Neurotransmitter systems and neurotrophic factors in autism: association study of 37 genes suggests involvement of DDC

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Abstract

Objectives. Neurotransmitter systems and neurotrophic factors can be considered strong candidates for autism spectrum disorder (ASD). The serotoninergic and dopaminergic systems are involved in neurotransmission, brain maturation and cortical organization, while neurotrophic factors (NTFs) participate in neurodevelopment, neuronal survival and synapses formation. We aimed to test the contribution of these candidate pathways to autism through a case–control association study of genes selected both for their role in central nervous system functions and for pathophysiological evidences.

Methods. The study sample consisted of 326 unrelated autistic patients and 350 gender-matched controls from Spain. We genotyped 369 tagSNPs to perform a case-control association study of 37 candidate genes.

Results. A significant association was obtained between the DDC gene and autism in the single-marker analysis (rs6592961, \( P = 0.00047 \)). Haplotype-based analysis pinpointed a four-marker combination in this gene associated with the disorder (rs2329340C – rs2044859T – rs6592961A – rs11761683T, \( P = 4.988 \times 10^{-5} \)). No significant results were obtained for the remaining genes after applying multiple testing corrections. However, the rs167771 marker in DRD3, associated with ASD in a previous study, displayed a nominal association in our analysis (\( P = 0.023 \)).

Conclusions. Our data suggest that common allelic variants in the DDC gene may be involved in autism susceptibility.

Key words: Genetics, autistic disorder, serotonin, dopamine, DDC gene

Introduction

Autism is a childhood-onset neurodevelopmental disorder characterised by impairment in reciprocal social interactions, communication and repetitive and stereotyped behavioural patterns (Lord et al. 2000). Autism is part of a larger group of neuropsychiatric conditions.
disorders defined as PDDs (pervasive developmental disorders) that also include Asperger syndrome, childhood disintegrative disorder and pervasive developmental disorder not otherwise specified (PDD-NOS). The disorder is approximately four times more frequent in males than in females. Prevalence estimates are around 0.2% for autism and 0.6–0.7% for PDDs, making it one of most prevalent disorders in childhood (Fombonne 2009). Although twin and family studies provide strong evidence for a genetic basis in PDD, only a few rare and highly penetrant mutations have been found to be involved in autism in several synaptic genes: NLGN3, NLGN4, NRXN1, SHANK3, SHANK2 and PITCH1 (Jamain et al. 2003; Durand et al. 2007; Szatmari et al. 2007; Noor et al. 2010; Pinto et al. 2010). These genes have also been associated with other neuropsychiatric disorders. It is likely that the autistic phenotype results from the combined effect of penetrant rare variants, such as structural variants or point mutations, and common susceptibility alleles of modest effect.

Recent findings support the hypothesis that synaptogenesis is disrupted in autism (Bourgeron 2009), although other pathways may also have a role in autism susceptibility. In this regard, neurotransmission systems and neurotrophic factors have been proposed to be involved in the disorder on the basis of numerous pathophysiological and genetic evidences (Pardo and Eberhart 2007; Cuscó et al. 2009; Nickl-Jockschat and Michel 2010).

Serotonin and dopamine are neurotransmitter monoamines involved in modulating adult cortical plasticity and known to have a critical role in early cortex development by regulating proliferation, migration and neuronal differentiation (Vitalis and Parnavelas 2003). Serotonin acts via seven families of receptors (5-HT1–5-HT7) and is related to sleep, mood, memory, learning, muscle contraction homeostasis and endocrine functions (Haavik et al. 2007). Dopamine acts through five receptors (D1–D5) and modulates multiple brain functions, including reward response, motivation, memory, attention, problem solving and is critical to control voluntary movements (Haavik et al. 2007). The metabolism of these two neurotransmitters is complex and tissue-type specific, both sharing the biosynthetic enzyme DOPA decarboxylase (DDC) and the catabolic enzymes monoamine oxidase A (MAOA) and B (MAOB).

Many findings show that both the serotoninergic and the dopaminergic systems may be considered as strong candidate pathways for autism: First, elevated levels of serotonin in blood and urine have been observed in approximately one-third of autistic individuals (Cook and Leventhal 1996; Croonenberghs et al. 2000; Burgess et al. 2006), whereas normal peripheral levels of dopamine have been reported in the majority of studies performed so far (McDougle et al. 2005). Second, selective serotonin reuptake inhibitors (SSRIs) and dopamine receptor antagonists have a role in reducing specific associated symptoms in autism: aggression, self-injury and compulsive behaviours (Nikolov et al. 2006). And third, neuroimaging studies with positron emission tomography (PET) have shown abnormal asymmetry of serotonin synthesis in frontal, temporal and parietal cortex in autistic individuals compared to controls (Chandana et al. 2005). In addition, the dopaminergic activity seems to be altered in the anterior, medial and prefrontal cortex of autistic individuals (Rumsey and Ernst 2000).

The serotonin transporter gene SLC6A4-HTT is one of the most studied genes in autism, with evidence of linkage to 17q11–12 reported in several genome-wide scan studies for autism (Auranen et al. 2002; Yonan et al. 2003; McCauley et al. 2005). Two variations, an insertion/deletion polymorphism in the promoter region and a Variable Number of Tandem Repeats (VNTR) in intron 2 have been analysed in many autistic samples through case-control and family-based association studies (Huang and Santangelo 2008). The role of this gene in autism susceptibility is still unclear due to discrepancies among different studies, although a recent meta-analysis failed to find an overall association (Huang and Santangelo 2008). Other serotonin-related genes, such as HTR1B, HTR2A, HTR3A and HTR5A, that encode the serotonin 5HT1B, 5HT2A, 5HT3 and 5HT5A receptors, have been proposed to contribute to autism susceptibility (Cho et al. 2007; Coutinho et al. 2007; Orabona et al. 2008; Anderson et al. 2009) as well as genes encoding the dopamine receptors D1 and D3 (Hettinger et al. 2008; de Krom et al. 2009).

Neurotrophic factors (NTFs) and their receptors represent another group of candidate genes for autism. NTFs are crucial during neurodevelopment, regulating many functional and structural aspects of the central nervous system (CNS), including differentiation, neuronal survival, synaptogenesis, synaptic plasticity and axonal and dendritic outgrowth (Reichardt 2006). Several studies suggest that NTFs may be at the basis of the pathophysiology of several neuropsychiatric disorders, such as schizophrenia and depression (Durany and Thome 2004; Hashimoto et al. 2005; Otsuki et al. 2008). The potential contribution of NTFs to autism has also been investigated. Interestingly, some reports described elevated levels of BDNF and NTF4/5 and low levels of NT3 in serum of autistic patients, suggesting that the corresponding genes may be deregulated in autism (Miyazaki et al. 2004; Connolly et al. 2006; Nelson et al. 2006).
However, it is still unclear whether the changes observed in NTFs reflect a primary pathogenic mechanism or are secondary to cortical abnormalities in ASD. Recently, genetic studies have also provided evidence for the involvement of NTFs in autism: association with *BDNF* has been reported in two independent studies and a common variant of *NTRK1* has been associated with autistic traits (Nishimura et al. 2007; Chakrabarti et al. 2009; Cheng et al. 2009). These data support the hypothesis that neuronal survival, differentiation and growth may be at the basis of autism aetiology.

The aim of this study was to identify common alleles of modest effect involved in autism. We genotyped 369 single nucleotide polymorphisms (SNPs) that tag most allelic variability of 37 functional candidate genes involved in the serotoninergic and dopaminergic neurotransmission or encoding neurotrophic factors and their receptors to perform a population-based association study in 326 ASD patients and 350 gender-matched controls from Spain.

### Methods

#### Subjects

The autism cohort under study included 326 individuals of singleton families that met DSM-IV-TR criteria for autism, Asperger disorder and PDD-NOS based on ADI-R (Autism Diagnostic Interview-Revised) and ADOS-G (Autism Diagnostic Observation Schedule-Generic) diagnostic instruments (Lord et al. 1994, 2000a, 2000b) (see Table I). The sample was recruited from different hospitals of Northern and Southern Spain (Catalonia and Andalusia). Cytogenetic abnormalities and positive Fragile X test were considered as exclusion criteria. The control sample consisted of 350 healthy donors, sex-matched with the case sample, recruited from the Blood and Tissue Bank at Hospital Universitari Vall d’Hebron (Barcelona). To minimize ethnic heterogeneity we have included only Spanish Caucasian cases and controls in our study. The study was approved by the relevant ethical committee of each participating institution and written informed consent was obtained from all parents/guardians.

| Description of autism spectrum disorder (ASD) patients included in our study. |
|---------------------------------|----------------|----------------|-----------------|----------------|
| Autism (56%) | Aperger (29%) | PDD-NOS (15%) | All ASD |
| Males | 85% | 86% | 72% | 83% |
| Mental retardation* | 73% | 0% | 74% | 51% |
| Average age (years) | 17 | 10 | 27 | 17 |

*Mean IQ < 70. PDD-NOS, pervasive developmental disorder not otherwise specified.*

### DNA isolation and quantification

Genomic DNA was isolated from peripheral blood lymphocytes using the salting out method (Miller et al. 1998) or magnetic bead technology with the Chemagic Magnetic Separation Module I and the Chemagic DNA kit, according to the manufacturer’s recommendations (Chemagen AG, Baesweiler, Germany). The double-stranded DNA concentrations of all samples were determined on a Gemini XPS fluorometer (Molecular Devices, Sunnyvale, CA, USA) using the PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, OR, USA), following the manufacturer’s instructions.

### Selection of genes and SNPs

We selected 38 functional candidate genes encoding the serotonin receptors (*5HT1A, 5HT1B, 5HT1D, 5HT1E, 5HT2A, 5HT2B, 5HT2C, 5HT3A, 5HT3B, 5HT4, 5HT5A, 5HT6, 5HT7*), the serotonin and dopamine transporters (*SLC6A4* and *SLC6A3*), enzymes involved in the serotonin and dopamine metabolic pathways (*TH, TPH1, DDC, MAOA, MAOB, COMT* and *DBH*), the dopamine receptors (*DRD1, DRD2, DRD3, DRD4, DRD5*), neurotrophic factors (*NGF, BDNF, NTF3, NTF4/5, CNTF*) and their receptors (*NTRK1, NTRK2, NTRK3, NGFR, CNTFR*) (Supplementary Table S1 available online).

The SNPs selection was based on genetic coverage criteria, by considering linkage disequilibrium (LD) patterns within the candidate genes. SNPs covering each gene plus 3–5 kb flanking sequences were picked from the CEU panel of the HapMap database (www.hapmap.org, release 20). We used the LD-select software (droog.gs.washington.edu/ldSelect.html) to evaluate LD of the genomic regions in order to minimize redundancy between the selected SNPs (Carlson et al. 2004). A total of 400 tagSNPs were selected with the following criteria: $r^2 < 0.85$ from any other SNP according to CEU HapMap data and a minor allele frequency (MAF) $> 0.15$ for genes with less than 15 tagSNPs and MAF $> 0.25$ for those genes with more than 15 tagSNPs in the serotoninergic and dopaminergic systems. However, we considered a MAF $> 0.10$ for genes encoding...
neurotrophic factors and their receptors as they were part of a previous design that followed distinct criteria (Ribasés et al. 2008) (Supplementary Table S1 available online). Some additional non-synonymous SNPs (nsSNPs) were included in our selection as potential functional polymorphisms: rs1007211 (NTRK1 exon 1, NP_001012331.1:p.Gly18Glu), rs1058576 (SHT2A exon 3, NP_000612.1:p.Ser421Phe), rs6318 (SHT2C exon 4, NP_000859.1:p.Cys23Ser), rs2228673 (SLC6A4 exon 5, NP_001036.1:p.Lys201Asn) and rs6265 (BDNF exon 2, NP_001137277.1:p.Val66-Met).

Plex design, genotyping and quality control

A total of 400 tagSNPs were initially selected in our study, of which 31 did not pass through the SNPplex design pipeline (ms.appliedbiosystems.com/snplex/snplexStart.jsp), resulting in a design rate of 92%. Eight SNPplex genotyping assays including 369 SNPs were designed: two for the serotoninergic system (48 and 47 SNPs), two for the dopaminergic system (46 and 45 SNPs) and four for the neurotrophic factors and their receptors (45, 48, 46 and 44 SNPs). The possible presence of population stratification that could lead to false positive results in the population-based association study was tested by genotyping an additional plex of 48 unlinked SNPs distributed across different chromosomes and located at least 100 Kb distant from known genes (Sanchez et al. 2006).

Genotyping was performed at the Barcelona node of the National Genotyping Center (CeGen, www.cegen.org) using the SNPlex technology (Applied Biosystems, Foster City, CA, USA) (Tobler et al. 2005). Two CEPH samples (NA11992 and NA11993) were included in the different genotyping assays, and a concordance rate of 100% with HapMap data was obtained. In addition, no differences were found in the genotypes of two replicas. No heterozygote calls were obtained in SNPs of candidate X-linked genes (SHT2C, MAOA, MAOB) in the male sample.

Statistical analysis

All the individuals with less than 85% of successful genotyping rate were excluded from the analysis that finally included 326 autistic patients and 350 controls. From the 369 SNPs that were genotyped, 307 were finally analyzed in the population-based association study, whereas 62 SNPs (17%) were excluded for one of the following reasons: more than 15% missing genotypes, $\chi^2 > 0.85$ from any other studied SNP in our control sample, monomorphic SNPs and deviation from Hardy–Weinberg equilibrium (HWE; threshold set at $P<0.01$ in our control population). The only SNP that was genotyped in the DRD4 gene was excluded from the statistical analysis because it did not overcome the quality control filters.

The analysis of study power was estimated post hoc with the Power Calculator for Genetic Studies software (sph.umich.edu/csg/abecasis/CaTS) (Skol et al. 2006), assuming an odds ratio (OR) of 1.5, disease prevalence of 0.07, significance level (\(\alpha\)) of 0.05 and the minimum MAF value in our sample, 0.10, under the additive model.

Potential genetic stratification was assessed using the STRUCTURE v2.3 software (Pritchard et al. 2000) by analysing 46 unlinked SNPs in HWE out of the 48 that were genotyped. The analysis was performed under the admixture model, with a length of the burning period and a number of MCMC repeats of 100,000 and performing five independent runs at each K value (from 1 to 5), with K referring to the number of groups to be inferred.

Single-marker analysis

The analysis of HWE and the case-control association study were performed with the SNPassoc R package (Gonzalez et al. 2007). For the case–control study we analyzed all single markers under the additive model using the Cochran–Armitage Trend Test (ATT) (Supplementary Table S1 available online). In our analysis we considered also the dominant (11 vs. 12 + 22) and recessive (11 + 12 vs. 11) models only for those SNPs that reached nominal $P$-values in the ATT test. Chromosome X markers were analyzed separately with an allelic test using the Haploview v4.2 software (Barrett et al. 2005). A Q-Q plot was generated with the ggplot2 R library (Wickham 2009). For the multiple comparison correction, we considered all tests performed and assumed a false discovery rate (FDR) of 15% with the $Q$-value R library (Storey 2002), which corresponds to a significance threshold of $P<4.7e-04$.

Multiple-marker analysis

To minimize multiple testing and type I errors (\(\alpha\)), we decided a priori to restrict the haplotype-based association study to those genes associated with autism in the single markers analysis after FDR correction. We ascertained the best two-marker haplotype from all possible combinations, rather than simplifying the study only to physically contiguous SNPs. Likewise, additional markers (up to four) were added in a stepwise manner to the initial two-SNP haplotype. The risk haplotype was identified
among those haplotypes defined by two-, three- or four-markers showing the highest OR values. In all cases, significance was estimated using 10,000 permutations with the UNPHASED 3.0 software (Dudbridge 2003). Since the expectation-maximization algorithm implemented in the UNPHASED software does not accurately estimate low haplotype frequencies, haplotypes with frequencies < 0.05 were excluded.

**Results**

We genotyped 369 tagSNPs in 326 autistic patients and 350 controls to assess the potential role in autism of 38 candidate genes encoding proteins involved in the serotoninergic and dopaminergic neurotransmission and also neurotrophic factors and their receptors. After quality control procedures, 62 tagSNPs were discarded from the statistical analysis for the following reasons: 32 SNPs with genotyping call rate < 85%, 14 SNPs that were in strong LD in the control group with other SNPs in the same candidate gene ($r^2 > 0.85$), 10 SNPs with MAF < 0.1 or monomorphic, 6 SNPs showing significant deviation from HWE in the control sample ($P<0.01$) (Supplementary Table S1 available online). These filters left the DRD4 gene out of the analysis, so the final number of genes included in the case-control study was 37.

The statistical power of our sample was 71% under the additive model. Population stratification was excluded using the Structure software on 46 unlinked SNPs that were genotyped in cases and controls (Supplementary Table S2 available online).

**Single-marker analysis**

In summary, 676 individuals were successfully analysed for 307 tagSNPs that finally passed through the quality control filters with an average genotyping efficiency of 97%. A quantile-quantile plot representation of all the results under the additive model is shown in Figure 1.

The results of our case-control analysis found nominal associations ($P<0.05$) under the additive model in 21 SNPs located in the following 10 genes: 

- **DDC** (rs1982406, rs6592961, rs3823674)
- **COMT** (rs2020917, rs933271)
- **DRD1** (rs251937)
- **DRD2** (rs4630328, rs4245146, rs17529477)
- **DRD3** (rs167771)
- **BDNF** (rs1491851)
- **NTF3** (rs6489630, rs7956189)
- **NTF4/5** (rs17206784)
- **NTRK3** (rs7176520, rs3784406, rs12440144, rs1435403)
- **CNTFR** (rs2381164, rs2381165, rs2274592)

The $P$-values of these SNPs are shown in Table II, together with those under the recessive and the dominant models. However, after applying corrections for multiple testing using a 15% FDR ($P_{FDR}=4.7e-04$) only the rs6592961 marker ($P_{FDR}=4.7e-04$, $OR_{FDR}=1.79$ [1.29–2.49]) in the **DDC** gene remained significant under the dominant model (Figure 2). No SNP remained significant after the restrictive Bonferroni correction.

**Multiple-marker analysis**

The analysis of possible risk haplotypes in our study was considered only for the **DDC** gene, the only one in which a marker remained associated after correcting for multiple testing. The LD patterns observed in our sample were similar to those in the HapMap CEU sample, indicating that the tagSNPs for this study were properly selected. The haplotype analysis performed in the **DDC** gene tested all possible SNP combinations and identified a risk haplotype of four markers (rs2329340–rs204859–rs6592961–rs11761683) associated with autism (best adjusted $P=9.9e-05$) (Table III). The allelic combination C-T-A-T was over-represented in the autistic sample (OR = 2.44, 95% CI = 1.55–3.83, Table III), while the haplotype defined by the allelic combination C-T-G-T was more frequent in controls (OR = 0.65, 95% CI = 0.50–0.83).

**Discussion**

Several lines of evidence suggest that genes of the serotoninergic and dopaminergic systems and...
<table>
<thead>
<tr>
<th>Gene (System)</th>
<th>SNP</th>
<th>Genotypes cases N (%)</th>
<th>Genotypes controls N (%)</th>
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<th>Genotype 11 + 12 vs. 22</th>
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</thead>
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<tr>
<td></td>
<td>(P&lt;0.05)</td>
<td>Sum</td>
<td>Sum</td>
<td>P value</td>
<td>OR (95% CI)</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Sum</td>
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<td>12</td>
<td>22</td>
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<td>120 (44)</td>
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<td>137 (42)</td>
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</tr>
</tbody>
</table>

Only those markers with a nominal association (P < 0.05) after applying the Cochran–Armitage's trend test are shown; association results under dominant and recessive models are also displayed.

CI, Confidence Interval; OR, Odds Ratio.

*Statistically significant P value after applying a FDR (false discovery rate) of 15% (P<0.00047).
neurotrophic factors and their receptors represent good candidates for autism susceptibility. Numerous association studies have been performed in past years in selected candidate genes of these systems, but results are controversial. Here, we have designed a comprehensive gene-system association study including 37 genes that participate in these pathways to identify common allelic risk variants. We analyzed 307 SNPs in 326 autistic patients and 350 sex-matched controls, all of them Spanish and Caucasian to minimize the possible effects of genetic heterogeneity. The SNPs have been selected to cover the target genes in terms of LD.

The results of our case–control association study identified a single SNP (rs6592961) in the DOPA decarboxylase gene (DDC, 7p12.2) that remained significantly associated with autism after correction for multiple tests. A previous family-based study did not find association between autism and two polymorphisms (rs11575267 and rs3837091) at 5’UTR of the DDC gene, although the sample size was small (90 trios) and gene coverage was limited to the promoter region (Lauritsen et al. 2002). The DDC gene encodes the enzyme that catalyzes the last step of the biosynthesis of three essential neuromolecules: the decarboxylation of L-3,4 dihydroxyphenylalanine (L-DOPA) to dopamine, 5-hydroxytryptophan (5HTP) to serotonin and L-tryptophan to tryptamine. Hence, it represents a good candidate gene for autism: the gene product is crucial to synthesize serotonin and dopamine, and interestingly both neurotransmitter systems have been proposed to be altered in autism at different levels.

Noteworthy, some evidences indicate that the serotonergic and dopaminergic neurotransmitter systems are involved in aggressive behaviours (Chen et al. 2005; Seo et al. 2008). In this regard, drugs such as the atypical antipsychotics (AAPs) and the selective serotonin reuptake inhibitors (SSRIs), acting on the dopaminergic and serotonergic systems, are widely used in autism to target symptoms like aggression or self-injury and compulsive rituals (Nikolov et al. 2006). Recently, DDC has been suggested to be involved in anger and aggression traits in suicide behaviours (Giegling et al. 2008). Positive associations of this gene with other neuropsychiatric disorders, such as Bipolar Affective Disorder (BPAD) and Attention-Deficit/Hyperactivity Disorder (ADHD) have also been reported (Børglum et al. 2003; Ribasés et al. 2009). Replication studies performed in several Caucasian populations make the association of DDC with ADHD consistent (Lasky-Su et al. 2008). Interestingly, autism and ADHD are known to share some clinical features. Rommelse et al. (2010) reported that 20–50% of children with ADHD fulfill diagnostic criteria for autism spectrum disorders (ASD) and 30–80% of ASD children meet criteria for ADHD. In addition, in both autism and ADHD, aggressive and self-injury traits are commonly described.

A wide range of mutations that abolish the activity of DDC have been described in aromatic L-amino-acid decarboxylase deficiency (AADD), a recessively inherited disease with a severe neurological condition characterized by developmental delay, oculogyric crises and autonomic dysfunction (Lee et al. 2009). To our knowledge, no rare variants in the DDC gene with a potential etiologic role have been described in any of the neuropsychiatric disorders in which association has been reported, and no further evidence arises from CNVs studies. However, some reports described chromosomal aberrations in
the region encompassing the DDC gene: a duplication (dup(7)p11.2–p12) in a three-generation family, in which patients show mild cognitive deficiencies and limited IQ (Leach et al. 2007) and a de novo inverted duplication of 7p11.2–p14.1 in a patient that meets diagnostic criteria for autism (Wolpert et al. 2001). All these evidences suggest that common variants of modest effect in the DDC gene may be involved in the genetics of autism.

Association studies in autism have been performed in the past on several candidate genes included in the present analysis, but results did not clearly implicate any of them. These previous studies were performed in one or few candidate genes. Although different populations and statistical approaches were used, we compared all the positive findings obtained by several association studies with those obtained in our target systems to determine if coincident results in independent samples may pinpoint one or more susceptibility genes in autism (Table IV). Nominal associations have been found in our analysis in three susceptibility genes in autism (Table IV). Nominal associations have been found in our analysis in three susceptibility genes in autism (Table IV). Nominal associations have been found in our analysis in three susceptibility genes in autism (Table IV). Nominal associations have been found in our analysis in three susceptibility genes in autism (Table IV).

In this regard, de Krom et al. (2009) investigated 132 candidate genes for autism in a two-stage design association study. SNP rs167771 was the only marker showing association in a first Dutch autistic sample (P = 2.0e–04) and in a second British sample of 125 individuals (P = 0.0011). In our study we also found (nominal) association with this marker. The replication in several European cohorts suggests that DRD3 may also be involved in autism spectrum disorders. The DRD3 gene encodes the subtype D3 of the dopamine receptor, highly expressed in brain and involved in cognitive, emotional and endocrine factors. Recently, association studies have been performed to elucidate the role of this gene also in schizophrenia and major depression (Light et al. 2006; Dominguez et al. 2007; Nunokawa et al. 2010). Interestingly, risperidone and AAPs, that act as agonists of the dopamine receptor D3, are used in autism to alleviate manifestations such as aggression, self-injury, compulsive and repetitive behaviors (Nikolov et al. 2006). Noteworthy, the rs167771 SNP has also been associated with extrapyramidal symptoms in patients treated with risperidone (Gassó et al. 2009). Recently, Allen-Brady et al. (2009) performed a high-density genome-wide linkage analysis in an extended pedigree with autism. In this study suggestive linkage was detected in five genomic regions that include the 7p14.1–p11.22 and 3q13.2–q13.31 loci, where DDC and DRD3 map. However, these two regions do not represent the top linkage regions replicated by several autism consortia (Abrahams and Geschwind 2008).

Both rs6592961 (DDC) and rs167771 (DRD3) are intronic polymorphisms located in poorly conserved regions, although rs6592961 is placed in a potential regulatory region identified by ESPERR (Evolutionary and Sequence Pattern Extraction through Reduced Representations, www.bx.psu.edu/projects/esperr) (Taylor et al. 2006). In both cases the minor allele is associated with autism. However, no data are available to clarify whether these alleles have functional effects or are rather in linkage disequilibrium with the actual risk alleles. For the two markers, no differences in the MAF values were observed among the different groups of ASD (Autism, Asperger and PDD-NOS) when compared to the group of controls (data not shown). We did not re-sequence the coding or regulatory regions of the DDC gene in our sample, so it is possible that we have missed novel variants involved in the disease. In our study we achieved a reasonable SNP coverage, although we cannot exclude the presence of other variants involved in the susceptibility to autism not captured by our SNPs selection.

Table III. Haplotype analysis of 11 SNPs in 326 autistic patients and 350 controls in the DDC gene using the UNPHASED software.

<table>
<thead>
<tr>
<th>Marker haplotype</th>
<th>Global P value</th>
<th>Risk allele combinations</th>
<th>Risk haplotype P value (adjusted P value)</th>
<th>Risk haplotype OR (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-8</td>
<td>0.00038</td>
<td>T-A</td>
<td>0.00054 (0.0012)</td>
<td>1.66 (1.24–2.19)</td>
</tr>
<tr>
<td>5-8-10</td>
<td>0.000697</td>
<td>T-A-T</td>
<td>0.0005943 (0.00019)</td>
<td>2.02 (1.32–3.10)</td>
</tr>
<tr>
<td>2-5-8-10</td>
<td>0.000087</td>
<td>C-T-A-T</td>
<td>0.000049 (0.00009)</td>
<td>2.44 (1.55–3.83)</td>
</tr>
<tr>
<td>Haplotype 2-5-8-10</td>
<td>Cases (%)</td>
<td>Controls (%)</td>
<td>Haplotype specific P value</td>
<td>0.19</td>
</tr>
<tr>
<td>C-C-G-T</td>
<td>85 (18.3)</td>
<td>101 (16.1)</td>
<td></td>
<td>0.000049</td>
</tr>
<tr>
<td>C-T-A-T</td>
<td>54 (12)</td>
<td>33 (5.3)</td>
<td></td>
<td>0.000057</td>
</tr>
<tr>
<td>C-T-G-G</td>
<td>181 (40)</td>
<td>317 (50.6)</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>T-G-G-C</td>
<td>80 (17.7)</td>
<td>120 (19.2)</td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td>T-A-C</td>
<td>52 (11.5)</td>
<td>55 (8.8)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Marker Haplotype: 2-rs2329340; 5-rs2044859; 8-rs6592961; 10-rs11761683. Best multiple marker combination is indicated in bold. CI, confidence interval; OR, odds.
Table IV. Previous association studies between autism and genes encoding neurotrophic factors and receptors, and proteins of the serotoninergic and dopaminergic neurotransmission systems that displayed significant results. Comparison with the SNPs tested in our study.

<table>
<thead>
<tr>
<th>Genes</th>
<th>System</th>
<th>No. of polymorphisms per gene</th>
<th>Marker/s associated</th>
<th>P value</th>
<th>Cases (Ethnicity)</th>
<th>Ref.</th>
<th>No. of SNPs per gene</th>
<th>SNP (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HTR1B</td>
<td>SER</td>
<td>4</td>
<td>rs11568817-rs130057-&lt;br&gt;rs130058-rs6296 (H)</td>
<td>FB</td>
<td>252 (Brazilian EA)</td>
<td>Orbona et al. (2008)</td>
<td>2</td>
<td>rs130058 (P = 0.53);&lt;br&gt;rs6296 (P = 0.86)</td>
</tr>
<tr>
<td>HTR2A</td>
<td>SER</td>
<td>2</td>
<td>rs6311-rs6313 (H)</td>
<td>FB</td>
<td>126 (korean)</td>
<td>Cho et al. (2007)</td>
<td>18</td>
<td>NC</td>
</tr>
<tr>
<td>HTR3A</td>
<td>SER</td>
<td>1</td>
<td>rs1150220</td>
<td>FB</td>
<td>403 (American EA)</td>
<td>Anderson et al. (2009)</td>
<td>4</td>
<td>NC</td>
</tr>
<tr>
<td>HTR5A</td>
<td>SER</td>
<td>2</td>
<td>rs1800883</td>
<td>FB</td>
<td>186 (Portuguese)</td>
<td>Coutinho et al. (2007)</td>
<td>5</td>
<td>NC</td>
</tr>
<tr>
<td>SLC6A4</td>
<td>SER</td>
<td>2</td>
<td>5-HTTLPR and STin2VNTR</td>
<td>Several studies</td>
<td>Several populations</td>
<td>Huang et al. (2008)</td>
<td>2</td>
<td>NC</td>
</tr>
<tr>
<td>DRD1*</td>
<td>DOP</td>
<td>3</td>
<td>rs265981</td>
<td>CC</td>
<td>112 (American)</td>
<td>Hettinger et al. (2008)</td>
<td>7</td>
<td>rs11749676* (P = 0.98)</td>
</tr>
<tr>
<td>DRD3*</td>
<td>DOP</td>
<td>11</td>
<td>rs167771</td>
<td>CC</td>
<td>261 (Dutch-British)</td>
<td>de Krom et al. (2009)</td>
<td>7</td>
<td>rs11749676* (P = 0.98);&lt;br&gt;rs167771 (P = 0.023)</td>
</tr>
<tr>
<td>MAOA</td>
<td>DOP</td>
<td>5</td>
<td>rs5906883-rs1137070-&lt;br&gt;rs3027407 (H)</td>
<td>CC</td>
<td>151 (Korean)</td>
<td>Yoo et al. (2009)</td>
<td>2</td>
<td>NC</td>
</tr>
<tr>
<td>BDNF*</td>
<td>NER</td>
<td>4</td>
<td>rs56164415</td>
<td>CC</td>
<td>124 (Chinese)</td>
<td>Cheng et al. (2009)</td>
<td>6</td>
<td>NC</td>
</tr>
<tr>
<td>NTRK1</td>
<td>NER</td>
<td>4</td>
<td>6337</td>
<td>CC</td>
<td>174 (British)</td>
<td>Chakrabarti et al. (2009)</td>
<td>9</td>
<td>NC</td>
</tr>
</tbody>
</table>

SER, serotoninergic system; DOP, dopaminergic system; NER, neurotrophic factors and their receptors; H, haplotype; FB, family-based association study; CC, case–control association study; EA, European ancestry; NC, markers not considered in our study.

*Gene found nominally associated in our single-marker association study: DRD1, rs251937 (P = 0.017); DRD3, rs167771 (P = 0.023); BDNF, rs1491851 (P = 0.012)

*When the SNP found associated by other authors was not directly analyzed in our study, we considered the P value of a marker with $r^2 > 0.95$ (HapMap CEU population).
In conclusion, our findings provide new insights into the genetics of autism, showing for the first time a significant association with DDC. Further investigations are needed to establish the role of this gene in autism with replications in larger cohorts.

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Statement of Interest

L.A.P.J. is a member of the scientific advisory board of qGenomics. No other author reported any biomedical financial interests or potential conflicts of interest.

References


Supplementary material available online

Table S1. Description of the 400 SPNs initially selected for the SNPlex genotyping assays in all gene systems.

Table S2. Assessment of population stratification using 46 unlinked SNPs in our sample of 326 cases and 350 controls with the STRUCTURE v2.3 software.