64, 37.5%) compared to the published reports (3/86, 3.4%; Black et al. 1991) (34/129, 26.4%; Hotamisligil et al. 1994) or observed in the random sample of controls (18/114, 15.8%). Of the 32 mothers examined for the MAOA dinucleotide repeat polymorphism, 7 were homozygous for the 122bp allele, 10 were heterozygous and 18 had two other alleles. Assuming the control population to be in Hardy-Weinberg equilibrium, we would expect on the basis of observed allele frequencies to observe only one 122bp homozygous individual, 9 heterozygous and 22 with two other alleles. Thus genotypes containing the 122bp allele
Table II. Allele frequencies at Monoamine Oxidase A VNTR/CA polymorphism

Reported allele frequencies are taken from Hinds et al. (1992).

<table>
<thead>
<tr>
<th>Allele</th>
<th>Reported</th>
<th>Observed in controls</th>
<th>Observed in mothers of male MPX sibships</th>
<th>Observed in mothers of female MPX sibships</th>
<th>Observed in mothers of male SPX</th>
<th>Observed in mothers of female SPX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=112</td>
<td>N=115</td>
<td>N=64</td>
<td>N=28</td>
<td>N=28</td>
<td>N=18</td>
</tr>
<tr>
<td>A1</td>
<td></td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B1</td>
<td>1</td>
<td></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B2</td>
<td>18</td>
<td></td>
<td>28</td>
<td>28</td>
<td>8</td>
<td>6</td>
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<td>B4</td>
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<td></td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>B5</td>
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<td></td>
<td>0</td>
<td>0</td>
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</tr>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B7</td>
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<td></td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>C2</td>
<td>6</td>
<td></td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C3</td>
<td>10</td>
<td></td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>C4</td>
<td>50</td>
<td></td>
<td>60</td>
<td>29</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>C5</td>
<td>2</td>
<td></td>
<td>2</td>
<td>9</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>D1</td>
<td>1</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E1</td>
<td>2</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
are more frequent in these mothers than expected in the general population, although not significant at the level of α=0.05 ($\chi^2=4.83$, 2 df, p=0.0985).

We have argued that male multiplex families represent a subset of the autistic/PDD population which is enriched for an X-linked disorder gene. To address this question we also examined MAOA allele frequencies in mothers with both an affected son and daughter as well as mothers with a single affected child, male or female. The microsatellite polymorphism in MAOA was not tested in these groups, due to the ambiguous results when comparing across different control groups. No significant difference in allele frequencies at the MAOA VNTR/CA polymorphism (Hinds et al. 1991), particularly in the frequency of the B2 allele, was observed in any of these three groups compared to controls or reported frequencies (Table II). The allele distributions are also not significantly different when compared to that observed in mothers of male MPX sibships (Table II). The lack of statistical significance in any of these comparisons is not surprising, given the small numbers of females genotyped in each of these groups. The B2 allele does not appear to be as frequent in mothers with single affected individuals of either sex or with affected children of both sexes, as it is in the male MPX group, however.

**Linkage disequilibrium between MAOA alleles in autism/PDD**

Given the tight linkage between the two MAOA polymorphisms, recombination was assumed to be very rare. The combinations of alleles at the two loci passed to the sibs was therefore taken as indicative of the phase of those alleles in the mothers. All
mothers carrying the 122bp allele also carried an equal number of B2 alleles, i.e. 122bp homozygotes were also homozygous for the B2 allele. In all cases where the B2 and 122bp alleles were both present (17/17), the B2 and 122bp alleles appeared to be transmitted as a haplotype. This B2-122bp haplotype occurred significantly more often than expected from the observed frequencies of these alleles in the male MPX mothers (Table IV; $\chi^2=44.3$, p$<<0.001$), suggesting that the two alleles are in linkage disequilibrium in these women. The high frequency of this B2-122bp haplotype compared to the general population strongly suggests its association with autism/PDD in these families.

Allele frequencies at MAOB

As the MAOA and MAOB genes are closely linked, it is possible that the altered allele frequencies observed in mothers of male multiplex sibships at MAOA are the result of linkage disequilibrium between the B2/122bp haplotype in MAOA and a causal mutation in MAOB, or that both genes contribute to the etiology of autism/PDD. Thus we examined sib pair concordance and allele frequencies at a highly polymorphic microsatellite marker located in the MAOB gene (Grimsby et al. 1992). No significant increase in concordance from the expected 50% was observed in male multiplex sibships, but a significant difference in the distribution of alleles was detected between mothers of male multiplex sibships and controls ($\chi^2=32.72$, 8 df, p$<0.0001$) or reported frequencies (Grimsby et al. 1992; $\chi^2=30.63$, 8 df, p$<0.0001$) (Table V). The two control groups also differed significantly from each other ($\chi^2=19.18$, 8 df, p$=0.0016$). Unlike the two loci in
Table IV: Linkage disequilibrium in MAOA in mothers of male MPX sibships

Expected haplotype frequencies were calculated from the observed allele frequencies in mothers of male multiplex sibships at the MAOA VNTR/CA and the MAOA CA repeat, using the groupings of ‘B2’ and ‘other’ (+) and ‘122bp’ and ‘other’ (+). Expected and observed frequencies were compared using the $\chi^2$ statistic.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Observed in male MPX mothers</th>
<th>Expected from observed allele frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>VNTR/CA</td>
<td>CA</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>122bp</td>
<td>23</td>
</tr>
<tr>
<td>B2</td>
<td>+</td>
<td>17</td>
</tr>
<tr>
<td>+</td>
<td>122bp</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>64</td>
</tr>
</tbody>
</table>

$\chi^2=44.3$, p<0.005
Table V. Allele frequencies at Monoamine Oxidase B dinucleotide repeat polymorphism
Reported frequencies are taken from Grimsby et al. (1992).

<table>
<thead>
<tr>
<th>Allele</th>
<th>Reported N=64</th>
<th>Observed in controls N=114</th>
<th>Observed in mothers of male MPX sibships N=64</th>
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<tbody>
<tr>
<td>195</td>
<td>0</td>
<td>2</td>
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<td>197</td>
<td>8</td>
<td>6</td>
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</tr>
<tr>
<td>199</td>
<td>19</td>
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<td>3</td>
</tr>
<tr>
<td>201</td>
<td>4</td>
<td>19</td>
<td>4</td>
</tr>
<tr>
<td>203</td>
<td>9</td>
<td>16</td>
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<td>205</td>
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<td>35</td>
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<td>0</td>
<td>13</td>
</tr>
<tr>
<td>209</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>211</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>
MAOA, in which the differences could be attributed to the excess of a particular allele and the reduced frequency of the normally most common allele, the three distributions compared for the MAOB polymorphism were quite dissimilar. For example, the 207 bp allele was much more frequent in the mothers of male MPX sibships (13/64 or 20.3%) than in the previously reported control group (Grimsby et al. 1992; 1/64 or 1.6%) and was not observed in the male newborn controls (0/86). Conversely, the 201 bp allele is much more frequent in the Guthrie spot controls (19/86 or 22.1%) than in the other two groups (4/64 or 6.3%), and the 199 bp allele is more frequent in the reported controls (19/64 or 29.7%) than in the Guthrie spot controls (8/86 or 9.3%) or the mothers of male MPX sibships (3/64 or 4.7%). These results suggest that allelic association may exist with MAOB in autism/PDD, and that MAOB might play a role in the etiology of the disorder as well. However, the allele(s) associated with the disorder is not clear. Of the 11 mothers carrying a 207 bp allele (2 homozygous, 9 heterozygous), only 8 also carried the MAOA B2 allele and 6 carried the MAOA 122 bp allele. The 207 bp allele did not cosegregate with the B2 or 122 bp alleles to the affected siblings in some cases. Overall, linkage disequilibrium between the MAOB 207 bp allele and alleles at MAOA appears to be weaker than between alleles at the two MAOA loci in these families, due to the greater recombination distance between the two genes.

Discussion

The data at the MAOA locus, while preliminary, are noteworthy for a number of reasons. The monoamine oxidases are logical candidates for several neuropsychiatric
disorders, including autism/PDD, as a consequence of their biochemical function in the enzymatic degradation of the monoamine neurotransmitters serotonin, dopamine and norepinephrine. Defects in serotonergic and dopaminergic systems have long been proposed in the etiology of autism, on the basis of the motor and behavioural characteristics of the disorder and the altered serum levels of these neurotransmitters in autistic individuals and their parents (Anderson 1987, Martineau et al. 1994). Thus genetic variation in MAOA or MAOB function could contribute to the etiology of autism/PDD.

Two observations support the possible involvement of MAOA in autism/PDD. Brunner et al. (1993a) have described a family in which affected members demonstrated mental retardation, aggressive behaviour and violent tendencies. The disorder has been traced to the presence of a point mutation in the MAOA gene resulting in a lack of MAOA expression (Brunner et al. 1993b), supporting the notion that mutations in MAOA may give rise to behavioural disturbances. Second, two brothers with atypical Norrie disease have been described, who in addition to the blindness normally present in Norrie disease also exhibited mental retardation and "autistic-like" features (de la Chapelle et al. 1985). The Norrie disease gene has been mapped to Xp11.4, very close to the MAO genes, and upon cytogenetic and molecular examination these two brothers both had a deletion which encompassed MAOA and MAOB as well as the Norrie syndrome gene (Sims et al 1989). Thus disruption of this chromosomal region may give rise to "autistic-like" phenotypes. More recent examination of these and similar deletion carriers have indicated that behavioural abnormalities are primarily associated with
MAOA deletion; MAOB-deficient patients do not exhibit abnormal behaviour or mental retardation (Lenders et al. 1996).

The significant differences in allele distributions between different control groups, particularly in the case of the MAOA microsatellite locus, reflect the importance of identifying a valid control group for allelic association studies. Differences in the ethnic composition or geographic origin of case and control groups may lead to statistically significant differences in allele frequencies, without any underlying biological significance of the test locus. For this reason, interpretations of association studies must be cautious, in the absence of replicating studies. The Canadian control group of unrelated newborn males is likely to be the best ethnic and geographic match for the patient group under study, and so statistical comparisons between these two groups are most useful for interpretation. The significant differences in allele distributions observed in MAOA and MAOB between mothers of male affected sib pairs and Canadian controls therefore suggests the involvement of these loci in the etiology of autism/PDD.

The results we report here for MAOA are difficult to reconcile with any standard genetic model. The increased frequency of the B2-122 bp haplotype in the mothers of male multiplex sibships cannot simply reflect the fact that a susceptibility allele associated with this haplotype is transmitted to the affected sibs, as few sib pairs were concordant for the haplotype. Rather, it would appear that it is the mother's genotype itself which is important for the etiology of autism/PDD in these sibships, in that the presence of the B2-122 bp haplotype increases the likelihood that a woman will have autistic/PDD sons.
Interestingly, our laboratory has obtained similar results at another candidate locus, dopamine beta hydroxylase (DBH), mapped to 9q34 (Robinson 1996). DBH is the enzyme responsible for the conversion of dopamine to norepinephrine. In the case of DBH, a 19bp deletion allele is more frequent in the same mothers of male multiplex sibships (Robinson 1996). Thus two enzymes, both involved in biogenic amine metabolism, appear to contribute to the etiology of autism/PDD via a similar mechanism. While with DBH, low serum enzyme activity levels have been shown to correlate with the number of 19bp deletion alleles (Robinson 1996), no direct correlation has been made between MAOA VNTR or microsatellite alleles and enzyme activity levels, as MAOA is not expressed in serum.

We propose that altered metabolism of one or all of the biogenic amine neurotransmitters, resulting from genetic variation at the DBH and MAOA genes and possibly others, results in abnormal levels of these neurotransmitters in maternal serum. Exposure of the fetus to these abnormal levels represents a stressful uterine environment which may lead to problems in development resulting in autism/PDD. Thus MAOA and DBH represent maternal effect genes, similar to the phenomenon of maternal phenylketonuria, in which exposure of the fetus to high maternal serum phenylalanine levels results in profound developmental disability (Mabry et al. 1963).

Clearly this model needs to be refined, in order to explain most of the peculiarities and complexities exhibited by the segregation pattern of autism/PDD. Alone, it cannot explain the excess of affected males in the autistic population, the low risk to sibs of an affected individual, the relatively low concordance in twins, or the broad phenotypic
range displayed by this group of disorders. Given the complex natures of neurochemistry, maternal-fetal interactions and early neuronal development, however, it would not be surprising for such a process to be modulated and mediated by sex-specific effects, fetal genotype factors (fetal susceptibility loci), and environmental influences.

The male multiplex sibships clearly represent a selected subset of the autistic population, as no significant increase in the frequencies of the VNTR/CA B2 allele was observed in mothers with only one affected child, or with both sons and daughters who are affected. Given the small number of mothers tested in these groups, we cannot exclude the possibility of increased MAOA B2-122bp haplotype frequency in these mothers. We have proposed that male multiplex sibships represent a more homogeneous subgroup which is enriched for the presence of an X-linked disorder locus and would therefore expect that the male simplex group be more heterogeneous, including a mixture of genetic and non-genetic etiologies. This is consistent with our inability to detect a statistically significant increase in the frequency of the MAOA haplotype in the mothers of male simplex sibships. While some mothers carried the B2-122bp MAOA haplotype in this group, and maternal MAOA activity levels may therefore have contributed to the disorder in their affected children, this proposed mechanism clearly does not account for most of the affected individuals in this group, and other etiological mechanisms must be at work in other types of families. Similarly, these other mechanisms may account for those male multiplex sibships in which the mothers lack the MAOA haplotype or the DBH deletion allele. It is plausible to suggest that other genetic and non-genetic influences affecting exposure of the developing fetus to maternal monoamine levels could
represent alternative mechanisms for the etiology of autism/PDD. These could include a number of genes involved in regulating monoamine levels, as well as perinatal stresses or pharmacological agents which affect neurotransmitter levels during pregnancy. Thus this general mechanism for the etiology of autism/PDD, in which exposure of the fetus to abnormal monoamine neurotransmitter levels in utero leads to abnormal brain development, could account for a much larger proportion of the autism/PDD population than the specific maternal effects proposed for MAOA and DBH in the male multiplex subgroup. Given the complexity of the autistic spectrum disorders, however, it is also possible that other, independent mechanisms may give rise to an autistic/PDD phenotype as well. In any case, the maternal effect mechanism proposed here may represent the first viable and testable model for the genetic etiology of autism and the related pervasive developmental disorders. Support for such a model awaits the replication of our results for MAOA and DBH by other groups, as well as the identification of the other genetic and environmental factors at work.
Chapter IV:

Increased concordance rates for maternal alleles of the serotonin and dopamine transporters (SERT, DAT1) in pairs of affected sibs suggest that altered biogenic amine transport confers increased susceptibility to autism/PDD

Abstract

While family and twin studies have consistently shown a strong genetic component to the etiology of autism and the related pervasive developmental disorders (autism/PDD), little progress has been made in identifying the genes involved. The unusual inheritance pattern and considerable clinical variation observed within families suggest a complex genetic mechanism. Several studies have implicated the involvement of neurotransmitter systems, particularly the biogenic amines. We report here an investigation of the role of biogenic amine transporters in autism/PDD. In 32 sibships with two affected males, significantly increased concordance for maternal alleles was observed at microsatellite repeat loci linked to the serotonin transporter (SERT) mapped to 17q11.2-q21 ($\chi^2=7.35$, 1 df; $p<0.01$) and the dopamine transporter (DAT1) mapped to 5p15.3 ($\chi^2=6.24$, 1 df; $p<0.025$). No increase in concordance was observed for paternal alleles at these loci, nor at a locus linked to the norepinephrine transporter (NET) located in 16q12.2. The increased concordances for SERT and DAT1 suggest a role for these transporter genes in conferring susceptibility to autism/PDD. The parent-of-origin effects observed may implicate a role for genomic imprinting at these loci.
Introduction

Biochemical and pharmacological studies of autism may provide suggestions regarding candidate genes. A number of lines of evidence have pointed to biogenic amine neurotransmitter systems as being implicated in autism, including responses to pharmacological agents (Anderson 1987) and altered levels of transmitters and their metabolites in the blood and urine of affected individuals and their relatives, although these have been inconsistent (Anderson 1987, Martineau et al. 1994). Thus the genes involved in these pathways are interesting when considering candidates conferring susceptibility to autism/PDD. Our results from genetic studies of dopamine beta hydroxylase (DBH; Robinson 1996) and monoamine oxidase A (MAOA; see Chapter III), two enzymes involved in the metabolism of the monoamines, have implicated abnormal monoamine levels in maternal serum as contributing factors in the etiology of autism/PDD. It is therefore of interest to examine some of the other genes involved in this pathway for possible involvement in the genetics of these disorders.

We have investigated the possible roles of the biogenic amine transporter proteins 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 in 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. It is reasonable to surmise that 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 NET1 located in 16q13-21 (Gelernter et al. 1993), DAT1 in 5p13.3 (Vandenbergh et al. 1992) and SERT in 17q11.2 (Gelernter et al. 1995). The three proteins share a similar structure and functional properties, with 12 transmembrane domains (Melikian et al. 1994). These transporters exhibit some affinity for the other monoamines in addition to their own substrate; for example, DAT1 has a higher affinity for NE than for DA, but is classified as a DA transporter on the basis of its pharmacological characteristics (Ramamoorthy et al. 1992).

The mapping of these transporter genes allows the selection of closely linked highly polymorphic markers for use in genetic studies of these genes in various disorders. We have studied the degree of allelic concordance at genetic loci near these transporter genes, in 32 pairs of siblings affected with autism/PDD, with the expectation 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.
Acknowledgements

Genotyping of families at the D16S398, D17S250 and THRA-1 loci was performed by Ms. Diana Polley. Genotyping of families at the DAT1 and D5S117 loci was carried out by Ms. Polley and Mr. José Monzon.

Materials and Methods

The families and inclusion criteria used in this study have been described previously (Szatmari et al. 1996). Genomic DNA was extracted from peripheral lymphocytes of affected individuals and all available parents. In some cases genomic DNA was obtained from dried blood spots (Holden et al. 1996b).

The polymorphic markers used for analysis of concordance rates for the three regions containing transporter genes are listed in Table I. 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 for this gene. A second, more highly variable locus linked to DAT1, D5S117, was examined as well. Highly polymorphic loci linked to NET1 and SERT were used to examine concordance rates for these two genes, as suitable intragenic polymorphisms were not available.

Polymerase chain reactions (PCRs) for D16S398, D17S250, THRA-1 and D5S117 were carried out using 50 ng template DNA in 10 µl volumes in thin-walled amplification tubes in a Perkin-Elmer Cetus 400 or 480 thermal cycling
Table I: Polymorphic loci used for affected sib pair analysis of monosmine transporter loci.

Test loci were selected on the basis of their proximity to the genetic map locations of the SERT, NET and DAT1 genes and their level of polymorphism.

<table>
<thead>
<tr>
<th>Candidate Gene</th>
<th>Chromosome Location</th>
<th>Test Locus</th>
<th>%HET</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERT</td>
<td>17q11.2-q21</td>
<td>D17S250</td>
<td>83</td>
<td>Weber et al. (1990) NAR 18:4640</td>
</tr>
<tr>
<td></td>
<td></td>
<td>THRA-1</td>
<td>81</td>
<td>Futreal et al. (1992) Hum Mol Genet 1:66</td>
</tr>
<tr>
<td>DAT1</td>
<td>5p13.3</td>
<td>DAT1 VNTR</td>
<td>45</td>
<td>Vandenbergh et al. (1992) Genomics 14:1104</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D5S117</td>
<td>55</td>
<td>Weber et al. (1990) NAR 18:4035</td>
</tr>
</tbody>
</table>
apparatus. Cycle conditions were: 94°C for 30 sec, 53°C for 1 min, 72°C for 1 min, for 28 cycles. To amplify from blood spots, five additional cycles of amplification were used. Reactions incorporated α-32P-labelled dCTP, and the 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, but in 20 µl reaction volumes with an annealing temperature of 68°C, and were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining and ultraviolet illumination.

Concordance for paternal and maternal alleles was assessed separately for each locus, and compared to the 50% concordance expected assuming independent segregation, using the χ² statistic. Analyses were corrected for multiple testing by dividing the significance level α by the number of tests performed (Bonferroni correction; Holm 1979).

Results

The concordances for alleles at each of the five polymorphic loci studied are shown in Table II. Concordance of both maternal and paternal alleles are shown separately. Uninformative matings result from the homozygosity of a parent, the inability to deduce the genotype of an unavailable parent (the father in all cases) from those of the mother and offspring, or from the inability to distinguish the parental origin of a particular allele.

The proportion of alleles shared IBD were shown to be significantly different from independent segregation (50%) in two instances: 1) maternal alleles at THRA-1, linked to SERT (χ²=7.35, 1 df, p<0.01) and 2) maternal alleles at D5S117, linked to DAT1 (χ²=6.24, 1 df, p<0.025). Concordance for the paternal allele at these two loci was not significantly increased.
Table II. Concordance rates for loci linked to biogenic amine transporter genes NET, SERT and DAT1.

Data on genotyping of these loci was provided by Diana Polley and Jose Monzon. Siblings were scored as either concordant (C), discordant (D) or non-informative (NI) for the transmission of alleles from each parent. A sibling pair was scored as non-informative if a parent was homozygous, or if the parental origin of an allele in the offspring could not be determined.

<table>
<thead>
<tr>
<th>Candidate Gene</th>
<th>Test Locus</th>
<th>Maternal concordance</th>
<th>Paternal concordance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C  D  NI  %C</td>
<td>C  D  NI  %C</td>
</tr>
<tr>
<td>NET</td>
<td>D16S398</td>
<td>14 10 7 58.3</td>
<td>9 10 6 47.4</td>
</tr>
<tr>
<td>SERT</td>
<td>D17S250</td>
<td>12 9 9 57.1</td>
<td>9 5 9 64.3</td>
</tr>
<tr>
<td></td>
<td>THRA-1</td>
<td>18 5 7 78.3*</td>
<td>12 7 6 63.2</td>
</tr>
<tr>
<td>DAT1</td>
<td>DAT VNTR</td>
<td>7 3 21 70.0</td>
<td>5 4 17 55.6</td>
</tr>
<tr>
<td></td>
<td>D5S117</td>
<td>11 2 7 85*</td>
<td>6 4 4 60</td>
</tr>
</tbody>
</table>

* denotes p<0.05
The limited sample size prohibits the exclusion of increased paternal concordance at these loci, both of which show non-significant trends toward increases. Similarly, increased concordance cannot be statistically ruled out at the other loci tested, given the number of informative families in the sample.

Discussion

Our results suggest a possible role for the DAT1 and SERT proteins in the etiology of autism/PDD. The markers THRA-1 and D5S117 were selected for study because of their proximity to the SERT and DAT1 genes, respectively. The elevated concordances at these two loci imply that pairs of affected male sibs are more likely to share alleles at SERT and DAT1 than expected by chance. These results suggest that mutations in the SERT and DAT1 genes contribute to the autistic/PDD phenotype in these sibships. The lack of a significant increase in concordance at the VNTR locus in DAT1 reflects the low level of heterozygosity and thus the small number of informative families for this locus, and does not rule out a role for DAT1 in autism/PDD in these sibships. While it is formally possible that other genes linked to D5S117 and THRA-1 are responsible for the increased concordance rates observed, SERT and DAT1 are the most likely candidate genes in these regions based on their involvement in monoaminergic systems. Recently, Cook et al. (1997) have reported evidence for association of the shorter allele of a functional polymorphism within the SERT gene with autism. This result supports the suggestion of involvement of SERT in the etiology of autism/PDD, and this locus is being tested in the present sample as well.

Our laboratory has recently found evidence for the involvement of the dopamine beta
hydroxylase (DBH) and monoamine oxidase A (MAOA) genes in the etiology of autism/PDD in these same families (Robinson 1996; Chapter III). Both enzymes are involved in the biosynthetic or degradative pathways of the catecholamine and biogenic amine neurotransmitters. 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. 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 monoamine neurotransmitters. The importance to normal development of monoamine 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), and by 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 monoamine(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. In most cases, there appears to be a trend toward increased concordance for alleles from
both parents. Due to the small samples sizes, increased paternal concordances cannot be
excluded, and a larger number of families needs to be examined at each of these loci. Similarly,
the paternal concordance rate for D16S398, linked to NET, approaches significance at the p<0.05
level, and thus the present study lacks the statistical power to exclude the involvement of NET or
the 16q12 region. 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 result 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.
Chapter V:

A model for the genetics of autism and the related pervasive developmental disorders combining a maternal effect with susceptibility factors in the fetus

Abstract

The genetics of autism and the related pervasive developmental disorders (autism/PDD) are currently not well understood. The segregation pattern of this group of disorders is complex, exhibiting features such as an unusual sex ratio and a relatively low recurrence risk to sibs. It seems likely that this complexity arises from a complex genetic etiology, involving multiple loci and causal heterogeneity. Traditional lod-score based linkage analysis methods have been unsuccessful in identifying any genes involved in the etiology of autism/PDD. On the basis of our results from autistic families and the literature, we propose a model for the etiology of this disorder in at least some families. In this model, autism is caused by abnormal monoamine neurotransmitter levels in maternal serum resulting from mutations in the monoamine oxidase A (MAOA) and dopamine beta hydroxylase (DBH) genes. In other words, a maternal effect mechanism, dependent on the mother’s genotype, creates a potentially damaging uterine environment in which the fetus cannot develop normally, resulting in autism/PDD. This maternal effect is modulated by fetal-specific factors, which appear to include fetal genotypes at the monoamine transporter genes (serotonin and dopamine transporters), but may also include such elements as fetal gender and birth weight. The severity of the
maternal effect may also be mediated by other biochemical and genetic factors as well as by environmental influences. This maternal effect/fetal susceptibility model explains most of the unusual genetic features of autism/PDD.

Introduction

While an important role for genetic factors in the etiology of autism and the related pervasive developmental disorders is clear from family and twin studies, identification of the genes involved or of the mode of inheritance of this group of disorders has not been possible to date. The etiology of autism/PDD is clearly complex, involving both genetic and non-genetic heterogeneity as well as polygenic inheritance. Such complexity has made it difficult to locate single genes which are involved in causing an autistic/PDD phenotype, and has made the identification of etiologically more homogeneous subgroups a key issue for genetic studies. We have suggested that families with multiple affected males and no affected females represent such a subgroup. On the basis of our findings in these families at several candidate loci, combined with evidence from the literature, we propose here a model for the genetic etiology of autism/PDD which accounts for many of the unusual characteristics of this group of disorders.
The model: Autism/PDD results from exposure of the developing fetus to abnormal monoamine levels in utero

The fundamental components of the model we propose here for the etiology of autism/PDD are illustrated in Figure I, and elaborated below in two parts: A) Maternal effect; and B) Fetal susceptibility.

A. Maternal effect: Normal gene variations or mutations in the DBH and/or MAOA genes contribute to abnormal neurotransmitter levels in maternal serum, and that exposure in utero to these levels may result in developmental insults to the fetus, leading to autism/PDD.

We have evidence for the involvement of two functionally related enzymes in the etiology of autism/PDD. The first of these, dopamine beta hydroxylase (DBH), is the enzyme responsible for the synthesis of norepinephrine (NE) from dopamine (DA) in noradrenergic neurons, and is also present in serum (Weinshilboum et al. 1971). The gene encoding DBH has been mapped to 9q34 (Craig et al. 1988), in close proximity to one of the two known tuberous sclerosis genes (Povey et al. 1994). Thus, DBH is an interesting candidate on the basis both of its biochemical function and impact on DA and NE levels, and of its chromosomal location, since autism is known to be associated with tuberous sclerosis (Smalley et al. 1991). The DBH gene has been cloned and its structure characterized (Lamouroux et al. 1991). The 5' untranslated region of the DBH
Figure I. A model illustrating the maternal effect/fetal susceptibility model for autism/PDD in male multiplex families.

See text for detailed explanation. The fetus is exposed to altered monoamine levels (DA, NE, 5-HT) in maternal serum, as a result of: genetic variation in the mother leading to abnormal monoamine metabolism; stress; pharmaceutical exposure; diet; or other environmental factors. The effects of such exposure may be amplified or modulated by factors affecting transfer of monoamines across the placenta, such as: genetic variation in the function of placental monoamine transporters; exposure to drugs such as cocaine or alcohol; or gender-specific effects. Abnormal degradation of monoamines in the placenta may also play a role, as a result of improper MAOA function in the placenta.

Abbreviations used: 5-HT - serotonin; DA - dopamine; DAT1 - dopamine transporter; DBH - dopamine β hydroxylase; MAOA - monoamine oxidase A; NE - norepinephrine; NET - norepinephrine transporter; SERT - serotonin transporter.
gene contains a dinucleotide repeat polymorphism and a 19bp insertion/deletion polymorphism as well. By analyzing the genotypes at this locus of pairs of affected boys and their parents (male multiplex or MPX sibships), we have found that alleles with the 19bp deletion are much more frequent in the mothers of these sibships than in controls, with many mothers being homozygous for the deletion (Robinson 1996). The sib pairs themselves, however, did not necessarily both inherit the 19bp deletion allele from their mothers. Thus the increased frequency of this allele in mothers appears to reflect the importance of the maternal DBH genotype to the risk of having autistic/PDD sons, rather than merely selection of mothers with sons more likely to carry the deletion allele.

We have found similar results for the monoamine oxidase A (MAOA) gene (see Chapter III). The biochemical role of MAOA is to oxidatively degrade the biogenic amines, including DA, NE and serotonin (5-HT). MAOA is expressed at the synaptic clefts of serotonergic and catecholaminergic neurons, where it functions to clear excess neurotransmitter; it is also expressed in a number of peripheral tissues (Berry et al. 1994). The MAOA gene has been mapped to Xp11.23-11.4 (Ozelius et al. 1988), directly adjacent to MAOB, a highly similar gene whose product performs the same functions but has different pharmacological and substrate specificities and a different tissue distribution. The two genes are thought to have arisen from a gene duplication event (Grimsby et al. 1991). The excess of males in the autistic population has long been thought to be suggestive of an X-linked autism gene segregating in at least some families, so
MAOA is an attractive candidate due to both its biochemical importance in biogenic amine metabolism and its chromosomal position. Mutations in MAOA have been shown to result in behavioural abnormalities (Brunner et al. 1993a,b). Of particular note is a report of two brothers carrying a deletion spanning MAOA, MAOB and the closely-linked Norrie disease gene; in addition to the blindness typical of Norrie disease, these brothers exhibited mental retardation and "autistic-like" behaviour (Sims et al. 1989). Thus disruptions of this region may give rise to an autistic phenotype.

We found a significant increase in the frequency of a particular MAOA genotype in the same mothers of male MPX sibships used in the DBH study (see Chapter III). The genotype is a haplotype combining the B2 allele at the variable number of tandem repeats (VNTR)/ CA repeat polymorphism in the 5' untranslated region of the MAOA gene (Hinds et al. 1991) and the 122 bp allele at the CA repeat polymorphism in the first intron (Black et al. 1992). These alleles were found to be significantly more frequent in the mothers of male multiplex sibships than in a sample of unrelated controls, and co-occurred as a haplotype more often than predicted on the basis of their observed frequencies in these mothers. Again, however, this haplotype was not necessarily transmitted to the affected boys, nor were affected sibs necessarily concordant for the haplotype. Thus the high frequency of the B2-122bp haplotype again appears to reflect the importance of the mother's genotype at this locus, rather than that of the child, in determining autism/PDD.
As mentioned, the increased prevalence of the DBH deletion allele or the B2-122bp haplotype in male MPX sibships cannot be explained by linked disease susceptibility alleles causing autism/PDD in the affected individuals, since the sib pairs do not appear to be more concordant at these loci. In fact, the siblings’ genotypes at these two loci appear to be largely irrelevant to the phenotype. Rather, it appears to be the maternal genotypes at these two loci which are important to the causation of autism/PDD. We have proposed that these results are best explained by a maternal effect model, in which the maternal genotype determines the fetal phenotype (Robinson 1996; Chapter III). The best-known medical example of this phenomenon is maternal phenylketonuria, in which a genetic defect in the phenylalanine hydroxylase gene leads to high maternal phenylalanine levels, causing profound mental retardation in the child (Mabry et al. 1963). We have suggested that normal gene variations or mutations in the DBH and/or MAOA genes contribute to abnormal neurotransmitter levels in maternal serum, and that exposure in utero to these levels may result in developmental insults to the fetus, leading to autism/PDD. Mutations in linkage disequilibrium with the DBH 19bp deletion and the MAOA B2-122bp haplotype, or possibly these sequences themselves, causing altered enzyme expression or activity could account for such abnormal biogenic amine levels. Which of the biogenic amines are involved in this process remains unclear, given the complexity of neurotransmitter regulation and the broad specificity of MAOA.

We have demonstrated for DBH that low serum enzyme activity correlates
with the presence of the 19bp deletion, with deletion homozygotes showing 
lowest, heterozygotes showing intermediate, and non-deletion homozygotes 
showing the highest activity *in vitro*, and that this holds true for the mothers of the 
male MPX sibships as well (Robinson 1996). Thus low DBH activity levels, 
presumably leading to high DA and low NE levels, are clearly implicated in the 
maternal effect model. The case is not so straightforward for MAOA, as this 
enzyme is not expressed in blood cells. Serum metabolite measures or activity 
assays reflect MAOB activity. MAOA activity may be measured in fibroblasts, 
but no attempt to correlate fibroblast MAOA activity with VNTR or microsatellite 
genotype has been made, and the relevance of fibroblast activity levels to those in 
other tissues and hence to the maternal effect model would be unclear. Thus we 
can currently only speculate as to the nature of the MAOA mutation involved. 
Low MAOA activity would result in increased DA, NE and 5-HT, whereas high 
activity would lower the levels of all three neurotransmitters.

Similarly, it is unclear whether the DBH and MAOA pathways represent 
independent maternal effect mechanisms, or whether altered DBH and MAOA 
levels interact and combine to increase the probability of having autistic/PDD 
children. Given the high frequencies of both the DBH deletion allele and the 
MAOA B2-122bp haplotype in the same group of individuals, and the close 
proximity of DBH and MAOA in the catecholaminergic pathway, we favour an 
additive or threshold model in which each additional ‘deleterious’ allele at either 
locus increases the severity of the maternal effect and the likelihood of an affected
child. However, this remains to be examined further.

If the maternal effect model is essentially correct, it would explain the failure of past studies to detect linkage at either of these loci. The problem becomes one of classification, as the mothers rather than the autistic children would have to be considered ‘affected’ for the purposes of linkage analysis. It would be interesting to re-examine previously tested extended pedigrees with this in mind, in light of the maternal effect model.

Clearly, the maternal effect model as described above cannot explain all the unusual features exhibited by the genetics of autism/PDD. For example, the population prevalence of autism/PDD would have to be much higher, given the frequencies of the DBH 19bp deletion allele and MAOA B2-122bp haplotype in the general population. Second, the recurrence risk to sibs should be much higher, since all of a mother’s offspring would be exposed to the same maternal effect. This is even more relevant for monozygotic and dizygotic twins, since they are exposed to the same uterine environment at the same time. Third, the model fails to account for the excess of affected males, although it is known that males are more susceptible to insults \textit{in utero} than are females (Szatmari et al. 1993).

Other modulating and mediating factors must be involved, in order to explain these differences. Two categories of factors can be considered here. The first are those influences which affect the nature of the uterine environment and hence the severity of the maternal effect; these could include other maternal
genetic factors, behaviour, pharmacological effects, and other external environmental influences. Temporal factors could come into play here as well. The second category is those factors determining fetal susceptibility to the maternal effect, and could include the fetal genotype at other loci (maternal effect susceptibility loci), fetal gender, and perhaps factors such as birth weight and correlates of birth order (in twins). Beyond these factors, it must also be remembered that the maternal effect mechanism may only be relevant to some families; etiologic heterogeneity likely exists, confounding interpretations of the autistic/PDD population as a whole.

B. Fetal susceptibility: The probability that a fetus exposed to altered maternal monoamine levels will develop autism/PDD, and/or the severity of the disorder developed, are determined by factors modulating fetal susceptibility, including the monoamine transporter loci.

As pointed out above, altered maternal biogenic amine levels due to specific alleles or mutations in MAOA and/or DBH are insufficient in themselves to explain many aspects of the population prevalence and segregation patterns of autism/PDD. The low risk to sibs, low concordance rates in twins and unusual sex ratio demand a more complex scenario involving variation in fetal susceptibility to the maternal effect. Thus a more refined genetic model would postulate the involvement of other, fetally derived genes which modulate fetal
vulnerability to the maternal effect, with allelic variations between siblings accounting for discordant phenotypes.

The maternal effect model for autism/PDD outlined above proposes that exposure of the developing fetus to abnormal biogenic amine levels in utero results in abnormal development leading to autism/PDD. A key issue raised by this model is therefore the extent of fetal exposure to biogenic amines in maternal serum during normal development. If it could not be demonstrated that such exposure occurs, one would be forced either to infer an indirect mechanism for the maternal effect, or to reject the model entirely. Thus the identification of a potential mechanism for the transfer of biogenic amines across the placenta represents a crucial test of the model.

The monoamine transporters are a group of Na\(^+\) and Cl\(^-\) dependent transmembrane proteins which show high affinities for the biogenic amine neurotransmitters. The group includes the norepinephrine transporter (NET1), serotonin transporter (SERT) and dopamine transporter (DAT1), which share a similar general structure with twelve transmembrane domains (Melikian et al. 1994). Their function is primarily to accumulate and recycle released monoamine neurotransmitters from the synaptic cleft in order to terminate synaptic transmission. Thus NET1, SERT and DAT1 are expressed on the presynaptic terminals of noradrenergic, serotonergic and dopaminergic neurons, respectively, where they transport their associated neurotransmitters across the presynaptic membrane. In addition to their synaptic localizations, however, the monoamine
transporters are also expressed in peripheral tissues. Of particular note in the context of the maternal effect model for autism/PDD which we have proposed is the expression of NET1 and SERT on the brush border membrane of the mammalian placenta (Balkovetz et al. 1989, Ramamoorthy et al. 1993). As this membrane is in direct contact with maternal serum, the expression of these proteins here suggests a role for them in the transport of monoamines across the placenta, and indeed radiolabelling assays have demonstrated such movement of monoamine transmitters from maternal serum (Ramamoorthy et al. 1992). While DAT1 is not expressed on the placental membrane, it is noteworthy that NET1 exhibits a higher affinity for DA than for NE (Ramamoorthy et al. 1992). Thus a mechanism exists for the transport of all three biogenic amine neurotransmitters across the placenta, making it likely that the fetus is exposed to monoamines from maternal serum at some point during development.

The importance of maternal serum monoamine levels to normal fetal development is as yet poorly understood. Three lines of evidence suggest that transport of maternally derived monoamines across the placenta is of relevance to normal growth and development. The first involves transgenic DBH-deficient mice, i.e. mice in which both DBH alleles have been knocked out (DBH/-) and which are thus unable to synthesize NE from DA. Whereas all the DBH/- offspring of DBH/- mothers died, demonstrating that NE is essential for fetal development, some DBH/- offspring of DBH+/- mothers survived, suggesting that these survivors were rescued by the transfer of maternal catecholamines.
across the placenta (Thomas et al. 1995). The second argument for a role of maternal monoamines in fetal development comes from studies which suggest that a synergistic interaction between growth factors and neurotransmitters is required for the differentiation of catecholaminergic neurons (Du and Iacovitti 1995). This presents a dilemma in that catecholamines must be present in the fetus prior to the differentiation of catecholaminergic neurons. One way to resolve this apparent paradox is through the exposure of the developing fetus to maternally derived catecholamines transported across the placenta (Du and Iacovitti 1995). Finally, the norepinephrine transporter expressed on the human placenta has been shown to be cocaine-sensitive (Ramamoorthy et al. 1993). Babies exposed to cocaine and related drugs in utero have been reported with a variety of developmental abnormalities, including mental retardation and autism (Harris et al. 1995); the actions of these drugs at the level of placental monoamine transport may be responsible for these developmental abnormalities. All of these results are suggestive of a role for maternal monoamines in normal development, in particular in neural differentiation and development. Exposure to altered levels of these monoamines could then be responsible for abnormal neural development, leading to autism/PDD as we have proposed. Very little is known, however, about the relative amounts and importance of the different neurotransmitters, regulation of their levels during different developmental stages, or regulation of monoamine transporter expression and function at the placental membrane. Furthermore, it is difficult to draw parallels between mortality in utero in mice
and an autism/PDD phenotype in humans. Although the details of the model at this level remain speculative, the existence of a mechanism for the transport of monoamines from maternal serum to the fetus across the placenta is consistent with the model we propose.

The placenta is fetal tissue; proteins expressed on the placental membrane are therefore determined by the fetal genotype. Based upon their presence on the placental membrane and their role in transporting monoamines from maternal serum to the fetus, the monoamine transporters thus are prime candidates for fetal susceptibility genes which could modulate the effects of altered maternal serum levels, in the context of our maternal effect model. Further, the transporters could play a role in fetal development at the neuronal level as well as, or rather than, in placental transport. In addition, the genes encoding the monoamine transporters are good candidate loci for autism/PDD in their own right, based on the successes of pharmacological treatments which target these transporters in some psychiatric patients.

For these reasons, we investigated the segregation of alleles at the NET1 SERT and DAT1 loci in our sample of 32 male MPX sibships, again using the affected sib-pair method (see Chapter IV). We hypothesized that if one of these loci were involved in contributing increased susceptibility to autism/PDD, then pairs of affected brothers should be concordant for parental alleles at that locus more frequently than expected from independent segregation. Thus we tested
highly polymorphic marker loci closely linked to the three transporter genes: NET1, mapped to 16q12.2 (Gelernter et al. 1993); DAT1 (5p15.3; Vandenberghe et al. 1992); and SERT (17q11.2-q21; Gelernter et al. 1995). Markers for the DAT1 locus were examined despite the absence of DAT1 expression on the placenta, since this lack of placental expression does not rule out a role for DAT1 in the disease mechanism. Whereas no increase in allelic concordance from either parent was observed using markers from the 16q region linked to NET1, significantly increased concordance rates for maternally inherited alleles, but not for paternal alleles, at loci linked to SERT and DAT1 were found (see Chapter IV). These results need to be interpreted with caution, as they were obtained from a relatively small number of informative families, using markers which in some cases were located some distance from the transporter loci. Thus increased concordance rates for the NET1 region, or for paternal alleles for the DAT1 and SERT regions, could not be ruled out in the above study. Current work using more male MPX families and more closely linked markers is addressing these concerns. The observation of increased concordance in affected male sibs of maternal alleles in the DAT1 and SERT regions is encouraging, however, and is suggestive of a role for these genes in the etiology of autism/PDD in these sibships, as predicted by the maternal effect model. Thus allelic variation in these genes may well contribute to the vulnerability of a fetus to altered maternal serum monoamine levels and/or modulate severity of the disorder in an affected individual, consistent with the model.
The maternal effect/fetal susceptibility model for autism/PDD we propose does not predict the apparent parent-of-origin effects observed in the concordance rates of DAT1 and SERT. Usually, an allele contributes in the same way toward expression of the disorder phenotype, regardless of the parent of origin; thus affected sibs are equally likely to be concordant for a susceptibility allele from either parent. The significant increase in concordance of only maternal alleles at these two loci, if not simply an artifact of smaller informative sample size in the fathers, points to an importance of the maternal origin of the allele in increasing the risk of autism/PDD. A possible explanation for this parent-of-origin effect is genomic imprinting of the DAT1 and SERT loci, in which expression of these genes in the relevant tissue is preferential, with only the maternal allele being expressed. Since the paternal allele is not expressed, inheritance of a susceptibility allele from the father would have no effect on the manifestation of the disorder, and pairs of affected offspring would be no more likely to share a paternal allele identical by descent than would any other sib pair. Inheritance of the susceptibility allele from the maternal side, in contrast, would be followed by expression of that allele and a concomitant increase in fetal susceptibility, with pairs of affected sibs being more likely to be concordant for the susceptibility allele. In the context of the maternal effect/fetal susceptibility model for autism/PDD, it is interesting to note that many genes involved in signalling and maternal-fetal interactions across the placenta are in fact imprinted (DeGroot and Hochberg 1993). Hence it would not be altogether surprising to find the
imprinting process at work at those transporters expressed on the placenta (SERT, NET1). The imprinting of DAT1 would be more unexpected, since it does not appear to be localized to the placenta; but given the structural similarities of all three monoamine transporters, similarities in their regulation at the genomic imprinting level are perhaps not unlikely.

The importance of genomic imprinting at the DAT1 and SERT loci to the genetic etiology of autism/PDD is unclear at this point, and awaits more extensive studies with larger informative sample sizes. Similarly, a larger sample will be required to rule out the involvement of the NET1 locus. Further, more detailed examination of the chromosomal regions surrounding each of these loci is necessary in order to rule out other genes in these regions as candidates for susceptibility loci. However, the current data are consistent with a role for at least two of the three monoamine transporters in the genetic etiology of autism/PDD, as predicted by the maternal effect model we have proposed.

The model proposes that abnormal function or expression of these transporter proteins results in an increase or decrease in the amount of maternal monoamines transferred across the placenta and/or inappropriate response to these monoamines by the fetus, causing abnormal neural development leading to autism/PDD. Processes of neural differentiation and development are often initiated and controlled by delicate balances and precise gradients and ratios of neurotransmitters and growth factors (Lauder 1988); small deviations could well have major consequences for the development of specific cognitive, motor and
behavioural functions which would also explain the broad range of variation in clinical presentation, even among pairs of siblings.

Within the framework of the model, we can envision several possible generalized scenarios resulting in the exposure of the fetus to abnormal monoamine levels in utero, leaving aside any consideration of the specific effects of this exposure on development. The combination of high maternal serum monoamine levels with relatively high transport across the placenta results in abnormally high exposure leading to an elevated risk of having an affected child, or of greater severity, whereas high serum levels and relatively low transport results in lower exposure, comparatively less risk and/or lower severity. Alternately, low maternal serum levels and decreased rates of transport could result in insufficient exposure of the fetus to a required monoamine and thus lead to increased risk and/or severity, whereas high transport rates could compensate for low serum levels and decrease risk and/or severity. Without detailed information regarding the specific neurotransmitter(s) involved, the effects of the putative mutations in MAOA, DBH and the transporter(s) involved, and the relative amounts of maternal transmitters required for normal development, the maternal effect/fetal susceptibility model described here accommodates each of these possibilities equally well, and it is impossible to distinguish between them using the data available to date. Moreover, it is conceivable that defects in monoamine transporter function represent a separate and independent mechanism for the etiology of autism/PDD, although the findings of altered DBH and MAOA
allele frequencies, decreased DBH activity, and increased concordance rates at SERT and DAT1 all in the same sample of male MPX sibships suggests this is unlikely. Rather, the results we have presented support a model of positive interaction, in which each additional detrimental allele increases the risk of an affected child, and possibly the severity of the disorder if the child is affected.

A further issue in considering the maternal effect/fetal susceptibility model is the developmental timepoint or window during which the fetus is vulnerable. Recent reports of autism resulting from thalidomide (Strömland et al. 1994) or valproic acid exposure (Collins et al. 1991), as well as experiments exposing rat embryos to valproic acid (Rodier et al. 1996), suggest that lesions to the motor cranial nerve nuclei at the time of neural tube closure are associated with autism. Thus the fetus may be susceptible to the effects of abnormal monoamine neurotransmitter exposure during a fairly narrow window of time around closure of the neural tube, during the fourth week of gestation.

The maternal effect/fetal susceptibility model for autism/PDD as proposed here accommodates the unusual aspects of the complex genetics of the disorder to a significant degree. Based upon a maternal effect alone, the frequency of autism/PDD in the general population would be very high, given that the DBH 19bp deletion alleles and the MAOA B2-122bp haplotype are quite frequent and maternal effect genotypes therefore quite common. Without invoking other modulating factors, a strict maternal effect model in which exposure of the fetus
to altered monoamine levels was sufficient to cause autism/PDD would predict 100% penetrance and therefore 100% recurrence risk to sibs and an equal sex ratio of affected offspring, since all children of a mother expressing the maternal effect would be affected. Similarly, no difference in concordance would be expected between MZ and DZ twins, nor would we expect to see variation in the type or severity of the disorder. Clearly, this model does not fit with the observed population frequency of autism/PDD (4 in 10000), the relatively low risk to sibs of an affected individual (2.5-4%), the unusual sex ratio (four affected males for every female), the increased concordance in MZ vs. DZ twins (64% vs 9%: Smalley et al. 1988), or the variable phenotypic presentation of the disorder within families.

The requirement for a fetal genotype at the SERT, DAT1 and/or NET1 loci which increases vulnerability to the maternal effect addresses these disparities. Parents would have to contribute susceptibility alleles at these loci to the offspring, and thus the probability of a mating producing the necessary combination of genotypes is substantially lower. It is impossible to estimate this probability, since the susceptibility alleles have not been identified and their frequencies are therefore unknown, and since the number of susceptibility alleles required to produce an autistic phenotype is unclear, but even with relatively frequent alleles the probability of the necessary combination of maternal alleles at MAOA and/or DBH with fetal alleles at DAT1, NET1 and/or SERT becomes much lower. The possibility of a parent-of-origin effect at the two monoamine
transporter loci reduces this probability even further, since it would require
inheritance of the susceptibility allele(s) from the maternal side. A female would
thus require a rather rare combination of genotypes at three or four, and possibly
more, loci in order to be at risk for having autistic/PDD children.

It would appear, however, that a single susceptibility locus cannot be
sufficient to explain the segregation pattern of autism/PDD, since the recurrence
risk to sibs, assuming the maternal effect to be constant, could be no lower than
0.25 (for a homozygous recessive model), lower than 1.00 but clearly still too
high. A combination of two susceptibility loci, on the other hand, could allow for
recurrence risks to sibs as low as 0.0625 (homozygous recessive at both loci) or
around 6%, in the range observed in autistic sibships. Thus it seems possible that
a small number of susceptibility genes in conjunction with the two maternal effect
loci DBH and MAOA could be sufficient to account for the population frequency
and recurrence risk to sibs for autism/PDD.

The above calculations treat the disorder as a dichotomous trait
(affected/unaffected). To account for the variability of clinical expression and
broad phenotypic range observed in the autistic spectrum disorders, even between
siblings, more factors must be postulated which modulate expression. Similarly,
no number of autosomal susceptibility loci can account for the skewed sex ratio
among affected individuals, nor for the low rates of phenotypic concordance in
MZ twins.

Two types of factors may be thought to contribute to the unusual sex ratio
observed in autism/PDD. One type is sex-linked or sex-determined traits which might operate as additional fetal susceptibility loci. Expression of fetal susceptibility genes located on the X chromosome would have greater impact on males than on heterozygous females, resulting in increased risk or severity for a male fetus. One particularly interesting candidate in this regard is MAOA itself, mapped to Xp11.23-p11.4. In addition to its expression in neurons and elsewhere, MAOA is highly expressed in placenta, where its role is thought to be in the elimination of 5-HT from the uterine environment (Koren et al. 1965). It is noteworthy that intrauterine injection of the MAO inhibitor pargyline is effective in inducing abortion in both rats (Koren et al. 1965) and humans (Koren et al. 1966). Thus, adequate levels of functional MAOA in utero are clearly necessary for normal fetal development. Since, in the context of the maternal effect/fetal susceptibility model, a mutant MAOA allele is present in much higher frequency in at-risk mothers, the male fetus stands to inherit this allele and express it in the placenta. Whatever effect this mutation has on MAOA activity in the mother could be exacerbated by the expression of the same allele in the fetus. So, for example, if the maternal effect results from low MAOA activity and therefore from elevated maternal serum monoamine levels crossing the placenta, low fetal MAOA activity in the placenta would lead to even higher than normal exposure in the fetus, greater risk and/or greater severity. A fetus inheriting a higher-activity allele from his mother might be able to reduce monoamine levels in the placenta and reduce exposure. Female offspring would in most cases receive a normal
MAOA allele from their fathers, which could compensate for maternally inherited susceptibility alleles. While such logic makes MAOA an appealing candidate for a fetal susceptibility locus in addition to its role in creating a maternal effect, no evidence for increased allelic concordance was observed in pairs of affected brothers (see Chapter III). While the study lacked the statistical power to rule out increased concordance rates, the evidence to date suggests that if MAOA does play a role in fetal susceptibility, it may be more important in modulating severity than in determining affected status.

Another potential X-linked locus affecting fetal susceptibility could be located in Xq24. Hallmayer et al. (1996) have recently reported a small positive lod score (1.24) at the DXS 424 locus. If a gene linked to this locus indeed acts to increase fetal susceptibility it could also contribute to the excess males in the autism/PDD population. However, in our own affected sib pair study of X-linked loci, we were unable to detect a significant increase in concordance between affected brothers at the DXS424 locus (see Chapter II).

The other type of factor which could influence the sex ratio of affected individuals is the gender of the fetus itself. It is known that females are less vulnerable to a variety of insults in utero (Szatmari et al. 1993). Thus by virtue of being female a fetus might be inherently less susceptible to the effects of increased maternal serum monoamines, and might require a greater genetic load in order to develop the disorder. This notion is intriguing in that the disorder in affected brothers of an autistic girl tends to be very severe (Szatmari, pers.)
The final aspect of autism genetics for which the maternal effect/fetal susceptibility model needs to account is the low rate of phenotypic concordance in MZ twins. Since such twins are genetically identical and experience the same uterine environment simultaneously, the model predicts that both twins be affected equally. It is known, however, that one twin is typically larger and receives a greater proportion of maternal resources in utero than his or her co-twin (Machin 1996). We suggest that this differential effect might be responsible for the discordant expression in MZ twins, with one twin being exposed to the maternal effect more or less acutely and as a result being more or less severely affected. Uterine position effects may also play a role, as it has been demonstrated in rats that blood flow to the maternal portion of the placenta varies greatly depending on the position in the centre of the uterus or in either uterine horn. Differences in blood flow according to the sex of the fetus have also been observed (Even et al. 1994). Such differences in fetal size, sex and uterine position could well account for discordant phenotypes in twins. A second possibility is that differences between MZ twins arise as a consequence of epigenetic phenomena which occur postmeiotically, or after the splitting of the embryo. These could include such mechanisms as random inactivation of one allele at a susceptibility locus, or cytogenetic or genomic instability, such as trinucleotide repeat expansion. The latter is an attractive possibility, as mitotic instability at a trinucleotide repeat within a susceptibility gene could result in
different repeat lengths in otherwise identical twins and therefore in discordant phenotypes.

The maternal effect/fetal susceptibility model we have proposed accounts for many of the unusual characteristics of the genetics of autism/PDD. Predictions made by the model with respect to the involvement of monoamine transporters as fetal susceptibility factors appear to have been borne out, and the data which has been accumulated to date are consistent with it. A number of important details need to be elucidated to test and solidify the model: in particular, the nature of the putative mutations in DBH and MAOA need to be determined to establish their effects on serum bioamine levels; the relevant bioamine(s) responsible for the maternal effect must be identified; the monoamine transporters must be established as the fetal susceptibility loci in their chromosomal regions; and the effects of altered bioamine levels on fetal neurodevelopment must be established. Furthermore, the results obtained to date for MAOA, DBH and the monamine transporter loci must be replicated in other, more extensive samples of multiplex families. Only when some of these questions have been addressed will it be possible to design further critical tests of the model.

It is also vital to bear in mind that the maternal effect/fetal susceptibility model was constructed on the basis of observations from a relatively small number of selected families. Care must therefore be taken in extending the model beyond those families with multiple affected boys and no affected girl siblings.
The increased frequency of the MAOA B2 haplotype in mothers of male MPX mothers could not be detected in mothers of SPX families, for instance, suggesting that the compositions of these groups differ significantly and that the MPX sibships represent a selected subgroup of the autistic/PDD population. It is likely that etiologic heterogeneity exists, with other genetic or non-genetic factors causing autism/PDD independently of, or contributing to, the maternal effect/fetal susceptibility pathway. The contribution of the model to the overall prevalence of autism/PDD is at this point difficult to estimate, but it is hoped that the development of the model may lead to the definition of clinically and etiologically relevant subgroups in which these issues may be addressed.

In the meantime, however, the maternal effect/fetal susceptibility model represents the first satisfactory theoretical framework for the genetic etiology of autism/PDD. If it is supported by replication and future examination, it may well also provide a conceptual model for the genetic bases of many of the complex neuropsychiatric disorders which have for so long resisted elucidation.
Abstract

A gene responsible for X-linked mental retardation with macrocephaly and seizures (MRX38) in a family with five affected males in three generations was localized to Xp21.1-p22.13 by linkage analysis. Recombination events placed the gene between DXS1226 distally and DXS1238 proximally, defining an interval of approximately 14 cM. A peak lod score of 2.71 was found with several loci in Xp21.1 (DXS992, DXS1236, DXS997 and DXS1036) at a recombination fraction of zero. The map intervals of five X-linked mental retardation loci, MRX2 (Xp22.1-p22.2), MRX19 (Xp22), MRX21 (Xp21.1-p22.3), MRX29 (Xp21.2-p22.1) and MRX32 (Xp21.2-p22.1), and two syndromal mental retardation loci, Partington Syndrome (PRTS; Xp22) and Coffin-Lowry Syndrome (CLS; Xp22.13-p22.2), overlap this region. As none of these display the same phenotype seen in the family reported here, this X-linked mental retardation locus represents a new entity.
Introduction

The incidence of X-linked mental retardation has been estimated at approximately 1/600 male births. Of these, 25-40% represent cases of fragile X syndrome (Sutherland and Hecht, 1985). The remainder of X-linked mental retardation cases may be subdivided into other recognized syndromal forms, if consistent phenotypic characteristics are present, or non-specific forms, if mental retardation is the only consistent feature among affected individuals. The most recent comprehensive list of X-linked mental retardation includes 127 syndromal and non-specific entities (Neri et al., 1994). Due to the difficulties in clinical definition of mental retardation entities with inconsistent phenotypic characteristics, the grouping of families for the purpose of linkage analysis is unwise. For this reason, only mapping information from sufficiently informative single families (lod score +2.0 or greater) (Mulley et al., 1992) is used. The disease locus segregating in such a family is assigned a sequential MRX designation. Thus, a single mental retardation gene may acquire several different designations in independent families.

Of the MRX loci given a regional assignment by linkage analysis, the majority map to the pericentric region. We report here an X-linked mental retardation locus (MRX38) mapping to Xp21.1-p22.13, and examine the overlap in clinical characteristics and genetic locations between this and other mental retardation conditions mapped to this region.
Materials and Methods

Clinical Data

The pedigree of the family examined is presented in Figure 1. The pedigree shows the segregation of an X-linked recessive disorder, with transmission through unaffected carrier females to six affected males in three generations. All affected males show mild to moderate mental handicap with particular abnormalities with speech. No specific facial or other physical features were seen, with the exception of macrocephaly in all affected males who, in addition, experienced seizures at some stage in life, to varying degrees.

Photographs of the affected individuals in this family are shown in Figure 2. Individual II:10 (Figure 2a), aged 49 years, was always severely delayed, had some speech and poor hand use. Seizures began at 6 years of age but with treatment are now rare. He has not walked for several years and has lost all speech. He has marked pes cavus with high arches and clawed toes, and a large head circumference (59.5 cm; >98 centile), but no other phenotypic abnormalities.

Individual III:3 (Figure 2b), age 36 years, has had seizures from an early age; is mildly delayed; walks; talks with stuttering; and is able to perform some manual work. He has had no schooling. He has a large head circumference (58.4 cm; >98 centile).

III:6 (Figure 2c), aged 34 years, has education to Grade 7 and can read and write a little, had seizures only in childhood, and a head circumference of 58.4 cm (>98 centile).
Figure 1. Pedigree structure of the extended MRX38 family.
Fig. 1. Pedigree structure of the MFX38 family.
Figure 2. Five of the six affected males in family MRX38. a) Individual II:10 b) Individual III:3 c) Individual III:6 d) Individual III:13 e) Individual IV:2.
III:13 (Figure 2d), aged 24 years, has global developmental delay with particular speech problems. At 9 years he had several grand mal seizures and remains on anticonvulsants although he has been seizure-free for years. He attended school and reads and writes to some extent, has poor speech and stutters. He is cheerful, outgoing and athletic. His head circumference measures 60.9 cm (>98 centile).

IV:2, aged 14 years, has global delay, abnormal EEG pattern and consistently recorded macrocephaly, with a head circumference at age 7 years of 55.5 cm (>98 centile). He showed some evidence of early seizures but is now seizure-free, and is doing well in special education in Grade 7.

Clinical characteristics of the six affected males are summarized in Table I.

DNA Analysis

Genomic DNA was obtained from peripheral lymphocytes by standard phenol-chloroform extraction. The polymerase chain reaction was used to amplify microsatellite alleles from 50 ng genomic DNA, using specified conditions in a Perkin Elmer/Cetus 400 or 480 thermal cycler, with 28 cycles of denaturation (94C, 1'), annealing (52-60C, 1') and extension (72C, 1') followed by
Table 1. Phenotypic characteristics observed in affected individuals.

<table>
<thead>
<tr>
<th></th>
<th>Individual II:3</th>
<th>Individual II:10</th>
<th>Individual III:3</th>
<th>Individual III:6</th>
<th>Individual III:13</th>
<th>Individual IV:2</th>
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<tbody>
<tr>
<td>Age</td>
<td>deceased</td>
<td>49 years</td>
<td>36 years</td>
<td>34 years</td>
<td>24 years</td>
<td>14 years</td>
</tr>
<tr>
<td>Seizures</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OFC (centile)</td>
<td>not available</td>
<td>59.5cm &gt;98%</td>
<td>58.4cm &gt;98%</td>
<td>58.4cm &gt;98%</td>
<td>60.9cm &gt;98%</td>
<td>55.5cm at 7yrs &gt;98%</td>
</tr>
<tr>
<td>Height</td>
<td>not available</td>
<td>166cm &lt;3%</td>
<td>166cm &lt;3%</td>
<td>173cm 10%</td>
<td>118cm at 7yrs 3%</td>
<td></td>
</tr>
</tbody>
</table>
a final extension (72°C, 10'). Alleles at the FMR-1 and FRAXE loci were amplified using published conditions (references in Table 2). α²³P-dCTP was incorporated into the PCR products. Reaction products were separated on 6% polyacrylamide, 8M urea sequencing gels and detected by autoradiography. Alleles were numbered in order of decreasing size, or sized by comparison to a control sequence, for each locus.

Two-point linkage analyses were performed using MLINK of the LINKAGE software package, assuming X-linked recessive inheritance.

Results

The family was initially screened with a series of highly polymorphic microsatellite markers distributed along the entire length of the X chromosome. No evidence of linkage was observed at any of 12 markers in Xq, but linkage was suggested in Xp, leading us to use further microsatellite markers to refine the localization and identify key recombination events (Table 2). Pairwise lod scores are given in Table 3 for the mental retardation locus against 23 markers in Xp and 12 markers in Xq, ordered Xpter-Xcen-Xqter. The relative order of these markers has been determined by physical and genetic mapping (Willard et al., 1994; Wang
Table 2. X-linked PCR-based polymorphic loci used in linkage analysis. Loci are ordered Xpter-Xcen-Xqter.

<table>
<thead>
<tr>
<th>KAL.5</th>
<th>Xp22.32</th>
<th>Boudreux et al., Nucl. Acids Res. 15,19,5453 (1991)</th>
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<tr>
<td>DXS 987</td>
<td>Xq22.2</td>
<td>Gyapay et al., Nat. Genet. 7, 246-338 (1994)</td>
</tr>
<tr>
<td>DXS 207</td>
<td>Xq22.2</td>
<td>Oudej et al., I. Mol. Genet. 30, 300-303 (1992)</td>
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<tr>
<td>DXS 1053</td>
<td>Xq22.2</td>
<td>Gyapay et al., Nat. Genet. 7, 246-338 (1994)</td>
</tr>
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<td>5' DMD</td>
<td>Xp21.1</td>
<td>Hugnot et al., Nucl. Acids Res. 19, 11, 3159 (1991)</td>
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<td>DXS 7</td>
<td>Xp11.4</td>
<td>Moore et al., Nucl. Acids Res. 20, 4, 929 (1992)</td>
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<td>ARA</td>
<td>Xq12</td>
<td>Edwards et al., Genomics 12, 241-253 (1992)</td>
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<td>DXS 439</td>
<td>Xq13.1</td>
<td>Weber et al., Nucl. Acids Res. 18, 13, 4037 (1990)</td>
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<td>DXS 441</td>
<td>Xq13.2</td>
<td>Ram et al., Nucl. Acids Res. 20, 6, 1428 (1992)</td>
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<td>DXS438</td>
<td>Xq21.33</td>
<td>Weber et al., Nucl. Acids Res. 18, 13, 4037 (1990)</td>
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<td>DXS 454</td>
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<td>Weber et al., Nucl. Acids Res. 18, 13, 4037 (1990)</td>
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<td>DXS 294</td>
<td>Xq26.3</td>
<td>Godson et al., Nucl. Acids Res. 19, 18, 5087 (1991)</td>
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<td>FMR-1</td>
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<td>Fux et al., Cell 67,4, 1047-1058 (1991)</td>
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<td>FRAXE</td>
<td>Xq28</td>
<td>Sutherland et al., Hum. Mol. Genet. 1, 111-113 (1992)</td>
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<td>GABRA3</td>
<td>Xq28</td>
<td>Hicks et al., Nucl. Acids Res. 19, 14, 4016 (1991)</td>
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Table 3. Two-point lod scores for MRX38 with markers in Xp.

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<th></th>
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<th>0.05</th>
<th>0.10</th>
<th>0.15</th>
<th>0.20</th>
<th>0.30</th>
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<td>0.11</td>
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<td>0.14</td>
<td>0.34</td>
<td>0.43</td>
<td>0.43</td>
<td>0.28</td>
</tr>
<tr>
<td>DXS 987</td>
<td>-∞</td>
<td>-0.05</td>
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<td>0.57</td>
<td>0.63</td>
<td>0.58</td>
<td>0.36</td>
</tr>
<tr>
<td>DXS 207</td>
<td>-∞</td>
<td>0.67</td>
<td>0.83</td>
<td>0.86</td>
<td>0.83</td>
<td>0.65</td>
<td>0.38</td>
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<tr>
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<td>-0.05</td>
<td>0.39</td>
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<td>0.63</td>
<td>0.58</td>
<td>0.36</td>
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<tr>
<td>DXS 418</td>
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<td>-0.12</td>
<td>0.11</td>
<td>0.22</td>
<td>0.29</td>
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<td>DXS 999</td>
<td>-∞</td>
<td>0.11</td>
<td>0.32</td>
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<td>0.83</td>
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<td>0.51</td>
<td>0.46</td>
<td>0.41</td>
<td>0.29</td>
<td>0.16</td>
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<tr>
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<td>-∞</td>
<td>1.23</td>
<td>1.34</td>
<td>1.32</td>
<td>1.24</td>
<td>0.95</td>
<td>0.54</td>
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<td>0.60</td>
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<td>DXS 1061</td>
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<tr>
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<td>1.32</td>
<td>1.24</td>
<td>0.95</td>
<td>0.54</td>
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<tr>
<td>5' DMD</td>
<td>-∞</td>
<td>1.23</td>
<td>1.34</td>
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<td>1.24</td>
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<td>DXS 538</td>
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<tr>
<td>DXS 7</td>
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<td>0.23</td>
<td>0.20</td>
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</table>
et al., 1994). The highest lod score, $z_{\text{max}} = 2.71$ was obtained with the dinucleotide repeat markers DXS992, DXS1236, DXS997 and DXS1036, each at $\theta=0.00$. This is equal to the highest $z_{\text{max}}$ expected for this family using the samples available, as all obligate carriers were informative at these loci. Haplotype analysis was used to determine the most likely phases in the females, and to identify crossover events between marker loci (Figure 3). A recombination event in individual III:13 places the disease locus proximal to DXS1226 in Xp22.13, while another recombination event in individual IV:2 places the locus distal to DXS1238 in Xp21.1. Together these two boundaries define genetic and physical intervals of approximately 14 cM and 9 Mb, respectively.

**Discussion**

X-linked mental retardation is a heterogeneous disorder. Even once cases of fragile X syndrome are removed, a large number of mental retardation entities exist which appear to be distinct on the basis of either genetic localization or clinical presentation. The difficulties in defining clear, consistent phenotypes prevents the grouping of families demonstrating clinical similarities. The limited genetic information available even from fairly large single pedigrees, however, makes precise mapping of X-linked mental retardation loci difficult, resulting in
relatively large map intervals for MRX entities. In many instances, these intervals overlap for different MRX loci; more precise localization with new markers or ultimately cloning of MRX genes will be necessary in order to determine which MRX entities arise from mutations in the same genes.

The peak lod score obtained for the family described here exceeds +2.0; this locus thus obtained the designation MRX38. Recombination events in affected individuals define the candidate region between DXS1226 distally and DXS1238 proximally, in Xp21.1-p22.13. This region includes the 3' end of the gene responsible for Duchenne and Becker muscular dystrophies, and overlaps the map intervals for two syndromal MR entities, Partington Syndrome (PRTS) (Partington et al., 1988) and Coffin-Lowry Syndrome (CLS) (Temptamy et al., 1975), as well as five non-specific mental retardation loci, MRX2 (Hu et al., 1994), MRX19 (Donelly et al., 1994), MRX21 (Kozak et al., 1993), MRX29 (Hane et al., 1995) and MRX32 (Howard-Peebles et al., 1979; Hane et al., 1995). PRTS, described as a syndrome of mental retardation, dystonic movements of the hands and dysarthria, has been mapped between DXS365 and DXS28, with a peak lod score at DXS989 (Gedeon et al., 1994). The features of CLS include a characteristic facies and minor manifestations in females suggesting X-linked dominant inheritance. The CLS locus has been mapped between Afm291w5 and
DXS1683 in Xp22.13 (Biancalana et al., 1994). The mental retardation disorder described here does not show X-linked dominant inheritance or the physical characteristics of either CLS or PRTS, and is therefore unlikely to reflect a new family with either of these conditions.

The linkage interval for MRX19 is defined by KAL and DXS989, so that the overlap with MRX38 spans the approximately 3cM region between DXS1226 and DXS989. Since MRX19 results in mild mental retardation in carrier females (Choo et al., 1984), it does not appear to be the same entity as MRX38. The family in which MRX2 was mapped to the DXS365-DXS989 interval (Hu et al., 1994) contained two affected males with large heads and seizures, but also included females described as slow and macroorchidism in affected males (Proops et al., 1983), features which are not present in the family described here. The overlap between these two candidate regions is limited by DXS1226 and DXS989. Kozak et al. (1993) described a family with four male mentally retarded patients with a characteristic facies, two mildly retarded obligate carrier females with no phenotypic abnormalities. This locus, subsequently designated MRX21, was mapped to Xp21.1-p22.3, and on the basis of the characteristic facies and affected females would appear to represent a distinct entity from MRX38. The MRX29 locus has been mapped to Xp21.2-p22.1, and is described to cause severe
mental retardation (Hane et al., 1995), unlike the moderate phenotype observed in this family. MRX32 leads to mental retardation of variable severity, apparently without any other abnormalities, and has been localized to Xp21.2-p22.1 as well (Howard-Peebles et al., 1979; Hane et al., 1995). Phenotypically, MRX29 and MRX32 appear distinct from MRX38.

The disorder described here, consisting of mental retardation and macrocephaly as the only consistent features, and designated MRX38, thus appears to represent a new non-specific X-linked mental retardation entity, clinically distinct from other previously described mental retardation syndromes in the Xp21.1-Xp22.13 region. The actual number of loci in this region responsible for X-linked disorders with mental retardation as a feature is unknown, and awaits the more precise localization and cloning of X-linked mental retardation genes.
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Appendix I. Genotypes at X-linked loci for all typed individuals in male multiplex sibships.
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