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MAOA, DBH and *SLC6A4* variants in CHARGE: A case control study of autism spectrum disorders

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Abstract

Background—Genetic factors are established to contribute to the development of autism. We examined three loci, serotonin transporter (*SLC6A4*), dopamine hydroxylase (*DBH*) and the variable number of tandem repeat promoter of the monoamine oxidase A (*MAOA*) for association with autism in participants from the CHARGE (CHildhood Autism Risks from Genetics and the Environment) Study, the first large-scale population-based case-control investigation of both environmental and genetic contributions to autism risk.

Methods—Among male children enrolled in the CHARGE study we tested associations between each of the three polymorphisms and autism (AU) (n=119), or a combined group of autism and other autism spectrum disorders (AU+ASD, which includes an additional n=53) as compared with typically developing controls (TD, n=137).

Results—The case-control association analysis showed neither *SLC6A4* nor *DBH* to be statistically significantly associated with AU or ASD. However, the male children carrying 4 tandem repeats in the promoter region of the *MAOA* gene showed a 2-fold higher risk of AU (or AU+ASD) than those carrying allele 3, adjusted for confounders (OR = 2.02, 95% CI = 1.12, 3.65, p = 0.02 for AU vs. TD, and OR = 2.05, 95% CI = 1.19, 3.53, p = 0.01 for ASD vs. TD). In addition, mothers homozygous for the 4 tandem repeat allele showed at least a 3-fold higher risk of AU (or AU+ASD) than mothers homozygous for allele 3 (OR = 3.07, 95% CI = 1.19, 7.91, p = 0.02 for AU vs. TD, and OR = 3.26, 95% CI = 1.35, 7.89, p = 0.009 for AU+ASD vs. TD).

Conclusions—These results suggest a potential role of the functional MAOA promoter alleles in the male child, the mother, or both in autism spectrum disorders.

Keywords

ASD; polymorphisms; *SLC6A4*; *MAOA*; *DBH*

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INTRODUCTION

Autism spectrum disorders (ASD) are complex, behaviorally defined, pervasive developmental disorders of early childhood characterized by impaired social relationships and communication, language deficits or disorders, and restricted, repetitive, or stereotyped behaviors. The incidence and the prevalence of ASD in the general population has dramatically increased in the last few decades, with a recent CDC Center for Disease Control and Prevention prevalence estimate of ASD at about one in 110, varying by geographic location and source of the diagnostic information [Autism and Developmental Disabilities Monitoring Network Surveillance Year 2002 Investigators, 2007]. A male to female ratio of approximately 4:1 has been described; however the ratio varies considerably in different populations [Fombonne, 2003; Gillberg and Coleman, 2000; Yeargin-Allsopp et al., 2003]. Although the majority of children with autism are diagnosed because they fail to reach the established developmental milestones, approximately 30–50% show regression in language or social skills after an initial period which may be characterized by either typical or atypical development [Goldberg et al., 2003; Hansen et al., 2008; Ozonoff et al., 2010; Shinnar et al., 2001]. While environmental factors are likely to contribute to risk and severity [Amaral et al., 2011; Zimmerman et al., 2009], it is generally agreed that autism has high heritability [Buxbaum, 2009; Lamb et al., 2000; Wassink and Piven, 2000] based on recurrence rates in siblings of autistic children and the greater concordance of monozygotic as compared with dizygotic twins [Bailey et al., 1995; Hallett et al., 2010; Lichtenstein et al., 2010; Ritvo et al., 1985; Steffenburg et al., 1989]. Multiple loci are associated with an ASD phenotype [Bill and Geschwind, 2009; Freitag, 2007; Freitag et al., 2009; Muhle et al., 2004]. Recent genomic-wide association studies (GWAS) have identified several copy number variants (CNVs) associated with autism [Glessner et al., 2009; Ma et al., 2009; Marshall et al., 2008; Wang et al., 2009]. It is also becoming clear that in order to understand the genetics of autism, attention needs to be focused on the specific molecular pathways that are dysregulated in ASDs.

Elevated levels of serotonin have been found in 30–50% of autistic patients [Anderson et al., 1990; Anderson, 2002; Cook and Leventhal, 1996; Hranilovic et al., 2008; Owley et al., 2002; Piven et al., 1991]. Serotonin is a developmental regulator of neural circuits, and altered brain levels could contribute to the manifestation of autism [Cases et al., 1996; Gaspar et al., 2003; Nordquist and Oreland, 2010]. In addition, numerous studies have provided evidence that the serotonin transporter gene contributes to autism. A 22 bp VNTR located in the promoter region of the gene gives rise to 2 alleles, the long allele (L) with 16-repeats and the short allele (S) with 14-repeats [Heils et al., 1996]. However, results across studies are contradictory, with some reporting the S allele [Cook et al., 1997] others the L allele [Klauck, 1997] and still others neither allele [Maestrini et al., 1999; Persico et al., 2000] to be associated with autism, making its pathogenic role unclear [Bartlett et al., 2005]. Due to the small sample size of most of these individual studies, the results are imprecise and may reflect random fluctuations rather than underlying differences. Two recent larger studies (>350 each) showed contradictory results regarding transmission of the serotonin transporter in autism, one sample from the Collaborative Programs of Excellence in Autism (CPEA) network [Devlin et al., 2005], and the other a combined analysis from Autism Genetics Resource Exchange (AGRE) and Southwestern Autism Research Center [Ramoz et al., 2006]. In the past few years, meta-analyses have been conducted to overcome underpowered association studies. One meta-analysis [Guhathakurta et al., 2006] suggested a significant over-transmission of the S allele, whereas Devlin and colleagues [Devlin et al., 2005] found 12 studies that reported over-transmission but were almost equally divided as to which allele was more commonly transmitted in autism. Most recently, Huang and Santangelo conducted a meta-analysis of 14 family and population based studies and failed to find any association between the serotonin transporter gene and autism, even when

simplex studies (11 studies with a single affected child) and multiplex studies (3 studies with more than one affected child) were analyzed separately.

Finally, the use of selective serotonin reuptake inhibitors (SSRIs) has been therapeutically beneficial in treating ritualistic behavior and aggression in autistic patients [Gilman and Tuchman, 1995; Gordon et al., 1993; Hellings et al., 1996; McDougle et al., 1996; Pigott and Seay, 1999; Todorov et al., 2000]. Although some of these findings have not been reproduced in other studies [Williams et al., 2010] collectively these observations have stimulated considerable interest in the possible involvement of serotonin in the etiology of autism.

Allelic variation at two loci, the monoamine oxidase A (*MAOA*) and the dopamine β-hydrolylase (*DBH*) could also be modifiers of autism risk. *MAOA*, located on the short arm of the X chromosome, catalyzes the oxidation of three neurotransmitters implicated in the pathogenesis of neurobehavioral disorders, namely dopamine, norepinephrine and serotonin, whereas *DBH* catalyzes the conversion of dopamine to norepinephrine. Gene expression of *MAOA* correlates with a 5'-untranslated 30bp variable number tandem repeat (uVNTR) functional polymorphism, which is located in the promoter region of the gene, such that the 3 allele is associated with decreased and the 4 allele with increased transcriptional activity [Deckert et al., 1999; Sabol et al., 1998]. The 4 allele has been found in association with ADHD and anxiety [Deckert et al., 1999; Roohi et al., 2008; Samochowiec et al., 2004]. Cohen et al. [2003] reported an association between the 3 allele and more severe symptoms of autism spectrum disorders (ASD) as well as a tendency toward lower intellectual abilities [Cohen et al., 2003; Yirmiya et al., 2001], however these findings were not replicated by Jones and collaborators [Jones et al., 2004]. Moreover, in a follow-up to their original work, Cohen and coworkers [Cohen et al., 2010] showed that the maternal MAOA uVNTR genotype was a significant modifier of the association between the male child's *MAOA* and several autistic traits. A recent case control study of Korean family trios with children diagnosed with ASDs and neurotypical controls examined MAOA uVNTR and four single nucleotide polymorphisms (SNPs), revealing a significant difference in haplotype frequencies between ASD patients and controls [Yoo et al., 2009].

Previous studies have shown decreased DBH activity, which is essential for intravesicular synthesis of epinephrine and norepinephrine, in individuals with autism compared with age-matched controls [Lake et al., 1977]. One study suggested that lowered maternal serum DBH activity results in a suboptimal uterine environment, with decreased norepinephrine relative to dopamine, which, in conjunction with genetic susceptibility of the fetus, might result in ASD in some families [Robinson et al., 2001].

Given several lines of evidence suggesting that a dysregulation of serotonergic, and possibly dopaminergic, neurotransmission may be involved in the pathogenesis of autism and that their functional polymorphisms affect neurotransmitter metabolism and kinetics, we considered the serotonin transporter, the *MAOA* and the *DBH* genes to be compelling candidate genes for autism risk. Polymorphic variants of these three genes were investigated in a case-control population composed of 189 children who have a confirmed ASD diagnosis and 167 children confirmed to be typically developing (TD) to test possible associations.

MATERIALS AND METHODS

Study Participants

This investigation is a project within the CHARGE (CHildhood Autism Risks from Genetics and the Environment) study, a comprehensive, ongoing population-based case-control

investigation with participants sampled from three strata: children with autism (AU), children with developmental delay but not autism, and children from the general population [Hertz-Picciotto et al., 2006]. The overall goal of this study is to uncover environmental factors that increase the risk and severity of autism, or that interact with genetic predisposition to affect susceptibility for developing autism. This NIH-funded study is being conducted at the University of California, Davis M.I.N.D. (Medical Investigations of Neurodevelopmental Disorders) Institute and the Center for Children's Environmental Health. Children with autism or with other developmental delays were identified through the California Department of Developmental Services (DDS), which contracts with 21 Regional Centers that cover the State and that coordinate services to persons with eligible developmental disorders, regardless of socioeconomic level, citizenship or racial/ethnic group. For controls, children from the general population were identified in the state birth files, and a stratified random sample was generated by frequency matching to the projected distribution among cases of age, sex, and broad geographic area. For the current project, we focused on children with AU, other ASDs and TD. None of the children shared parents with any others in this sample; siblings were excluded from this candidate gene analysis.

Recruitment

Parents were contacted by letter and/or telephone to describe the study and invite participation. Recruitment began in April 2003, and for the current analyses, only children enrolled through 2007 were included. To be eligible for the CHARGE study, children must: a) be between the ages of 24 and 60 months, b) live with at least one biologic parent, c) have a parent who speaks English or Spanish, d) have been born in California, and e) currently reside in the catchment areas of a specified list of DDS Regional Centers in California. Families of the children meeting these criteria are invited to the M.I.N.D. Institute where trained staff administer informed consent, conduct a battery of tests and a physical examination, and draw blood samples. Whenever possible, the blood is drawn on not only the child, but also the parents and siblings. A telephone interview is also conducted to obtain information about the mother's medical conditions; reproductive, residential and occupational history; medications; use of household products and other exposures during pregnancy and the lactation period; and the child's environment in early life. Further details on the CHARGE Study recruitment and data collection protocols have been previously reported [Hertz-Picciotto et al., 2006].

Final Sample Selection

Eligibility for the genetic studies required blood samples from the index child and both parents. Our final sample included 356 index children, comprised of 128 cases with AU, 61 cases with other ASDs, and 167 TD. The protocols for this study were approved by institutional review boards of the University of California in Davis and Los Angeles and the State of California. Informed consent was obtained from each child‘s parent, prior to participation.

Details on the confirmation of the diagnosis of autism, other clinical assessment and genotyping are reported in a supplementary section.

Statistical analysis

The term "AU" is used to refer to individuals with confirmed autism, "AU+ASD" to refer to those having confirmed AU or ASD, and "TD" to refer to children confirmed to be typically developing. Separate statistical analyses were conducted for the narrower AU and the broader AU+ASD case definitions, and a binary code was used to designate each. Due to the rather small number of female children in the AU and ASD groups, we focused on male

children only when conducting analyses and reporting results in the paper, and presented descriptive statistics for female children in the Appendix.

Association with autism risk

We tested for differences between cases and controls (AU vs. TD, AU+ASD vs. TD) with regard to genotype and to specific alleles. The observed genotype distributions were tested for departures from Hardy-Weinberg equilibrium in unrelated TD children. Logistic regression was used to examine the case status vs. gene marker association and to estimate odds ratios and 95% confidence intervals. Multiple logistic regression models were used to investigate genotype prevalence odds ratios comparing cases *vs.* TD, adjusting for ethnicity and maternal age, with stratification by gender. Only White and Hispanic children were used in the logistic regression models due to the low frequency of other race/ethnic groups (< =8%). To address whether *SLC6A4* genotype and the *MAOA* 4 allele might show a synergistic relationship to risk of autism, which might be hypothesized to be mediated via human platelet serotonin levels, we also examined the combination of the LL and the 4 allele in children. Additionally, we examined the child's case status in relation to the maternal *MAOA* genotype.

Association with regression and cognitive and adaptive functions

We also determined whether specific gene polymorphisms were associated with subphenotypes within the case groups. Associations with regression status (regressive autism/ASD vs. non-regressive) were tested by simple and multiple logistic regression models (adjusted for race/ethnicity, and maternal age) among White and Hispanic AU and AU+ASD children. The associations between the gene markers and cognitive function were examined using linear regression models of the MSEL scores on genotypes, with and without adjustment for race/ethnicity, and maternal age. Associations between gene markers and adaptive function, measured by Vineland scores, were assessed similarly.

The logistic and linear regression analyses were carried out assuming additive, dominant, recessive, and general (2 degrees of freedom) genetic models where appropriate. All statistical tests were two sided and $P < 0.05$ was considered statistically significant. Case control association analyses were conducted using version 9.1.3 and version 9.2 of the SAS programming language (SAS Institute, Cary, NC) and R (http://www.r-project.org/). All multivariate models were adjusted for race/ethnicity and maternal age.

RESULTS

A total of 309 male index children were genotyped, 119 with AU, 53 with ASD, and 137 with TD. Table 1 summarizes the attributes of these male participants, including ADI-R and ADOS scores in each domain for AU and ASD groups. For each diagnostic group the majority of the index children were White (43–55%) or Hispanic (28–36%), with 4–8% reporting to be Black, Asian or Pacific Islander (Table 1). The mean paternal age was 38.5 years old and it was not significantly different for AU vs. TD groups or AU+ASD vs. TD. Mean maternal age was slightly, but insignificantly higher in AU (35.8 years old) or AU +ASD groups (35.9 years old) than in the TD group (34.7 years old). The AU/ASD children were also slightly, but significantly older (3.7 and 3.8 years in AU and AU+ASD respectively vs. 3.4 years among TDs).

Association with autism risk

Distributions of the *SLC6A4* and *DBH* genes did not show significant departure from Hardy Weinberg Equilibrium in TD control children (p-values = 0.21 for *SLC6A4*, 0.71 for *DBH*). Table 2 presents the genotype and allele frequency of the markers in each of the study

groups, and also the results from chi-square tests for frequency comparisons between groups. The minor alleles for *SLC6A4* and *DBH* were S (45.6%) and D (46.4%) respectively, based on the TD group. Neither genotype nor allele frequencies of *SLC6A4* and *DBH* were significantly different comparing AU or AU+ASD with TD children (Table 2).

Four allelic variants of the *MAOA* gene (2 repeat, 3 repeat, 4 repeat and 5 repeat alleles) were observed in the male index children and 3.5 repeat was seen in one male TD. Alleles 2, 3.5 and 5 were omitted when conducting chi-square tests due to their low frequency (< 4% for all three combined). After this adjustment, about 64% AU or AU + ASD male participants have allele 4 for the *MAOA* locus, as compared with 46% in TD children ($p = 0.004$ for AU vs. TD, $p=0.001$ for AU+ASD vs. TD).

Due to their small numbers, Black, Asian and Pacific Islander were excluded from the logistic regression models, resulting in analyses total sample sizes of 208 (251) for the logistic regression analyses of AU (AU+ASD), inclusive of White and HIspanic children only. Results from logistic regression did not show a significant association between case status and either *SLC6A4* or *DBH* regardless of which genetic model was assumed ($p > 0.05$ for all models of both genes) (Table 3), with or without adjusting for race/ethnicity and maternal age. However, a significant association was found for *MAOA* and the risk of AU or AU+ASD among male children in both unadjusted and adjusted analyses. As compared with allele 3, allele 4 of the *MAOA* gene was significantly more common among children with AU (OR = 2.02, 95% CI = 1.12, 3.65, $p = 0.02$) and AU+ASD (OR = 2.05, 95% CI = 1.19, 3.53, $p = 0.01$), even after adjusting for ethnicity and maternal age (Table 3). Results for all the markers remained similar when we further adjusted for children's age and geographic area in logistic regression models.

We also investigated the association between autism risk and the mother's MAOA genotype. Genotype and allele frequencies are presented in Table 2 before excluding the rare genotypes and alleles. When conducting association tests, only alleles 3 and 4, and genotypes 33, 34 or 43 and 44 were used, as all other alleles or genotypes were too rare. We found a significantly higher frequency of mothers homozygous for the 4 allele in both the AU (49%) and AU+ASD (45%) groups compared to the TD group (30%) (p-value = 0.007 for both AU vs. TD and AU+ASD vs. TD).

The association between mothers' MAOA genotype and risk for autism was further confirmed by fitting multiple logistic regression models under additive, dominant, recessive and general genotypic models, adjusting for race/ethnicity and maternal age (Table 4). Autism risk was significantly associated with mothers' *MAOA* under all the genetic models. Especially under the general genotypic model, compared with mothers homozygous for the allele 3, children whose mothers were homozygous for the allele 4 had a much higher risk for AU (OR = 3.07, 95% CI = 1.19, 7.91, $p = 0.02$) and AU+ASD (OR = 3.26, 95% CI = 1.35, 7.89, $p = 0.009$).

We also examined the combination of the *SLC6A4* LL genotype and the *MAOA* 4 allele in cases and controls to address a possible synergistic relationship to risk of autism. The frequency of individuals with this gene combination did not differ significantly comparing AU or AU+ASD with TD children (data not shown).

Association with regression and cognitive functions

Regression was present in 60 (50.4%) male children with AU and 80 (46.5%) with AU +ASD. No significant association was found between regression status and the three polymorphisms regardless of which genetic model was assumed. Under a recessive genetic model, AU children with the genotype SS of *SLC6A4* had higher cognitive (MSEL) scores

than TD children (coefficient estimate = 9.45, 95% CI = 2.84, 16.07, p = 0.006), adjusting for race/ethnicity and maternal age. This association became insignificant when including ASD children (AU + ASD). For *DBH*, under a recessive genetic model with adjustment for confounders, AU+ASD children homozygous for the D allele had lower cognitive scores (coefficient estimate = −6.52, 95% CI = −13.18, 0.14) as compared with those heterozygous or homozygous for the I allele (p =0.054). No significant association between *MAOA* and cognitive scores was found in either AU or AU+ASD children.

Adaptive function was not significantly related to *SLC6A4* or *DBH* in either the AU or the AU+ASD group. However, the 4 allele for the *MAOA* locus was associated with lower Vineland scores, at close to the significant level (coefficient estimate = −4.29, 95% CI = -8.72, 0.15, p = 0.06) in males with AU, and the association became significant when ASDs were also included (coefficient estimate = −6.35, 95% CI = −12.57, −0.13, p = 0.046), adjusting for race/ethnicity and maternal age.

DISCUSSION

Dysregulated catecholamine and serotonin neurotransmitters are suspected to etiologically contribute to ASD because of their involvement in neurodevelopment and because the drugs that affect these neurotransmitters can improve many of the symptoms of autism. Although such findings have stimulated considerable interest in the possible involvement of serotonin in the etiology of autism the results are quite contradictory. Our analysis is consistent with these null results for an association between the *SLC6A4* and autism. Based on the published literature and our own findings, it would appear that if *SLC6A4* and/or *DBH* are associated with risk of autism, the association is probably rather small, such that any future study attempting to quantify it might require thousands of participants. Thus, at this stage, the accumulated evidence for a role in autism risk remains unconvincing.

The *MAOA* gene is located on chromosome X and due to its role in metabolism of both catecholamine and serotonin, a connection to autism is plausible. In particular, the MAOA enzyme preferentially acts on serotonin and norepinephrine. The promoter region of the *MAOA* contains a 30bp variable number of tandem repeats (uVNTR) with alleles of 2, 3, 3.5, 4, 5 and 6 repeats reported, although the 3 and 4 repeat variants represent more than 97% of all alleles [Cases et al., 1996; Filic et al., 2005] (96% of our sample). The 3 allele is associated with decreased transcription and, hence reduced activity compared to the other alleles, suggesting that regulation via repeat length could have a phenotypic effect [Cases et al., 1996; Denney et al., 1999]. Reduced activity of the MAOA enzyme has been associated with abnormal behavior, but many of these studies involve alcoholism, which has not been associated with autism in well-conducted studies. Cohen et al. [2003] looked at 41 boys aged 12 or under with autism, and observed lower IQ and more severe autistic behavior (as measured by teachers and parents reports) in children with the low activity *MAOA* 3-allele than in children with the higher activity 4-allele. However, in a larger case series, the same group demonstrated behavioral differences according to the maternal genotype beyond those associated with the child's genes. In particular, although those boys with the 3 allele had more sensory and social-pragmatic difficulties, boys who received their 4 allele from a homozygous mother were at highest risk for ritualistic behaviors/resistance to change (comparing with both boys having the 3 allele, and boys having the 4 allele with a heterozygous mother) and also for fears, though this finding came short of statistical significance. Compared with boys inheriting the 4 allele from a heterozygous mother, those inheriting the same allele but from a homozygous mother scored worse on the autism composite score, social approach-withdrawal, ritualistic behaviors, and aggressiveness/ negative mood states. These authors additionally demonstrated deficits in expressive language and semantic pragmatic language for boys carrying the 4 repeat vs. the 3 repeat

allele [Cohen et al., 2010]. Thus, our finding of higher risk for autism in boys with homozygous mothers is consistent with evidence from Cohen's work showing greater severity of symptoms in several domains. In contrast to the influence of maternal zygosity on boys with the 4 repeats, they observed no such heterogeneity in boys who inherited the 3 repeat allele. Together, these results may suggest an effect of maternal MAOA activity during the gestational period.

An alternative explanation for the high prevalence of homozygotic mothers is population stratification whereby the admixture in cases differs from that in controls and some aspect of ethnicity is responsible for this excess maternal genotype. In this study, the proportions Hispanics and non-Hispanics (defined by self report) was similar in cases was very close to those in controls, and maternal MAOA genotypes did not differ comparing these two ethnic groups in the controls (nor in either the broad or narrow case group). Notably, a large population-based study demonstrated a high correlation between ancestry markers and self-reported Hispanic ethnicity [Sweeney et al., 2007]. Nevertheless, population stratification cannot be entirely excluded.

Interpretation of the association of genotype with autism depends on how allelic variants in the promoter region of the *MAOA* affect MAOA enzyme activity and how such enzyme activity influences development or persistence of autistic behaviors. Some reports indicate that the 3-repeat allele is involved with 'externalizing'-type behaviors; longer alleles are associated with more 'internalizing' (withdrawal) behaviors [Buckholtz and Meyer-Lindenberg, 2008; May et al., 2009], though the results of Cohen [2010] suggest it is more complex.

Our findings of higher prevalence among cases of mothers homozygous for allele 4 suggests the possibility that mother's genotype at the *MAOA* locus may act either as the primary influence on prenatal brain development (the OR was larger for a homozygous mother with the high activity 4 allele than for a male child with the 4 allele), or as a modifier in combination with the child's genotype, on the autism phenotype, perhaps through influences on the intrauterine environment. Interestingly, another team reported gene/protein expression levels associated with allelic variants in several loci including *MAOA* and *SLC6A4* in human placenta. Thus, a direct impact of genetically programmed maternal neurotransmitters on fetal growth and development is plausible [Zhang et al., 2010].

MAOA interactions with other genes have been documented, for instance, DRD TaqI-A polymorphism [Wang et al., 2007] and ALDH2 [Lee et al., 2009], both of which involve the *MAOA* uVNTR 4-allele. It is also possible that this *MAOA* functional polymorphism is in linkage disequilibrium with another, yet unidentified gene, which directly influences the risk of autism. Finally, environmental factors could be interacting with the different allelic variants to play a role in the behavioral outcomes; in support of this possibility, a number of common environmental factors affect *MAOA*, such as pesticides [Rao and Rao, 1993] and medications [Ravindran and Stein, 2010].

Interestingly, Hranilovic et al. [2008] observed higher platelet serotonin levels in the autistic subjects carrying the *MAOA* 4 allele compared to those carrying the 3 allele, which suggests a potential role of this functional polymorphism in the development of hyperserotonemia; elevated levels of whole blood or platelet serotonin have been observed in approximately one third of autistic patients [Anderson et al., 1987; Anderson et al., 2002; Cook Jr, 1990; Lesch et al., 1996]. In addition, studies of the functional effects of *SLC6A4* genotype indicate that the deletion form (S) results in lower mRNA levels, reduced transporter protein expression, and decreased uptake rates in transfected and native lymphoblastoid cell lines. Indeed, a higher rate of serotonin uptake has been observed in individuals with the LL

genotype compared to individuals with the SS or SL genotype (on average 50% higher uptake rate) [Anderson, 2002; Lesch et al., 1996]. These findings support the notion that *SLC6A4* genotype, through its effects on transporter expression and uptake rate, is a determinant of platelet 5-HT content but given no difference in genotype for affected vs. typically developing children, other factors must be operating. Though *SLC6A4* probably does not contribute completely to the elevation in platelet 5-HT levels, altered uptake may still play a role.

Focusing on phenotypic variation among cases, we observed a few associations for cognitive or adaptive developmental scores and child's genotype, but most were of borderline significance. The *MAOA 4*-allele was associated with lower adaptive scores. Other studies, using different instruments or case confirmation methods, have been mixed [Yirmiya et al. 2002] [Jones et al., 2004]. These were small studies.

Replication of our results in an independent sample is needed. The GWAS studies are powerful but those conducted to date do not adequately serve as a replication source for polymorphisms based on variable tandem repeats, as is the case for the *MAOA promoter locus*. The lack of replication, in two GWAS-autism studies using quite different study designs [Ma et al., 2009]. Wang and colleagues [2009], of genes repeatedly linked to autism in candidate gene studies, underscores that there are fundamental differences between candidate gene and GWAS approaches. Both should be viewed as valid, each having a different set of strengths. The trade-off in GWAS studies is tight control of Type I errors but increased rates of Type II errors, especially for effects that are not exceptionally large.

The other gene we examined is *DBH*, which encodes a protein that oxidizes dopamine resulting in norepinephrine synthesis. This protein is expressed in neurons and neuro-endocrine cells and therefore represents a marker for adrenergic and noradrenergic neurons in CNS. Decreased serum DBH enzyme activity has been reported in autistic children [Lake et al., 1977] as well as in mothers with 1 or 2 children with autism [Robinson et al., 2001]. The insertion/deletion polymorphism has been associated with low plasma DBH activity, which is associated with psychotic symptoms in several psychiatric disorders [Cubells et al., 2000]. In our study we did not see any difference in allelic frequencies of the *DBH* 5'-Ins/Del of the *DBH* gene between AU or AU +ASD and TD children, nor any association between maternal genotype and having a child with AU/ASD. Given heterogeneity of ASD, possibly this genotype is important only for certain phenotypic subsets, which may be consistent with our finding of lower Mullen scores associated with the homozygous D allele genotype. DBH may be influential only in the presence of other genes or some environmental factor.

This study was moderate in size, and if the effects of *SLC6A4* or *DBH* are effectively small for autism or ASD, then power may have been low. However, this investigation had numerous strengths: the population-based control group, a similar distribution of ethnicity in cases and controls, confirmation of all diagnoses using two research reliable instruments (ADOS and ADI-R), a well-trained assessment team, detailed characterization of phenotype which permitted examination of cognitive and adaptive skills as well as regression, the multivariate analysis with control for confounders, and the high quality laboratory protocols utilized in this study.

The *MAOA* 4-repeat allele findings we report may provide a clue regarding a mechanism that may influence risk of developing autism and/or plasma serotonin levels in children with autism. Further work is needed to quantify the influence of the gene on circulating serotonin, specifically during early life, to determine if this could represent an etiologic pathway. A large body of literature emphasizes allelic variants (either SNPs or single tandem repeats) as

modulating the gene function in dopamine and serotonin pathways (for a review see [D'Souza and Craig, 2008]) leading to human behavioral disorders and traits. Further research that addresses the molecular mechanisms linked to functional polymorphism variants in the neurotransmitter pathways and the role of environmental factors in these mechanisms will help to elucidate how these polymorphisms can influence the behavioral outcome.

CONCLUSIONS

Our findings indicate that in the population-based CHARGE Study, the serotonin transporter and the dopamine hydroxylase loci are not associated with autism spectrum disorders. However, the prevalence of the high activity allele 4 at the *MAOA* promoter locus in the child and the prevalence of mothers homozygous for this allele were higher for those with autism spectrum disorders as compared with their typically developing counterparts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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This work is dedicated to the memory of Matteo.

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Table 1

Demographic and clinical characteristics in male children.

	AU (N=119)		ASD (N=53)		AU+ASD (N=172)		TD (N=137)			
Variable	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**P1**	**P2**
Race/Ethnicity									0.51	0.60
White	66	55.5	24	45.3	90	52.3	66	48.2		
Hispanic	34	28.5	19	35.9	53	30.8	42	30.7		
Others*	9	7.6	4	7.5	13	7.6	6	4.3		
Missing	10	8.4	6	11.3	16	9.3	23	16.8		
	mean	**SD**	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**		
Age	3.7	0.9	3.9	0.9	3.8	0.9	3.4	0.8	0.002	0.0001
Father age	38.5	6.3	38.9	6.0	38.6	6.2	38.1	7.3	0.76	0.66
Mother age	35.8	5.4	36.1	5.1	35.9	5.3	34.7	5.5	0.12	0.06
ADI-R scores^										
ADRtotalAs	19.8	4.2	15.8	5.2	18.5	4.9				
ADRtotalCs	6.3	2.1	4.5	2.8	5.7	2.5				
ADRtotalDs	4.2	0.9	3.8	1.0	4.1	0.9				
ADRtotalNVBs	11.4	2.1	9.9	2.4	11.1	2.2				
ADRtotalVBs	15.6	3.4	12.6	3.4	14.4	3.7				
ADOS scores^										
AM1tcomm	5.7	1.6	3.8	1.7	5.1	1.8				
AM1tcomsoc	16.3	2.8	11.3	3.7	14.8	3.8				
AM1tsocial	10.7	1.8	7.5	2.6	9.7	2.5				
AM2tcomm	6.5	1.3	4.3	0.9	5.7	1.6				
AM2tcomsoc	15.8	2.5	10.2	1.9	13.9	3.5				
AM2tsocial	9.3	1.6	5.9	1.6	8.1	2.2				

P1, P2: p-values for AU vs TD and AU+ASD vs TD respectively after excluding missing values.

*Others include Black, Asian and Pacific Islander.

^ADI-R and ADOS scores are only available for AU and ASD participants.

ADRtotalAs: ADIR A. Social Interaction Total (0 – 30)

ADRtotalVBs: ADIR B. Verbal Communication Total (0 – 26)

ADRtotalNVBs: ADIR B. Non-Verbal Communication Total (0 – 14)

ADRtotalCs: ADIR C. Stereotyped Behaviors Total (0 – 5)

ADRtotalDS: ADIR D. Onset <36 mo Total (0 – 5)

AM1tcomm: ADOS Module 1. Communication Total (0 – 10)

AM1tsocial: ADOS Module 1. Social Interaction Total (0 – 14)

AM1tcomsoc: ADOS Module 1. Communication + Social Interaction Total (0 – 24)

AM2tcomm: ADOS Module 2. Communication Total (0 – 10)

AM2tsocial: ADOS Module 2. Social Interaction Total (0 – 14)

AM2tcomsoc: ADOS Module 2. Communication + Social Interaction Total (0 – 24)

Table 2

Genotype and allele frequencies of HTT, DBH and MAOA in male children and MAOA mothers.

	AU (N=119)		ASD (N=53)		AU+ASD (N=172)		TD (N=137)			
Marker	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**P1**	**P2**
HTT										
Genotype									0.72	0.45
LL	33	27.7	15	28.3	48	27.9	44	32.1		
SL/LS	58	48.7	31	58.5	89	51.7	61	44.5		
SS	28	23.5	7	13.2	35	20.4	32	23.4		
allele									0.61	0.88
L	124	52.1	61	57.6	185	53.8	149	54.4		
S	114	47.9	45	42.5	159	46.2	125	45.6		
DBH										
Genotype									0.98	0.99
DD	26	21.9	12	22.6	38	22.1	31	22.6		
DI/ID	56	47.1	26	49.1	82	47.7	65	47.5		
II	37	31.1	15	28.3	52	30.2	41	29.9		
allele									0.83	0.92
D	108	45.4	50	47.2	158	45.9	127	46.4		
I	130	54.6	56	52.8	186	54.1	147	53.7		
MAOA*										
Allele									0.004*	0.001*
2	0	0	0	0	0	0	1	0.7		
3	42	35.3	19	35.9	61	35.5	73	53.3		
4	74	62.2	34	64.1	108	62.8	61	44.6		
5	3	2.5	0	0	3	1.7	1	0.7		
3.5	0	0	0	0	0	0	1	0.7		
Mother's *MAOA**										
Genotype									0.007*	0.007*

	AU (N=119)		ASD (N=53)		AU+ASD (N=172)		TD (N=137)			
Marker	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**P1**	**P2**
23	0	0	0	0	0	0.0	1	0.8		
33	14	11.8	4	7.7	18	10.5	27	20.9		
34/43	44	37.0	29	55.8	73	42.7	61	47.3		
35/53	0	0.0	0	0.0	0	0.0	1	0.8		
3.5,4	0	0.0	0	0.0	0	0.0	1	0.8		
44	55	46.2	19	36.5	74	43.3	37	28.7		
45/54	5	4.2	0	0.0	5	2.9	0	0.0		
55	1	0.8	0	0.0	1	0.6	1	0.8		
Allele									0.002*	0.002*
2	0	0.0	0	0.0	0	0.0	1	0.4		
3	72	30.3	37	35.6	109	31.9	117	45.4		
4	159	66.8	67	64.4	226	66.1	136	52.7		
5	7	2.9	0	0.0	7	2.1	3	1.2		
3.5	0	0.0	0	0	0	0.0	1	0.4		

P1, P2: p values from chi-square test for vs TD and AU+ASD vs TD, respectively.

*For MAOA, p values were calculated after deleting rare alleles and genotypes.

Table 3

Results from multiple logistic regression for male children.

	AU			AU+ASD		
Marker	**OR**	**95% CI**	**P**	**OR**	**95% CI**	**P**
HTT						
Additive	0.97	0.66,1.42	0.87	0.89	0.62,1.28	0.53
Dominant	1.16	0.63,2.15	0.63	1.13	0.64,1.99	0.68
Recessive	0.77	0.40,1.48	0.43	0.61	0.33,1.13	0.12
General			0.51			0.16
Het	1.34	0.69,2.63	0.39	1.41	0.76,2.62	0.27
Hom	0.91	0.42,1.95	0.81	0.74	0.36,1.52	0.41
DBH						
Additive	0.97	0.65,1.44	0.86	1.05	0.73,1.51	0.80
Dominant	0.98	0.53,1.80	0.93	1.08	0.61,1.91	0.78
Recessive	0.93	0.46,1.87	0.83	1.04	0.55,1.98	0.91
General			0.98			0.96
Het	1.00	0.52,1.92	0.99	1.08	0.59,1.98	0.80
Hom	0.92	0.41,2.07	0.85	1.09	0.52,2.29	0.82
MAOA	2.02	1.12,3.65	0.02	2.05	1.19,3.53	0.01

Multiple logistic regression of disease and markers adjusted for ethnicity and maternal age, using White and Hispanic index children only.

Additive refers to additive genetic model for which the OR shows the effects of heterozygous genotype (eg. LS or SL for HTT and DI or ID for DBH) on autism risk as compared to those of homozygous genotype for the major allele (eg. LL for HTT and II for DBH); the effects of homozygous genotype (eg. SS for HTT and DD for DBH) will be 2*OR.

Dominant refers to dominant genetic model where subjects with heterzygous or homozygous genotype for the minor allele (eg. S for HTT and D for DBH) were compared with those with homozygous genotype for the major allele (eg. L for HTT and I for DBH).

Recessive refers to recessive genetic model where subjects with homozygous genotype for the were compared to those with either hyterozygous or homozygous genotype for the major allele.

General refers to the general genetic model where subjects with heterozygous and homozygous for the minor allele were compared with those with homozygous genotype for the major allele.

Table 4

Results from multiple logistic regression for maternal MAOA of male children.

	AU			AU+ASD		
MAOA	**OR**	**95% CI**	**P**	**OR**	**95% CI**	**P**
Additive	1.76	1.13,2.74	0.01	1.72	1.14,2.60	0.009
Dominant	2.24	0.93,5.39	0.07	2.59	1.15,5.83	0.02
Recessive	2.03	1.10,3.72	0.02	1.78	1.01,3.12	0.04
General			0.04			0.03
Het	1.71	0.68,4.32	0.26	2.15	0.92,5.04	0.08
Hom	3.07	1.19,7.91	0.02	3.26	1.35,7.89	0.009

Multiple logistic regression of disease and marker adjusted for ethnicity and maternal age, using White and Hispanic index children only.

Additive refers to additive genetic model for which the OR shows the effects of heterozygous genotype (eg. 34 or 43) on autism risk as compared to those of homozygous genotype for the major allele (eg. 33); the effects of homozygous genotype (eg. 44) will be 2*OR.

Dominant refers to dominant genetic model where subjects with heterozygous or homozygous genotype for allele 4 were compared with those with homozygous genotype for allele 3.

Recessive refers to recessive genetic model where subjects with homozygous genotype for allele 4 were compared to those with either hyterozygous or homozygous genotype for allele 3.

General refers to the general genetic model where subjects with heterozygous and homozygous genotypes for allele 4 were compared with those with homozygous genotype for allele 3 respectively.

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