ORIGINAL ARTICLE

Exploration of 19 serotoninergic candidate genes in adults and children with attention-deficit/hyperactivity disorder identifies association for *5HT2*A, *DDC* and *MAOB*

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Attention-deficit/hyperactivity disorder (ADHD) is a common psychiatric disorder in which different genetic and environmental susceptibility factors are involved. Several lines of evidence support the view that at least 30% of ADHD patients diagnosed in childhood continue to suffer the disorder during adulthood and that genetic risk factors may play an essential role in the persistence of the disorder throughout lifespan. Genetic, biochemical and pharmacological studies support the idea that the serotonin system participates in the etiology of ADHD. Based on these data, we aimed to analyze single nucleotide polymorphisms across 19 genes involved in the serotoninergic neurotransmission in a clinical sample of 451 ADHD patients (188 adults and 263 children) and 400 controls using a population-based association study. Several significant associations were found after correcting for multiple testing: (1) the *DDC* gene was strongly associated with both adulthood (*P* = 0.00053; odds ratio (OR) = 2.17) and childhood ADHD (*P* = 0.0017; OR = 1.90); (2) the *MAOB* gene was found specifically associated in the adult ADHD sample (*P* = 0.0029; OR = 1.90) and (3) the *5HT2A* gene showed evidence of association only with the combined ADHD subtype both in adults (*P* = 0.0036; OR = 1.63) and children (*P* = 0.0084; OR = 1.49). Our data support the contribution of the serotoninergic system in the genetic predisposition to ADHD, identifying common childhood and adulthood ADHD susceptibility factors, associations that are specific to ADHD subtypes and one variant potentially involved in the continuity of the disorder throughout lifespan.
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Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a highly prevalent psychiatric disorder characterized by severe impairment in attention, hyperactivity and/or impulsivity that affects up to 6% of school-age children and from 3 to 6% of adults.[1–4] Several follow-up studies have shown that at least 30% of ADHD patients diagnosed during childhood continue

to suffer the disorder during adolescence and adulthood.[5–8] The etiology of ADHD is complex, with the involvement of both genetic and environmental factors. Twin, family and adoption studies, however, support the view that there is a major genetic component in its susceptibility. Higher concordance rates in monozygotic than in dizygotic ADHD twins have been reported, showing a mean estimated heritability of 76%.[4,9,10] In addition, several family studies have shown increased prevalence of ADHD among relatives of patients with ADHD[10] and adoption studies also suggest a major genetic contribution in the predisposition to ADHD, with biological relatives of ADHD children being more likely to have ADHD than adoptive relatives.[11] Although genetic studies have mainly focused on ADHD children,

several evidences point to the existence of an even stronger genetic component in adult ADHD: (1) there is a higher risk for ADHD among children of ADHD parents than among relatives of children with ADHD, (2) ADHD is more prevalent among relatives of persistent ADHD probands than relatives of remitted ADHD probands and (3) relatives of adolescent probands show greater risk of ADHD than relatives of child probands.[12–15] These data suggest that persistence of ADHD into adolescence and adulthood is influenced by genetic factors and these might be greater in the etiology of persistent than remitting ADHD.

Studies of genetic susceptibility to ADHD have mainly focused on the dopaminergic neurotransmitter system. However, biochemical, pharmacological and molecular studies support an important role for serotonin neurotransmission, in addition to dopamine, in the etiology of the disorder.[16] In the first place, early biochemical evidence suggested low serotonin levels and reduced serotoninergic uptake in platelets from ADHD patients.[17,18] Altered cerebrospinal fluid concentrations of 5-hydroxyindoleacetic acid, a serotoninergic metabolite, are consistently associated with hyperactivity, aggression and impulsive behavior, common characteristics in ADHD.[19–21] In addition, it has been reported that tryptophan depletion, the essential amino acid for brain serotonin synthesis, impairs learning and memory in healthy controls.[22]

Pharmacological studies have also provided insight into the role of serotonin in ADHD susceptibility. Although the most effective treatments for ADHD are based on the administration of stimulant drugs, there is evidence that some serotonin agonists, including selective serotonin reuptake inhibitors, tricyclic antidepressants and monoamine oxidase inhibitors, also reduce ADHD symptoms.[23–26] Moreover, pharmacological studies in humans and rodents support a contribution of the serotoninergic system to psychostimulant-mediated behavior, such as locomotor stimulatory and rewarding effects.[27–33]

Animal models emphasize the potential importance of serotonin in ADHD too. The decreased locomotion in response to psychostimulants in mice lacking the gene encoding the dopamine transporter (SLC6A3; DAT-KO) depends on serotoninergic neurotransmission rather than a direct effect on dopamine reuptake. Once administered, serotonin agonists, which include 5-hydroxytryptophan, L-tryptophan and selective serotonin reuptake inhibitors, markedly attenuate hyperactivity in the DAT-KO mice.[34] In addition, mice lacking the 5HT1B receptor show hyperactivity, increased aggression and behavioral deinhibition[35–39] and 5HT4 receptor knockout mice show attenuated novelty-induced exploratory activity.[40] Finally, 5HT2 receptor agonists modulate hyperlocomotor activity in rats, and monoamine oxidase inhibitors reduce impulsiveness in animal models of ADHD.[41,42]

Association studies also provide growing evidence for the involvement of serotoninergic genes in ADHD.

Of these, *SLC6A4, 5HT2A* and *5HT2B* genes are the most widely studied ones. Several case–control and family-based studies have demonstrated association between different *SCL6A4* functional polymorphisms, which include the promoter 5HTT-linked polymorphic region and the 5HTTVNTR in intron 2, and ADHD or ADHD-related psychopathological traits[43,44] (Supplementary Table 1). Other research groups aimed to determine the involvement of the *5HT2A* and *5HT2B* genes in ADHD in several data sets and reported contradictory results (Supplementary Table 1). Finally, it has been reported that SLC6A4 together with the serotonin receptor *5HT1A* and the tryptophan hydroxylase gene (*TPH1*) accounted for 3.09% of ADHD variance.[45]

Based on all these data, we hypothesized that alterations in serotonin neurotransmission may be involved in genetic susceptibility to ADHD, and that common risk variants may participate in both childhood and adulthood ADHD. To address these issues, we ran a population-based association study in 451 ADHD patients (188 adults and 263 children) and 400 controls, with single nucleotide polymorphisms (SNPs) covering 19 candidate genes that encode serotonin receptors (5HT1A, 5HT1B, 5HT1D, 5HT1E, 5HT1F, 5HT2A, 5HT2B, 5HT2C, 5HT3A, 5HT3B, 5HT4, 5HT5A, 5HT6 and 5HT7), the serotonin transporter (SLC6A4) and enzymes involved in serotonin synthesis (TPH1 and DDC) and degradation (MAOA and MAOB).

Materials and methods

Subjects
The clinical sample consisted of 451 Caucasoid patients with ADHD recruited from three centers in the Barcelona area (Spain) between 2004 and 2006. All subjects met DSM-IV (Diagnostic and Statistical Manual of Mental Disorders) criteria for ADHD and consisted of 188 adult cases (67% combined ADHD, 29.3% inattentive ADHD and 3.7% hyperactive-impulsive ADHD patients) and 263 children (73% combined ADHD, 22% inattentive ADHD and 5% hyperactive-impulsive ADHD patients). As two child samples were sons of two adult patients, the children were excluded when all the samples were appraised together. Seventy-eight percent of patients were males (72.8% of adults and 82.1% of children). Diagnosis was blind to genotype. The control sample, consisting of 400 Caucasoid-unrelated adult subjects in whom DSM-IV ADHD symptoms have been excluded retrospectively, matched for sex the ADHD clinical group. The average age at assessment was 29.86 years (s.d. = 11.86) for adult ADHD patients, 9.18 years (s.d. = 2.56) for child ADHD patients and 42.38 years (s.d. = 13.6) for the controls. The study was approved by the ethics committee of each participating institution and written informed consent was obtained from all adult subjects, children and their parents.

Clinical assessment

Adulthood ADHD. All adult ADHD patients were recruited from the Department of Psychiatry of the Hospital Universitari Vall d'Hebron. The diagnosis of ADHD in adulthood was evaluated with the Structured Clinical Interview for DSM-IV Axis I and II Disorders (SCID-I and SCID-II) and the Conners' Adult ADHD Diagnostic Interview for DSM-IV (CAADID Parts I and II).[46] Severity of ADHD symptoms was evaluated with the long version of the Conners' ADHD Rating Scale (self-report form CAARS-S:L and observer form CAARS-O:L),[47] the ADHD Rating Scale (ADHD-RS),[48] the ADHD Screening Checklist[49] and the Wender Utah Rating Scale (WURS)[50] for retrospective symptomatology. The level of impairment was measured with the Clinical Global Impression (CGI), included in the CAADID Part II, and the Sheehan Disability Inventory (SDI).[51] For the evaluation of psychiatric symptoms, patients were filled in the Beck Depression Inventory (BDI), the State Trait Anxiety Inventory (STAI) and the Millon Clinical Multiaxial Inventory (MCMI-II). Full-Scale IQ was estimated with the Vocabulary and Block Design subtests of the WAIS-III. Patients also completed the digit span, arithmetic, Letter-Number Sequencing and Symbol Search subtests of the WAIS-III, the Conners' Continuous Performance Test (CPT),[52] the California Verbal Learning Test (CVLT), the Logical Memory I–II and Visual Memory I–II of the WMS-R and the trail-making test (Parts A and B).

Childhood ADHD. The same diagnostic procedures were applied in all three Institutions (Department of Psychiatry, Hospital Universitari Vall d'Hebron; Child and Adolescent Mental Health Unit, Hospital Mútua de Terrassa; Unitat de Neuropediatria, Hospital de Sabadell). The diagnosis of ADHD in children was evaluated with the present and lifetime version of the Schedule for Affective Disorders and Schizophrenia for School-age children (K-SADS-PL). Rating scales used to evaluate ADHD symptoms were the Long Version of the Conners' Parent Rating Scale (CPRS-R:L) and the Long Version of the Conners' Teacher Rating Scale (CTRS-R:L). General psychiatric symptomatology was evaluated using the Achenbach System of Empirically Based Assessment (ASEBA), the Child Behavior Checklist (CBCL) for parents, the Teacher Report Form (TRF) for teachers, the parent and teacher versions of the Strengths and Difficulties Questionnaires (SDQ) and the Clinical Global Impressions of ADHD (CGI). Neuropsychological evaluation included the Wechsler Intelligence Scale for Children (WISC-R: WISC IV). Reading and writing performance was evaluated with the TALE (Spanish reading and writing test, primary education), TALEC (Catalan reading and writing test, primary education) and PROLEC-SE (Catalan reading and writing test, secondary education).[53,54] Parents completed a general questionnaire on the socio-demographic and general health information of their child and parents'

family psychiatric history. A self-scored questionnaire of ADHD symptoms was filled out by both parents,[49] who also completed a scale of pregnancy and perinatal information on their child. Parenting style was assessed by the perceived parental rearing practices questionnaire (EMBU).

Exclusion criteria for both adults and children included IQ < 70, schizophrenia or other psychotic disorders, ADHD symptoms due to mood, anxiety, dissociative or personality disorders, adoption, sexual or physical abuse, birth weight < 1.5 kg, and other neurological or systemic disorders that might explain ADHD symptoms.

DNA isolation and quantification

Genomic DNA was isolated from peripheral blood lymphocytes by the salting-out procedure[55] or using 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. Subsequently, all DNA samples were normalized to 75 ng μl^{-1}.

SNP selection

We selected 19 candidate genes involved in the serotoninergic neurotransmission pathway that encode 14 serotonin receptors (*5HT1A*, *5HT1B*, *5HT1D*, *5HT1E*, *5HT1F*, *5HT2A*, *5HT2B*, *5HT2C*, *5HT3A*, *5HT3B*, *5HT4*, *5HT5A*, *5HT6* and *5HT7*), the serotonin transporter (*SLC6A4*), and four enzymes involved in serotonin synthesis (*TPH1* and *DDC*) and degradation (*MAOA* and *MAOB*) (Supplementary Table 2). For the SNP selection, we used information on the Centre d'Etude du Polymorphisme Humain (CEPH) panel from the HapMap database (www.hapmap.org; release 20, January 2006[56]). To minimize redundancy of selected markers and ensure full genetic coverage of candidate genes, we evaluated with the linkage disequilibrium (LD)-select software,[57] the LD pattern of the region spanning each candidate gene plus 3–5 kb flanking sequences. TagSNPs were selected at an r^2 threshold of 0.85 from all SNPs with minor allele frequency (MAF) > 0.15 for genes with fewer than 15 tagSNPs (*5HT1A*, *5HT1B*, *5HT1D*, *5HT1E*, *5HT1F*, *5HT2B*, *5HT2C*, *5HT3A*, *5HT3B*, *5HT5A*, *5HT6*, *5HT7*, *SLC6A4*, *TPH1*, *MAOA* and *MAOB*) and MAF > 0.25 for those genes with more than 15 tagSNPs (*5HT2A*, *5HT4* and *DDC*). A total of 129 tagSNPs (69 in multi-loci bins and 60 singletons) were chosen with these criteria. Three additional SNPs located within exons were included in the analysis: rs1058576 in exon 3 of the *5HT2A* gene, rs6318 in exon 4 of the *5HT2C* gene and rs2228673 in exon 5 of the *SLC6A4* gene.

74

Plex design, genotyping and quality control

We assessed the 132 selected SNPs with the automated assay design pipeline at ms.appliedbiosystems.com/snplex/snplexStart.jsp. Two SNPlex genotyping assays of 47 and 48 SNPs were designed, 27 SNPs were included in two other assays with additional SNPs not related to this project and a proper design could not be achieved for 10 SNPs, which translates into a design rate of 92.4%. To detect population stratification, 48 unlinked anonymous SNPs located at least 100 kb distant from known genes were also genotyped.[58] All SNPs were genotyped using the SNPlex platform (Applied Biosystems, Foster City, CA, USA) as described.[59] Briefly, DNA was fragmented by heating at 99 °C for 10 min. Fragmented DNA (150 ng) was dispensed in a 384 microplate and the manufacturer's specifications were followed through phosphorylation, oligonucleotide ligation, exonuclease cleanup, PCR and hybridization steps using liquid-handling robots. Finally, the specifically bound fluorescent probes were eluted and analyzed on an Applied Biosystems 3730xl DNA Analyzer. Two HapMap samples (NA11992 and NA11993) were included in all genotyping assays and a concordance rate of 99.98% with HapMap data was obtained. In addition, no differences were found among four replicate samples.

Statistical analyses

To better understand the genetic predisposition to adult and childhood ADHD, we first analyzed independently both clinical samples. Then, and only when a potential common susceptibility factor was identified, the two data sets were analyzed together. To test the hypothesis that the serotoninergic system may confer predisposition on the ADHD clinical subtypes by different mechanisms and to reduce heterogeneity, ADHD patients were also subdivided into two main diagnostic groups, combined ADHD and inattentive ADHD. The hyperactive-impulsive ADHD group could not be considered due to its small sample size ($n = 20$). The analysis of minimal statistical power was performed *post hoc* using the Genetic Power Calculator software,[60] assuming an odds ratio (OR) of 1.5, prevalence of 0.05, significance level of 0.05 and the lowest MAF of 0.153. We tested potential genetic stratification in our sample by analyzing the SNPs in Hardy–Weinberg equilibrium from the 48 anonymous SNP set with three different approaches: (1) the STRUCTURE software (version 2.0)[61,62] was used under the admixture model, with a length of the burning period and a number of Markov Chain Monte Carlo (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; (2) the Fst coefficient was calculated using the Weir and Cockerham approach with the FSTAT software and the 95% confidence interval was determined by bootstrapping[63,64] and (3) the method by Pritchard and Rosenberg[65] was implemented to test whether the genotype distributions at each marker loci (under codominant, dominant and recessive models) are the same in the case and control groups. As ADHD children were collected by three clinical groups, genetic heterogeneity among them was evaluated by comparing allele frequencies of the 88 autosomic SNPs included in the case–control study using χ^2 tests with the statistical package SPSS 12.0.

Single-marker analysis. The analysis of Hardy–Weinberg equilibrium (threshold set at $P < 0.01$) and the comparison of both genotype and allele frequencies between cases and controls was performed using the SNPassoc R package.[66] Dominant (11 vs 12 + 22) and recessive (11 + 12 vs 22) models were only considered for those SNPs displaying nominal association when either genotypes under a codominant model or allele frequencies were taken into account. All tests were adjusted for sex. Genotype frequencies of SNPs within genes located on chromosome X were considered in the sample of females, whereas in the comparison of allele frequencies both males and females were analyzed. For the multiple comparison correction, we considered all tests performed and assumed a false discovery rate (FDR) of 15%, which corresponds to a significant threshold of $P < 0.00191$, using the Q-value R package.[67]

Multiple-marker analysis. To minimize multiple testing and type I errors (α), we decided *a priori* to restrict the haplotype-based association study to those genes associated with ADHD in the single-marker analyses after correction for multiple comparisons. For each of these genes, rather than simplifying the study to physically contiguous SNPs, the best two-marker haplotype from all possible combinations was identified in the adult sample. Likewise, additional markers (up to four) were added in a stepwise manner to the initial two-SNP haplotype. The best two-, three- and four-marker haplotypes identified within each gene were subsequently evaluated in the child and adult + child samples in order to test the initial association in the other data sets. In all cases, significance was estimated by a permutation procedure using 10 000 permutations with the UNPHASED software.[68] Since the expectation-maximization algorithm implemented in the UNPHASED software does not accurately estimate low haplotype frequencies,[69] haplotypes with frequencies < 0.05 were excluded. To evaluate potential additive and epistatic effects between the identified risk haplotypes, we first assigned specific estimated haplotypes to individuals considering cases and controls separately with the PHASE 2.0 software.[70] Then, we implemented a stepwise logistic regression procedure using the statistical package SPSS 12.0. Epistasis analysis was performed by taking genes two by two and comparing two different regression models by a likelihood ratio test. In the first model, we took the affection status as a

dependent variable and the two risk haplotypes as predictive variables. In the second model, we included the interaction between haplotypes as an independent variable in the logistic regression model.

Results

We studied tagSNPs in 19 candidate genes encoding proteins involved in the synthesis, degradation, transport and signaling of the serotonin neurotransmitter system in 451 ADHD cases (188 adults and 263 children) and 400 controls. Of the 132 SNPs initially selected for inclusion in the SNPlex assay, 25 were discarded (10 did not pass through the SNPlex design pipeline, 2 were monomorphic, 6 had genotype calls < 90%, 5 had MAFs < 0.15 in the control sample and 2 had significant departures from Hardy–Weinberg equilibrium in the control group ($P < 0.01$)). To avoid redundancies in genetic information, we determined the LD pattern in the control group and discarded seven additional SNPs that were in strong LD with other SNPs within the same candidate genes ($r^2 > 0.85$) (Supplementary Table 2). Thus, a total of 100 SNPs with an average genotype call rate of 98.8% (s.d. = 0.7) were used for our final analysis. Taking the SNP with the lowest MAF (0.153) and assuming an OR of 1.5, the adult ADHD sample showed minimum statistical powers of 61.2, 31.7 and 78.3% when the combined, inattentive and all ADHD patients were considered, respectively. The child ADHD sample had minimum statistical powers of 79.1, 33.1 and 90.0% for the combined, inattentive and all ADHD patients, respectively.

Analysis of single-markers
After excluding population admixture in our sample using the STRUCTURE software (Supplementary Table 3), the Fst coefficient (theta = 0 with a 95% confidence interval of 0.000–0.001) and the method by Pritchard and Rosenberg ($P = 0.1225$), we compared genotype and allele frequencies between all adult ADHD patients and controls. Nominal significant differences were found for eight SNPs located in five genes: *5HT2A*, *5HT6*, *5HT7*, *DDC* and *MAOB* (Table 1 and Supplementary Table 4). When we subdivided adult patients by ADHD clinical subtype, we identified 14 SNPs within 6 genes (*5HT2A*, *5HT2C*, *5HT3B*, *5HT7*, *DDC* and *MAOB*) displaying nominal association with combined ADHD and 5 SNPs in 4 genes (*5HT1F*, *5HT3A*, *5HT6* and *MAOB*) associated with inattentive ADHD (Table 1 and Supplementary Table S4). However, after correcting for multiple comparisons by applying an FDR of 15% ($P < 0.0019$), only rs6592961 in the *DDC* gene ($P = 0.00067$, OR = 1.89 (1.31–2.72)) and rs3027415 in the *MAOB* gene ($P = 0.00056$, 1.89 (1.32–2.70)) remained positively associated when all the adult ADHD samples were taken into account. In the combined ADHD clinical subtype, association was still significant for rs7984966 in the *5HT2A* gene

($P = 0.0012$, OR = 1.97 (1.30–2.99)) and rs6592961 in the *DDC* gene ($P = 0.00059$, OR = 2.09 (1.38–3.17)).

Genetic heterogeneity was first excluded in the childhood ADHD data set recruited from three different centers ($P > 0.05$). Subsequently, single-marker analysis identified seven SNPs in four serotoninergic genes (*5HT1B*, *5HT1D*, *5HT2A* and *DDC*) significantly associated with ADHD (Table 2 and Supplementary Table 4). We further considered the two ADHD clinical subtypes and observed 11 SNPs in 5 genes (*5HT1B*, *5HT1D*, *5HT2A*, *DDC* and *TPH1*) nominally associated with combined ADHD, and 2 SNPs in 2 genes (*5HT1D* and *DDC*) associated with inattentive ADHD (Table 2 and Supplementary Table 4). However, after applying an FDR of 15%, only rs6592961 within the *DDC* gene remained positively associated when all the childhood ADHD patients were considered ($P = 1.9e−06$, OR = 2.22 (1.60–3.08)). Applying this correction to the ADHD clinical subgroups, only rs7322347 in *5HT2A* ($P = 0.0015$, OR = 1.49 (1.16–1.92)) and the *DDC* rs6592961 sequence variant ($P = 7.2e−05$, OR = 2.09 (1.45–3.01)) were still associated with combined ADHD, and an SNP in the DDC gene (rs6592961, $P = 0.00061$, OR = 2.71 (1.54–4.76)) showed evidence of association with inattentive ADHD.

In summary, as shown in Figure 1, after correction for multiple testing, one SNP (rs6592961) within the *DDC* gene was associated with adult and child ADHD samples both when taking into account all clinical subtypes and when considering only the combined subtype. This SNP was also positively associated with inattentive ADHD in the child sample. In addition, two different SNPs in the *5HT2A* gene were associated with combined ADHD in both adult (rs7984966) and child (rs7322347) clinical groups. Finally, rs3027415 in the *MAOB* gene was found to be associated with ADHD only in the adult sample.

Analysis of multiple markers
To minimize multiple testing, only the genes that showed evidence for association in the single-marker analysis after correction for multiple comparisons (*DDC*, *5HT2A* and *MAOB*) were considered for the multiple-marker analysis in the relevant ADHD subtype and/or age group. All the associations described in the sections below remained significant after applying a multiple comparison correction by permutation (see adjusted P-values in Tables 3a, 4a and 5a).

DDC. The analysis of all possible SNP combinations within the *DDC* gene revealed a four-marker haplotype (rs11238131/rs6592961/rs1982406/rs2044859) associated with ADHD in adults (global $P = 0.0042$) (Figure 1). These results were independently replicated in children (global $P = 0.018$) and when both clinical samples were considered together (global $P = 0.00092$) (Table 3a). The evaluation of the contribution of individual haplotypes to the phenotype showed evidence of an

Table 1 Association study in 188 adult ADHD patients (126 with combined ADHD, 55 inattentive ADHD and 7 hyperactive-impulsive ADHD patients) and 400 controls

| Gene | SNP | Genotypes | | | | | | | | | Alleles | | | | | |
| | | Cases N (%) | | | | Controls N (%) | | | | P-value | Genotype 11 vs 12+22 | | Genotype 22 vs 11+12 | | Allele 2 vs allele 1 | |
		11	12	22	Sum	11	12	22	Sum		OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
All																
5HT2A	rs7984966	79 (42.0)	87 (46.3)	22 (11.7)	188	202 (50.5)	169 (42.2)	29 (7.2)	400	0.069	1.42[a] (1.00–2.01)	0.050	1.70 (0.95–3.05)	0.079	1.35 (1.04–1.75)	0.024
	rs9534495	72 (38.3)	90 (47.9)	26 (13.8)	188	119 (29.9)	208 (52.3)	71 (17.8)	398	0.10	1.47[a] (1.02–2.08)	0.042	1.35[a] (0.83–2.17)	0.22	1.30 (1.01–1.67)	0.043
	rs7322347	45 (24.2)	105 (56.5)	36 (19.4)	186	102 (26.0)	182 (46.4)	108 (27.6)	392	0.046	1.10[a] (0.73–1.64)	0.66	1.59[a] (1.04–2.44)	0.030	1.14[a] (0.89–1.45)	0.30
5HT6	rs9659997	63 (34.6)	81 (44.5)	38 (20.9)	182	174 (44.3)	167 (42.5)	52 (13.2)	393	0.026	1.49[a] (1.04–2.15)	0.029	1.72 (1.08–2.73)	0.023	1.43 (1.11–1.85)	0.0055
5HT7	rs11817364	111 (60.0)	58 (31.4)	16 (8.6)	185	265 (67.4)	111 (28.2)	17 (4.3)	393	0.074	1.37[a] (0.96–1.97)	0.086	2.07 (1.02–4.20)	0.046	1.41 (1.05–1.92)	0.024
	rs11186320	80 (42.8)	80 (42.8)	27 (14.4)	187	212 (53.8)	147 (37.3)	35 (8.9)	394	0.025	1.55[a] (1.09–2.21)	0.014	1.71 (1.00–2.93)	0.053	1.47 (1.12–1.89)	0.0049
DDC[&]	rs6592961[&]	108 (58.1)	72 (38.7)	6 (3.2)	186	288 (72.4)	98 (24.6)	12 (3.0)	398	0.0023	1.89[a] (1.31–2.72)	0.00067[&]	1.08 (0.40–2.92)	0.88	1.61 (1.18–2.17)	0.0029
MAOB[&]	rs3027415[&]	24 (48.0)	19 (38.0)	7 (14.0)	50	70 (72.2)	21 (21.6)	6 (6.2)	97	0.015	2.81[a] (1.38–5.72)	0.0041	2.47 (0.78–7.79)	0.12	1.89 (1.32–2.70)	0.00056[&]
Combined																
5HT2A[&]	rs1328684	67 (53.2)	47 (37.3)	12 (9.5)	126	167 (42.6)	195 (49.7)	30 (7.7)	392	0.049	1.27 (0.63–2.56)	0.51	1.67 (1.10–2.5)	0.014	1.23[a] (0.9–1.68)	0.19
	rs2770296	72 (58.1)	43 (34.7)	9 (7.3)	124	192 (48.1)	163 (40.9)	44 (11.0)	399	0.12	1.49 (0.99–2.22)	0.053	1.59[a] (0.75–3.33)	0.21	1.41[a] (1.02–1.95)	0.03
	rs2224721	82 (65.6)	42 (33.6)	1 (0.8)	125	242 (60.5)	134 (33.5)	24 (6.0)	400	0.019	1.25 (0.82–1.89)	0.30	7.69 (1.06–50)	0.0052	1.38 (0.96–1.99)	0.079
	rs7984966[&]	43 (34.1)	68 (54.0)	15 (11.9)	126	202 (50.5)	169 (42.2)	29 (7.2)	400	0.0040	1.97[a] (1.30–2.99)	0.0012[&]	1.73 (0.89–3.34)	0.11	1.61 (1.19–2.17)	0.0019[&]
	rs9534495	54 (42.9)	58 (46.0)	14 (11.1)	73	119 (29.9)	208 (52.3)	71 (17.8)	398	0.016	1.75 (1.16–2.63)	0.0079	1.75[a] (0.94–3.22)	0.064	1.51[a] (1.12–2.04)	0.0054
	rs7322347	33 (26.6)	70 (56.5)	21 (16.9)	124	102 (26.0)	182 (46.4)	108 (27.6)	392	0.039	1.03 (0.65–1.61)	0.91	1.85[a] (1.11–3.12)	0.014	1.25[a] (0.93–1.67)	0.13
5HT2C	rs518147	10 (33.3)	16 (53.3)	4 (13.3)	30	44 (45.4)	47 (48.5)	6 (6.2)	97	0.32	1.66[a] (0.70–3.92)	0.24	2.33 (0.61–8.89)	0.23	1.46 (1–2.13)	0.049
5HT3B	rs1672717	42 (33.6)	74 (59.2)	9 (7.2)	125	181 (46.2)	175 (44.6)	36 (9.2)	392	0.017	1.70[a] (1.11–2.59)	0.012	1.30[a] (0.61–2.78)	0.48	1.27 (0.94–1.69)	0.12
5HT7	rs11817364	78 (63.4)	31 (25.2)	14 (11.4)	123	265 (67.4)	111 (28.2)	17 (4.3)	393	0.026	1.20[a] (0.78–1.83)	0.41	2.85 (1.36–5.98)	0.0070	1.39 (0.99–1.96)	0.060
	rs11186320	56 (44.8)	47 (37.6)	22 (17.6)	125	212 (53.8)	147 (37.3)	35 (8.9)	394	0.023	1.44[a] (0.96–2.16)	0.077	2.21 (1.24–3.94)	0.0090	1.51 (1.11–2.04)	0.0079
	rs12259062	46 (37.4)	48 (39.0)	29 (23.6)	123	166 (42.2)	170 (43.3)	57 (14.5)	393	0.067	1.23[a] (0.81–1.86)	0.33	1.84 (1.11–3.05)	0.020	1.34 (1.0–1.80)	0.048
DDC[&]	rs1982406	58 (46.8)	58 (46.8)	8 (6.5)	124	233 (58.5)	134 (33.7)	31 (7.8)	398	0.033	1.61[a] (1.07–2.41)	0.022	1.22[a] (0.55–2.70)	0.62	1.30 (0.95–1.78)	0.10
	rs6592961[&]	69 (55.6)	50 (40.3)	5 (4.0)	124	288 (72.4)	98 (24.6)	12 (3.0)	398	0.0026	2.09[a] (1.38–3.17)	0.00059[&]	1.35 (0.47–3.91)	0.59	1.75 (1.25–2.50)	0.0018[&]
MAOB	rs3027415	14 (50.0)	10 (35.7)	4 (14.3)	28	70 (72.2)	21 (21.6)	6 (6.2)	97	0.089	2.59[a] (1.09–6.15)	0.031	2.53 (0.66–9.68)	0.19	1.72 (1.14–2.63)	0.011
Inattentive																
5HT1F	rs1503433	21 (38.2)	20 (36.4)	14 (25.5)	55	145 (36.4)	208 (52.3)	45 (11.3)	398	0.023	1.03 (0.57–1.85)	0.92	2.54 (1.28–5.05)	0.011	1.30 (0.87–1.96)	0.20
5HT3A	rs1150222	29 (52.7)	25 (45.5)	1 (1.8)	55	284 (71.0)	103 (25.8)	13 (3.2)	400	0.018	2.11[a] (1.19–3.76)	0.012	1.96[a] (0.25–14.29)	0.49	1.63 (1.01–2.63)	0.051
	rs1176717	25 (45.5)	28 (50.9)	2 (3.6)	55	248 (62.0)	136 (34.0)	16 (4.0)	400	0.069	1.90 (1.08–3.37)	0.027	1.12 (0.25–5.0)	0.88	1.51 (0.96–2.36)	0.078
5HT6	rs9659997	19 (34.5)	22 (40.0)	14 (25.5)	55	174 (44.3)	167 (42.5)	52 (13.2)	393	0.078	1.48[a] (0.82–2.68)	0.19	2.21 (1.13–4.36)	0.028	1.56 (1.04–2.32)	0.031
MAOB	rs3027415	9 (42.9)	9 (42.9)	3 (14.3)	21	70 (72.2)	21 (21.6)	6 (6.2)	97	0.041	3.46[a] (1.31–9.13)	0.012	2.53 (0.58–11.05)	0.24	2.17 (1.28–3.70)	0.0055

Abbreviations: CI, confidence interval; FDR, false discovery rate; OR, odds ratio; SNP, single nucleotide polymorphism.
[&]Statistically significant *P*-values after applying an FDR of 15% (*P* < 0.00191).
[a]When odds ratio < 1, the inverted score is shown.

Table 2 Association study in 263 child ADHD patients (192 combined ADHD, 58 inattentive ADHD and 13 hyperactive-impulsive ADHD patients) and 400 controls

		Genotypes										Alleles					
		Cases N (%)				Controls N (%)				P-value	Genotype 11 vs 12+22		Genotype 22 vs 11+12		Allele 2 vs allele 1		
Gene	SNP	11	12	22	Sum	11	12	22	Sum		OR (95% CI)	P-value	OR (95% CI)	P-value	OR (95% CI)	P-value
All																
5HT1B	rs6296	138 (53.7)	108 (42.0)	11 (4.3)	257	235 (59.9)	130 (33.2)	27 (6.9)	392	0.033	1.28[a] (0.93–1.77)	0.12	1.75[a] (0.85–3.70)	0.12	1.09 (0.84–1.41)	0.52
5HT1D	rs604030	111 (42.9)	124 (47.9)	24 (9.3)	259	149 (37.9)	183 (46.6)	61 (15.5)	393	0.046	1.22 (0.88–1.69)	0.22	1.82[a] (1.11–3.03)	0.015	1.28[a] (1.01–1.61)	0.038
5HT2A	rs7984966	118 (45.0)	112 (42.7)	32 (12.2)	262	202 (50.5)	169 (42.2)	29 (7.2)	400	0.073	1.26[a] (0.92–1.72)	0.15	1.78 (1.05–3.03)	0.033	1.28 (1.01–1.64)	0.039
	rs7997012	124 (47.9)	111 (42.9)	24 (9.3)	259	164 (41.8)	167 (42.6)	61 (15.6)	392	0.052	1.28 (0.93–1.75)	0.13	1.78[a] (1.07–2.94)	0.02	1.31 (1.04–1.67)	0.023
	rs7322347	86 (33.2)	123 (47.5)	50 (19.3)	259	102 (26.0)	182 (46.4)	108 (27.6)	392	0.033	1.39 (0.99–1.96)	0.061	1.56[a] (1.07–2.33)	0.018	1.35[a] (1.08–1.69)	0.0085
DDC[&&]	rs1982406	122 (46.9)	113 (43.5)	25 (9.6)	260	233 (58.5)	134 (33.7)	31 (7.8)	398	0.017	1.58 (1.15–2.17)	0.0044	1.28 (0.73–2.22)	0.39	1.39 (1.09–1.79)	0.0087
	rs6592961[&&]	142 (54.4)	109 (41.8)	10 (3.8)	261	288 (72.4)	98 (24.6)	12 (3.0)	398	1.0e-05[&&]	2.22a (1.60–3.08)	1.9e-06[&&]	1.31 (0.56–3.09)	0.54	1.82 (1.39–2.44)	2.03e−05[&&]
Combined																
5HT1B	rs6296	102 (54.3)	80 (42.6)	6 (3.2)	188	235 (59.9)	130 (33.2)	27 (6.9)	392	0.015	1.25[a] (0.88–1.78)	0.21	2.5[a] (1.01–6.25)	0.030	1.03 (0.77–1.37)	0.83
5HT1D	rs604030	84 (44.2)	88 (46.3)	18 (9.5)	190	149 (37.9)	183 (46.6)	61 (15.5)	393	0.074	1.28 (0.90–1.81)	0.17	1.81[a] (1.04–3.26)	0.030	1.31[a] (1.01–1.7)	0.039
5HT2A[&]	rs7984966	82 (42.9)	82 (42.9)	27 (14.1)	191	202 (50.5)	169 (42.2)	29 (7.2)	400	0.021	1.38 (0.97–1.95)	0.072	2.11 (1.21–3.69)	0.0097	1.41 (1.09–1.82)	0.011
	rs6561333	87 (45.8)	79 (41.6)	24 (12.6)	190	141 (35.9)	176 (44.8)	76 (19.3)	393	0.050	1.47 (1.04–2.13)	0.030	1.59[a] (0.96–2.63)	0.067	1.39[a] (1.07–1.78)	0.011
	rs6561332	46 (24.3)	85 (45.0)	58 (30.7)	189	134 (33.5)	177 (44.2)	89 (22.2)	400	0.048	1.50[a] (1.01–2.23)	0.040	1.51 (1.02–2.23)	0.94	1.38 (1.08–1.77)	0.0098
	rs7997012	101 (53.2)	71 (37.4)	18 (9.5)	190	164 (41.8)	167 (42.6)	61 (15.6)	392	0.021	1.59 (1.11–2.22)	0.011	1.69[a] (0.97–3.03)	0.053	1.48[a] (1.13–1.93)	0.0039
	rs7322347[&]	72 (37.9)	82 (43.2)	36 (18.9)	190	102 (26.0)	182 (46.4)	108 (27.6)	392	0.0092	1.69 (1.18–2.5)	0.0053	1.62[a] (1.05–2.44)	0.027	1.49[a] (1.16–21.92)	0.0015[&]
DDC[&&]	rs1982406	82 (43.2)	91 (47.9)	17 (8.9)	190	233 (58.5)	134 (33.7)	31 (7.8)	398	0.0024	1.85 (1.30–2.63)	0.00058	1.22 (0.65–2.27)	0.54	1.51 (1.15–1.96)	0.0031
	rs6944090	96 (50.3)	83 (43.5)	12 (6.3)	191	239 (60.1)	133 (33.4)	26 (6.5)	398	0.058	1.49[a] (1.05–2.12)	0.025	1.04[a] (0.51–2.13)	0.91	1.28 (0.97–1.69)	0.077
	rs6592961[&&]	107 (56.0)	78 (40.8)	6 (3.1)	191	288 (72.4)	98 (24.6)	12 (3.0)	398	0.00027[&]	2.09 (1.45–3.01)	7.2e-05[&&]	1.09 (0.40–2.98)	0.87	1.72 (1.27–2.38)	0.00053[&]
TPH1	rs1800532	58 (30.7)	95 (50.3)	36 (19.0)	190	161 (40.4)	174 (43.6)	64 (16.0)	399	0.053	1.57[a] (1.09–2.28)	0.015	1.23 (0.78–1.943)	0.37	1.32 (1.03–1.69)	0.030
Inattentive																
5HT1D	rs676643	43 (76.8)	12 (21.4)	1 (1.8)	56	254 (64.6)	122 (31.0)	17 (4.3)	393	0.14	1.85 (0.96–3.57)	0.054	2.5[a] (0.33–20)	0.31	1.77[a] (0.98–3.18)	0.045
DDC[&]	rs6592961[&]	28 (49.1)	26 (45.6)	3 (5.3)	57	288 (72.4)	98 (24.6)	12 (3.0)	398	0.0028	2.71[a] (1.54–4.76)	0.00061[&]	1.78 (0.49–6.52)	0.41	2.17 (1.37–3.33)	0.0014[&]

Abbreviations: CI, confidence interval; FDR, false discovery rate; OR, odds ratio; SNP, single nucleotide polymorphism.
[&]Statistically significant P-values after applying an FDR of 15% (P < 0.00191); [&&]Statistically significant P-values after Bonferroni correction (P < 8e-05).
[a]When odds ratio < 1, the inverted score is shown.
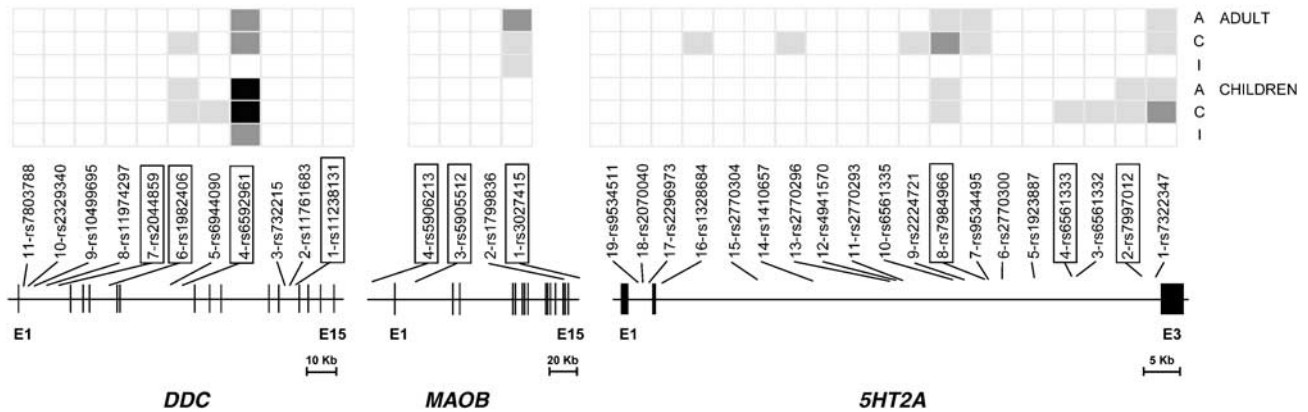
Figure 1 Diagram of the *DDC*, *MAOB* and *5HT2A* genes. The three genes, with their exon/intron structures, are drawn to different scales. All tagSNPs included in the study are shown on top of each gene. SNPs that conform haplotypes significantly associated with ADHD are boxed. On top, the level of significance of individual SNPs is indicated with different colors: white (no association), light gray (nominal association, $P < 0.05$), dark gray (significant association after a 15% FDR correction for multiple testing, $P < 0.00191$) and black (significant association after Bonferroni correction, $P < 8e-05$). A, all the ADHD sample; C, combined ADHD subtype; I, inattentive ADHD subtype. ADHD, attention-deficit/hyperactivity disorder; FDR, false discovery rate; SNP, single nucleotide polymorphism.

overrepresentation of the C-A-A-T haplotype in the ADHD group (adults: OR = 2.17 (1.42–3.33); children: OR = 1.90 (1.27–2.84) and adults + children: OR = 2.02 (1.42–2.88)) and a trend towards underrepresentation of the C-G-G-T allelic combination, which was only significant when all patients were considered (OR = 1.27 (1.01–1.59)) (Table 3b). Interestingly, the strong association between the C-A-A-T haplotype and ADHD was also detected in the combined ADHD subgroup (adults: OR = 2.25 (1.39–3.644); children: OR = 1.90 (1.23–2.93) and adults + children: OR = 2.04 (1.40–2.99) (Supplementary Table 5), but was not significant in the inattentive clinical subgroup after the multiple comparison correction (data not shown).

5HT2A. Consistent with the single-marker study, the analysis of the 19 *5HT2A* SNPs showed strong evidence of association between combined ADHD and a three-marker haplotype (rs7997012/rs6561333/rs7984966) (Figure 1) in the adult sample (global $P = 0.00013$), in the child sample (global $P = 0.0055$) and when all patients were considered together (global $P = 0.00062$) (Table 4a). One of the four allelic combinations that were observed, the G-C-C haplotype, was overrepresented in all combined ADHD groups when compared to controls (adults: OR = 1.63 (1.17–2.25); children: OR = 1.49 (1.12–1.97) and adults + children: OR = 1.55 (1.21–1.98)), whereas the G-C-T and A-T-T haplotypes were less frequent in adult (OR = 2.05 (1.42–2.96)) and child (OR = 1.56 (1.16–2.08)) combined ADHD patients, respectively (Table 4b). The underrepresentation of these allelic combinations was also observed when the adult and child groups were considered together (OR = 1.30 (1.02–1.66)). We also compared *5HT2A* haplotype frequencies between the combined and inattentive ADHD subgroups and observed nominal differences (global $P = 0.028$), due to an overrepresentation of the G-C-C risk haplotype in the combined ADHD sample

(OR = 1.61 (1.11–2.32)). However, these differences did not remain statistically significant after correcting by permutations.

MAOA and MAOB. As the rs3027415 variant associated with adulthood ADHD in the single-marker study is located between the *MAOA* and *MAOB* genes, we analyzed haplotypes for markers located in the region spanning both genes. We observed a strong association between ADHD and a three-marker haplotype of the *MAOB* gene (rs3027415/rs5905512/rs5906213; global $P = 0.0052$) (Figure 1 and Table 5a), due to overrepresentation of the C-G-C haplotype in the group of adult ADHD patients (OR = 1.90 (1.28–2.82)) (Table 5b).

Analysis of additive and epistatic effects

We evaluated the potential additive effects of the G-C-C (rs7997012/rs6561333/rs7984966), C-A-A-T (rs11238131/rs6592961/rs1982406/rs2044859) and C-G-C (rs3027415/rs5905512/rs5906213) risk haplotypes in the *5HT2A*, *DDC* and *MAOB* genes, respectively. Since the *MAOB* gene was associated only with adulthood ADHD, in the analysis of the adult ADHD data set, we considered haplotypes from these three serotoninergic genes, whereas for the childhood sample, we only analyzed the *DDC* and *5HT2A* risk variants. Assuming a simple additive model, we estimated that the combined effect of the three risk haplotypes contributes 5.2% of the adult ADHD phenotypic variance in our Spanish sample, whereas the *DDC* and *5HT2A* genetic variants account for 2.3% of child ADHD variability. We further evaluated possible interactions between the different risk haplotypes, but we found no evidence supporting the existence of epistatic effects between these serotoninergic genes in the risk to develop ADHD (data not shown).

Table 3 (a) Haplotype analysis of 11 *DDC* SNPs in a clinical sample of 188 adult ADHD patients, 263 child ADHD patients and 400 controls using the UNPHASED software; (b) haplotype distributions of the rs11238131, rs6592961, rs1982406 and rs2044859 *DDC* SNPs

(a)

Marker[b] *haplotype*	*Adults*			*Children*			*Adults + children*[a]		
	Global P-value	*Best haplotype-specific* P-value *(Adjusted* P-value*)*	*Haplotype-specific OR (CI)*	*Global* P-value	*Best haplotype-specific* P-value *(Adjusted* P-value*)*	*Haplotype-specific OR (CI)*	*Global* P-value	*Best haplotype-specific* P-value *(Adjusted* P-value*)*	*Haplotype-specific OR (CI)*
14	0.0075	0.0015 (0.0087)	2.12 (1.39–3.23)	0.00049	0.0006 (0.003)	1.69 (1.20–2.39)	0.00011	0.00012 (0.0004)	1.89 (1.33–2.70)
146	0.0058	0.0034 (0.019)	2.02 (1.29–3.18)	0.00039	0.00026 (0.0015)	1.86 (1.30–2.67)	3.95e-05	0.00010 (0.00050)	1.75 (1.27–2.42)
1467	**0.0042**	**0.00053 (0.0058)**	**2.17 (1.42–3.33)**	**0.018**	**0.0017 (0.009)**	**1.90 (1.27–2.84)**	**0.00092**	**2.62e−05 (0.0003)**	**2.02 (1.42–2.88)**

(b)

Marker[b] *haplotype*	*Adults*			*Children*			*Adults + children*[a]		
	Cases	*Controls*	*Haplotype-specific* P-value; *OR (CI)*	*Cases*	*Controls*	*Haplotype-specific* P-value; *OR (CI)*	*Cases*	*Controls*	*Haplotype-specific* P-value; *OR (CI)*
1 4 6 7									
CAAT	46 (17.3)	52 (8.8)	0.00053; 2.17 (1.42–3.33)	56 (15.5)	52 (8.8)	0.0017; 1.90 (1.27–2.84)	102 (16.3)	52 (8.8)	2.6 e−05; 2.02 (1.42–2.88)
CGAC	34 (12.7)	93 (15.7)	NS	55 (15.2)	93 (15.7)	NS	88 (14.0)	93 (15.7)	NS
CGGT	125 (47.0)	314 (53.0)	NS	172 (47.5)	314 (53.0)	NS	296 (47.3)	314 (53.0)	0.044; 1.27 (1.01–1.59)[c]
TGGC	61 (23.0)	133 (22.5)	NS	79 (21.8)	133 (22.5)	NS	140 (22.4)	133 (22.5)	NS

Abbreviations: ADHD, attention-deficit/hyperactivity disorder; CI, confidence interval; OR, odds ratio; NS, not significant; SNP, single nucleotide polymorphism.
[a]Two children with ADHD were excluded from the clinical sample when adults and children were analyzed together because they were relatives of some adult patients.
[b]1-rs11238131; 4-rs6592961; 6-rs1982406; 7-rs2044859. Best multiple-marker combination is indicated in bold.
[c]Down-represented in ADHD patients in comparison with controls.

Table 4 (a) Haplotype analysis of 19 *5HT2A* SNPs in a clinical sample of 126 adult and 192 child combined ADHD patients and 400 controls using the UNPHASED software; (b) Haplotype distributions of the rs7997012, rs6561333 and rs7984966 *5HT2A* SNPs

(a)

Marker[b] haplotype	*Adults*			*Children*			*Adults + children[a]*		
	Global P-value	*Best haplotype-specific P-value (adjusted P-value)*	*Haplotype-specific OR (CI)*	*Global P-value*	*Best haplotype-specific P-value (adjusted P-value)*	*Haplotype-specific OR (CI)*	*Global P-value*	*Best haplotype-specific P-value (adjusted P-value)*	*Haplotype-specific OR (CI)*
48	0.00012	3.925 e−05 (0.0027)	2.03 (1.42–2.91)	0.013	0.011 (0.029)	1.44 (1.10–1.89)	0.0026	0.0006 (0.0025)	1.50 (1.19–1.90)
248	**0.00013**	**2.39 e−05 (0.00060)**	**2.05 (1.42–2.96)**	**0.0055**	**0.0032 (0.013)**	**1.56 (1.16–2.08)**	**0.00062**	**0.00039 (0.0015)**	**1.55 (1.21–1.98)**

(b)

Marker[b] haplotype	*Adults*			*Children*			*Adults + Children[a]*		
	Cases	*Controls*	*Haplotype specific P-value; OR (CI)*	*Cases*	*Controls*	*Haplotype specific P-value; OR (CI)*	*Cases*	*Controls*	*Haplotype specific P-value; OR (CI)*
248									
ATT	75 (34.4)	243 (34.3)	NS	86 (25.1)	243 (34.3)	0.0032; 1.56 (1.16–2.08)[c]	160 (28.7)	243 (34.3)	0.038; 1.30 (1.02–1.65)[c]
GCC	77 (35.3)	178 (25.1)	0.0036; 1.63 (1.17–2.25)	114 (33.4)	178 (25.1)	0.0084; 1.49 (1.12–1.97)	191 (34.2)	178 (25.1)	0.00039; 1.55 (1.21–1.98)
GCT	44 (20.2)	242 (34.2)	2.39e-05; 2.05 (1.42–2.96)[c]	115 (33.6)	242 (34.2)	NS	159 (28.5)	242 (34.2)	0.030; 1.30 (1.02–1.66)[c]
GTT	22 (10.1)	45 (6.4)	NS	27 (7.9)	45 (6.4)	NS	48 (8.6)	45 (6.4)	NS

Abbreviations: ADHD, attention-deficit/hyperactivity disorder; CI, confidence interval; OR, odds ratio; NS, not significant; SNP, single nucleotide polymorphism.
[a]One child with ADHD was excluded from the clinical sample when adults and children were analyzed together because he was offspring of an adult ADHD patient.
[b]2-rs7997012; 4-rs6561333; 8-rs7984966. Best multiple-marker combination is indicated in bold.
[c]Down-represented in combined ADHD patients in comparison with controls.

Table 5 (a) Haplotype analysis of two SNPs within the *MAOA* gene and four SNPs within the *MAOB* gene in a clinical sample of 188 adult ADHD patients and 400 controls using the UNPHASED software; (b) Haplotype distributions of the rs3027415, rs5905512, rs5906213 *MAOB* SNPs

(a)

Marker[a] haplotype	Global P-value	Best haplotype-specific P-value (adjusted P-value)	Haplotype-specific OR (CI)
13	0.0013	0.0009 (0.0034)	1.91 (1.33–2.75)
134	**0.0052**	**0.0029 (0.012)**	**1.90 (1.28–2.82)**

(b)

Marker[a] haplotype	Cases	Controls	Haplotype specific P-value; OR (CI)
1 3 4			
CGC	57 (28.2)	74 (17.1)	0.0029; 1.90 (1.28–2.82)
TAT	101 (50.0)	237 (54.9)	NS
TGC	44 (21.8)	121 (28.0)	NS

Abbreviations: ADHD, attention-deficit/hyperactivity disorder; CI, confidence interval; OR, odds ratio; SNP, single nucleotide polymorphism.
[a]1-rs3027415; 3-rs5905512; 4-rs5906213. Best multiple-marker combination is indicated in bold.

Discussion

This is the first time, to our knowledge, that association between SNPs across genes coding for the main components of the serotoninergic system and both adulthood and childhood ADHD has been investigated. Specifically, we aimed to address the following issues: (1) identify genetic susceptibility factors involved in ADHD in the serotoninergic neurotransmission system; (2) test the existence of common genetic factors in childhood and adulthood ADHD; and (3) investigate if there are distinct genetic risk variants for the different ADHD subtypes.

DDC

A strong association between one SNP in the *DDC* gene and both childhood and adulthood ADHD was independently found, showing genetic evidence for common susceptibility factors being involved in both adulthood and childhood ADHD and suggesting the diagnostic continuity of ADHD throughout lifespan. Regarding childhood ADHD, previous studies reported preferential transmission of a 4 bp insertion in exon 1 to ADHD patients.[71,72] Moreover, Brookes *et al.* considered 41 SNPs within the *DDC* gene and found a positive association between rs1466163 located in intron 3 and combined ADHD. Although we did not analyze this sequence variant, our haplotype-based association analysis reflected an overrepresentation of a four-marker haplotype that contains an SNP (rs1982406) in modest LD with rs1466163 ($r^2 = 0.50$; $D' = 1.0$).[73] Interestingly, functional brain imaging studies showed evidence of altered DDC activity in children and adults with ADHD, also suggesting that *DDC* is a susceptibility

factor common to both adulthood and childhood ADHD.[74,75]

MAOB

The observation of positive association results between ADHD and the *MAOB* gene only in adults may reflect a higher genetic load in this clinical sample than in child patients. Childhood ADHD can be considered as a heterogeneous group that includes not only persistent patients that will become adults with ADHD, but also remitting patients. In this sense, *MAOB* may also confer susceptibility on the subset of our child ADHD sample in which the disorder will not remit, but this cannot be detected due to the impossibility of discerning between remitting and persistent ADHD patients in childhood. In this respect, Jiang *et al.* reported a positive association between childhood ADHD and the DXS7 locus, a microsatellite marker on chromosome X closely linked to *MAO* genes,[76–78] but no other studies support *MAOB* involvement in childhood ADHD.[73,78,79] A follow-up study of patients diagnosed during childhood would provide more insights into the role of *MAOB* in the persistence of ADHD through lifespan.

5HT2A

Another interesting finding arising from this study is the association of SNPs located in the *5HT2A* gene with only the combined ADHD clinical subgroup, both in adults and in children, suggesting the existence of some differential genetic components in different ADHD subtypes. This hypothesis is supported by other research groups that reported familial clustering of latent class and DSM-IV defined ADHD

82

subtypes.[80–83] In addition, different candidate genes, such as *5HT1B* or *SLC4A3*, have been associated with specific ADHD diagnostic groups.[84,85] In this respect, the combined subtype may represent a distinctive and more homogeneous phenotype that could facilitate the identification of genetic factors contributing to ADHD.

However, Brookes et al.[73] exhaustively investigated 32 markers within this candidate gene (including rs2770296, rs1328684, rs6561333 and rs7322347, which showed nominal significant *P*-values in our sample) in 776 combined ADHD cases and found no evidence of association, which disagreed with our findings. These discrepant results could be explained by differences in study design (TDT in Brookes et al. and case–control in our study) or between the populations under study (Brookes et al. recruited patients from eight different countries, whereas our study is based on patients from Spain). Alternatively, and given our limited sample size, we cannot exclude the possibility that the positive signals observed in our study arose by chance. Nevertheless, the fact that the association was consistent in both our child and adult data sets suggests that variants in this gene are susceptibility factors for ADHD, at least in the Spanish population. Other previous association studies that mainly focused on the analysis of rs6311, rs6313 and rs6314 variants reported inconsistent results (Supplementary Table 1). In this respect, we found no evidence of association between ADHD and rs9526246 that tags the rs6311 and rs6313 sequence variants.

Serotonin and dopamine system interactions
Interestingly, the three proteins encoded by the genes (*DDC*, *5HT2A* and *MAOB*) that we found associated with ADHD are also involved in dopamine neurotransmission, which is extensively implicated in ADHD etiology. DDC and MAOB are enzymes with an essential role in the synthesis and degradation, respectively, of both serotonin and dopamine.[86] 5HT2A receptors that localize on dopaminergic neurons inhibit dopamine firing, whereas 5HT2A antagonists induce dopamine release and reduce dopamine-induced hyperactivity in rodents.[87,88] The serotonergic modulation of dopaminergic function is also supported by the analysis of DAT-KO mice, in which hyperlocomotion is reversed by selective 5HT2A antagonists and the effects of psychostimulant treatments depend on the serotonin system.[34,88] Therefore, our results contribute to growing evidence, suggesting that the serotoninergic system may indirectly affect ADHD by modulating dopamine neurotransmission.[16]

Methodological considerations
The present case–control association study raises several methodological questions. First, our limited sample size (188 adults and 263 child patients) may have prevented us from detecting susceptibility loci with very subtle effects in the overall population of patients with ADHD. Our power decreased further when patients were subdivided according to DSM-IV clinical subtypes in order to reduce clinical heterogeneity. In this respect, our study had 31.7 and 33.1% power to detect a minimum OR of 1.5 for an SNP with an MAF of 0.153, in the adult and child inattentive ADHD samples, respectively, which could explain the negative results found between the *DDC* gene and the adult inattentive ADHD group. The hyperactive-impulsive subgroup of patients could not even be considered in this study, because it had insufficient power to detect meaningful effects.

Second, population stratification is a major concern in case–control association studies, because it can lead to false positive signals. In our study, several preventive measures were taken: (1) population stratification was discarded using 45 unlinked SNPs; (2) the patient population on which our study was based was clinically well defined and genetically homogeneous; and (3) both control and patient samples were recruited from the same restricted geographical area and matched for sex. Moreover, the combined ADHD-specific association observed for SNPs in the *5HT2A* gene argues against spurious results due to stratification within the control group. However, to further control for population stratification, we are currently recruiting case–parent trios that will allow the use of family-based methods.

Third, the high-throughput SNP analysis reported requires correction for multiple comparisons to reduce type I errors. We applied an FDR of 15%, which corresponds to a significant level of $P < 0.00191$. Under the more conservative Bonferroni correction, taking into account 100 SNPs, three diagnostic groups and both adult and children samples, the significant threshold would be set at $P < 8e-05$. Only rs6592961 within the *DDC* gene meets this strict criterion for childhood ADHD. Both corrections, however, may be too stringent to identify subtle genetic factors involved in the etiology of a complex disease such as ADHD.

Fourth, to limit the number of tests, we chose a systematic approach for the haplotype analysis: (1) we selected only the genes that showed significant evidence for association in the single-marker analysis after FDR corrections; (2) we identified the best SNP combinations in a stepwise manner taking only the adult sample; and (3) these combinations were subsequently tested in the child and adult + child data sets. Because of this strategy, SNPs in nine genes that showed nominal association with the ADHD phenotype and haplotypes that did not include the best two-, three- and four-marker combinations fixed in the adult sample were not further analyzed. Therefore, we cannot rule out having missed other haplotypes that might contribute to ADHD-specific traits or modulate the phenotype through interactions with other candidate genes.

Fifth, although we achieved adequate SNP coverage for many genes ($r^2 = 0.85$), gaps still exist in 9 genes, because 16 tagSNPs could not be tested due to experimental constraints. In this respect, it would

be particularly relevant to genotype by other methods those SNPs located in multiloci bins, such as rs12150214 (*SLC6A4*), rs11080121 (*SLC6A4*), rs7809758 (*DDC*) and rs2876827 (*DDC*). In addition, as uncommon SNPs (MAF < 0.15) were not considered, we cannot rule out the possibility of other rare genetic effects in these genes.

Finally, as the putative functional consequences of the SNPs were not taken into account in our SNP selection approach, most of the variants that we found associated with ADHD were located within introns, with the exception of rs5906213 and rs3027415 that localize upstream and downstream of the *MAOB* gene, respectively. This suggests that the identified risk haplotypes may not confer by themselves functional alterations, but that they are in LD with other unknown susceptibility variants (SNPs or other types of polymorphisms) directly involved in genetic vulnerability to ADHD.

In conclusion, our results provide evidence for *DDC* and *5HT2A* contribution to adult and child ADHD, and suggest that their effect is highly significant in the combined ADHD clinical subtype. We also identified a strong association between the *MAOB* gene and adult ADHD, suggesting its participation in the persistence of the disorder through lifespan. These three genes need further evaluation to improve the understanding of their relative importance in the different ADHD subtypes and age groups. In general, further research is required to establish replication by other groups in different populations and to identify the functional variants involved.

Acknowledgments

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)