



ORIGINAL INVESTIGATION

Candidate system analysis in ADHD: Evaluation of nine genes involved in dopaminergic neurotransmission identifies association with *DRD1*

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Abstract

Objectives. Several pharmacological and genetic studies support the involvement of the dopamine neurotransmitter system in the aetiology of attention-deficit hyperactivity disorder (ADHD). Based on this information we evaluated the contribution to ADHD of nine genes involved in dopaminergic neurotransmission (*DRD1*, *DRD2*, *DRD3*, *DRD4*, *DRD5*, *DAT1*, *TH*, *DBH* and *COMT*). **Methods.** We genotyped a total of 61 tagging single nucleotide polymorphisms (SNPs) in a sample of 533 ADHD patients (322 children and 211 adults), 533 sex-matched unrelated controls and additional 196 nuclear ADHD families from Spain. **Results.** The single- and multiple-marker analysis in both population and family-based approaches provided preliminary evidence for the contribution of *DRD1* to combined-type ADHD in children ($P = 8.8 \times 10^{-4}$; OR = 1.50 (1.18–1.90) and $P = 0.0061$; OR = 1.73 (1.23–2.45)) but not in adults. Subsequently, we tested positive results for replication in an independent sample of 353 German families with combined-type ADHD children and replicated the initial association between *DRD1* and childhood ADHD ($P = 8.4 \times 10^{-5}$; OR = 3.67 (2.04–6.63)). **Conclusions:** The replication of the association between *DRD1* and ADHD in two European cohorts highlights the validity of our finding and supports the involvement of *DRD1* in childhood ADHD.

Key words: Genetics, biological psychiatry, childhood ADHD, *DRD1*, association study

Introduction

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterized by

persistent and pervasive symptoms of hyperactivity, inattention and increased impulsivity that affects 5–6% of children (Polanczyk et al. 2007). Despite

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being one of the most prevalent childhood psychiatric disorders with persistence into adulthood in approximately 30–50% of patients, its aetiology is poorly understood (Faraone et al. 2000; Kessler et al. 2005; Kooij et al. 2005; Faraone et al. 2006; Polanczyk et al. 2007).

Several lines of evidence imply aberrant dopaminergic neurotransmission as one underlying pathological mechanism of ADHD. Psychostimulant drugs that show successful therapeutic effects in ADHD, such as amphetamines or methylphenidate, block dopamine reuptake from the synaptic cleft through the blockage of the dopamine transporter (*SLC6A3/DAT1*; Roman et al. 2002; Volkow and Swanson 2003). In addition, alterations in striatal *DAT1* have been identified in ADHD patients, and brain regions that are rich in dopamine activity, such as striatum, mid-brain and frontal cortex, are involved in the disorder (Castellanos et al. 1996; Ernst et al. 1998; Faraone and Biederman 1998; Vaidya et al. 1998; Dougherty et al. 1999; Ernst et al. 1999; Rubia et al. 1999; Dresel et al. 2000; Krause et al. 2000; Moll et al. 2000; Kim et al. 2002; Cheon et al. 2003; Jucaite et al. 2005; Spencer et al. 2005).

Animal studies also provided evidence for the implication of dopaminergic mechanisms in ADHD, pointing to genes encoding the dopamine receptors (*Drd1*, *Drd2*, *Drd3*, *Drd4* and *Drd5*), the dopamine transporter (*Dat1*) and dopamine β -hydroxylase (*Dbh*). Spontaneously hyperactive rats (SHR) show altered *Dat1* expression, and the selective blockage of the D2 receptor in the hyperactive mutant mouse *Coloboma* eliminates hyperactivity and blocks the amphetamine-induced reduction in locomotor activity (Watanabe et al. 1997; Leo et al. 2003; Fan et al. 2010). Interestingly, *Dat1* $-/-$ and *Drd3* $-/-$ knock-out mice show spontaneous hyperactivity (Accili et al. 1996; Giros et al. 1996), while reduced locomotor activity was observed in *Drd2* $-/-$ and *Drd4* $-/-$ mutant mice (Baik et al. 1995; Rubinstein et al. 1997). In addition, *Dbh* $-/-$ and *Drd4* $-/-$ mice display hypersensitivity to amphetamine (Weinshenker et al. 2002) or ethanol, cocaine and methamphetamine-induced hyperactivity, respectively (Rubinstein et al. 1997). Additionally, *Drd1* $-/-$ mutant mice show reduced locomotor stimulant effects of cocaine and *Drd5* $-/-$ knock-out mice exhibit lower levels of immobility and reduced response to the hyperactivity-inducing effects of dopaminergic agonists (Xu et al. 1994a; Holmes et al. 2001).

Variants in dopaminergic genes have also been identified as risk factors for ADHD through case-control and/or family-based association studies. Evidence for association has been reported by meta-analyses for variants in *DRD4*, *DRD5*, *DAT1* and

DBH (Gizer et al. 2009; reviewed in Thapar et al. 2005, 2007).

Our group has recently focused on the association of adult and childhood ADHD with genetic variants in several candidate gene systems, covering entire functional networks such as the serotonergic system (Ribasés et al. 2009b), neurotrophins and their receptors (Ribasés et al. 2008) and genes potentially involved in brain laterality (Ribasés et al. 2009a). Along this line, and based on previously reported pharmacological, neuroimaging and genetic information, we have investigated 61 common sequence variants within nine dopaminergic genes in childhood and adulthood ADHD. These genes encode the dopamine receptors (*DRD1*, *DRD2*, *DRD3*, *DRD4* and *DRD5*), the dopamine transporter (*DAT1*), the rate-limiting enzyme in dopamine synthesis (tyrosine hydroxylase, *TH*) and enzymes involved in dopamine degradation (dopamine β -hydroxylase, *DBH*, and catechol-*O*-methyl transferase, *COMT*) (Supplementary Table I; Supplementary Tables I–IV available online at <http://www.informahealthcare.com/wbp/doi/10.3109/15622975.2011.584905>). To address this issue we performed case-control and family-based association studies in 533 ADHD patients (322 children and 211 adults), 533 sex-matched unrelated controls and 196 nuclear ADHD families from Spain. The results were then tested for replication in an independent sample of 353 nuclear ADHD families with combined-type ADHD from Germany.

Methods

Subjects and clinical assessment

The clinical description of the sample of 2426 Caucasian subjects included in the present study is shown in Supplementary Table II. Diagnosis was blind to genotype. The study was approved by the ethics committee of each institution and informed consent was obtained from all subjects. A more detailed description of the different diagnostic instruments used was published previously (Ribasés et al. 2008, 2009ab).

Discovery cohort: Childhood ADHD sample. Three hundred and twenty-two children with ADHD (73.6% combined, 21.7% inattentive and 4.7% hyperactive-impulsive) and 322 sex-matched unrelated controls were recruited from two hospitals, Hospital Vall d'Hebron and Hospital Mútua de Terrassa, located in the Barcelona area (Spain). Seventy-nine percent of patients and controls were male. The average age at assessment was 9.3 years (SD = 2.6) for patients and 36.8 years (SD = 17.0)

for controls. Patients were evaluated with the Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version (KSADS-PL) reported by parents. ADHD symptoms were assessed using the Conners' Parent Rating Scale and the Conners' Teacher Rating Scale. For the subsequent family-based study, one or both unscreened parents of a subset of 196 affected children suffering from combined-type ADHD, were available (both parents: $n = 137$ and one parent: $n = 59$). Eighty-three percent of these ADHD cases were males. The average age at assessment was 9.1 years ($SD = 2.8$) for probands and 43 years ($SD = 7.9$) for parents.

Discovery cohort: Adulthood ADHD sample. The adulthood population consisted of 211 ADHD subjects (140 combined, 61 inattentive and 10 hyperactive-impulsive) and 211 sex-matched unrelated controls from Hospital Vall d'Hebron, Barcelona (Spain). Seventy-three percent of subjects were male. The average age at diagnosis was 29.8 years ($SD = 12.1$) for patients and 44.2 years ($SD = 14.7$) for controls. The ADHD diagnosis was based on the Structured Clinical Interview for DSM-IV Axis I and Axis II Disorders (SCID-I and SCID-II) and the Conners' Adult ADHD Diagnostic Interview for DSM-IV (CAADID). The level of impairment was measured by the Clinical Global Impression (CGI) included in the CAADID Part II and the Sheehan Disability Inventory.

Exclusion criteria for the adult and childhood Spanish patients cohorts were $IQ < 70$; pervasive developmental disorders; schizophrenia or other psychotic disorders; the presence of mood, anxiety or personality disorders that might explain ADHD symptoms; adoption; sexual or physical abuse; birth weight < 1.5 kg; and other neurological or systemic disorders that might explain ADHD symptoms. All controls consisted of Caucasian blood donors in which DSM-IV life-time ADHD symptomatology was excluded under the following criteria: (1) not having previously been diagnosed with ADHD and (2) answering negatively to the life-time presence of the following DSM-IV ADHD symptoms: (a) often has trouble keeping attention on tasks, (b) often loses things needed for tasks, (c) often fidgets with hands or feet or squirms in seat and (d) often gets up from seat when remaining in seat is expected. Due to ethics concerns, all subjects included as controls were adults.

Replication cohort. A total sample of 353 nuclear ADHD families from Germany (225 from the Department of Child and Adolescent Psychiatry,

Psychosomatics and Psychotherapy, University Hospital, Würzburg, and 128 from the Departments of Child and Adolescent Psychiatry, Saarland University Hospital, Homburg, and Neurobehavioral Genetics, Institute of Psychobiology, Trier) with at least one child suffering from combined-type ADHD was assessed for replication. DNA was available from both parents of 304 probands, one parent of 49 probands and 17 siblings. Six siblings had combined, three inattentive and three hyperactive-impulsive ADHD. The other five siblings were healthy. Parents were not screened for ADHD or any other mental disorder. Eighty-two percent of probands were males. The average age at assessment was 10.74 years ($SD = 2.47$) for probands. The index child and siblings were included when at least 6 years old. All children were either assessed by the Kiddie-SADS-PL-German Version or the Kinder-DIPS, and parent and teacher ADHD DSM-IV based rating scales were obtained to ensure pervasiveness of symptoms. Exclusion criteria were $IQ < 80$, co-morbid autistic disorders or somatic disorders (hyperthyroidism, epilepsy, neurological diseases, severe head trauma, etc.), primary affective disorders, Tourette syndrome, psychotic disorders or other severe primary psychiatric disorders, and birth weight below 2 kg. Full phenotypic assessment methods of the sample were published previously (Palmason et al. 2010).

DNA isolation

Genomic DNA was isolated either from saliva using the Oragene DNA Self-Collection kit (DNA Genotek, Kanata, Ontario, Canada) or from blood by the salting-out procedure or with magnetic bead technology with the Chemagic Magnetic Separation Module I and Chemagic DNA kit (Chemagen, Baesweiler, Germany). DNA concentrations were determined using the PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, Oregon).

SNP selection and genotyping

For the SNP selection, we used information from the Centre d'Étude du Polymorphisme Humain (CEPH) panel and from the HapMap database (www.hapmap.org; release 20) and considered 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 (*DRD1*, *DRD2*, *DRD3*, *DRD4*, *DRD5*, *TH* and *COMT*) and MAF > 0.25 for those genes with more than 15 tagSNPs (*DBH* and *DAT1*). A total of 68

tagSNPs (27 in multi-loci bins and 41 singletons) were chosen under these criteria with the LD-select software (Carlson et al. 2004). Additionally, rs2227850 located in exon 1 of *DRD5* was included in the analysis. The 69 selected SNPs were assessed with the automated assay design pipeline at ms.appliedbiosystems.com/snpflex/snpflexStart.jsp and a proper design could not be achieved for five SNPs, which translates into a design rate of 92.7%. All SNPs were genotyped using the SNPlex platform (Applied Biosystems, Foster City, CA, USA) as described (Tobler et al. 2005). Two HapMap samples were included in all genotyping assays and a concordance rate of 100% with HapMap data was obtained.

Statistical analyses

A two-stage association study was performed: (i) We first tested association between ADHD and 61 variants in nine dopaminergic genes by a population-based case-control association study in two Spanish samples of adult and childhood ADHD patients and control subjects. Parents from 61% of children with ADHD were also available and those genes showing positive signals in the population-based case-control study after applying the restrictive Bonferroni correction were analyzed using a family-based association approach (ii) Genes with SNPs showing significant association values were tested for replication in an independent sample of nuclear ADHD families from Germany. The analysis of minimal statistical power was performed post hoc using the Genetic Power Calculator software (Purcell et al. 2003), assuming an odds ratio (OR) of 1.3, prevalence of 0.05, significance level of 0.05, and the lowest MAF of 0.15. The presence of population substructures had previously been discarded in the Spanish case-control sample by means of genetic stratification testing using a panel of 45 unlinked non-genic SNPs (Ribasés et al. 2008, 2009a,b).

Case-control association study: single-marker analysis. The analysis of Hardy-Weinberg equilibrium (HWE) ($P > 0.01$) and the comparison of genotype and allele frequencies between cases and controls were performed using a Chi-square test with the SNPassoc R package (Gonzalez et al. 2007). Dominant and recessive models were considered for SNPs displaying nominal association when either genotypes under a codominant model or alleles were taken into account. Bonferroni correction for 244 tests in the initial association study, considering 61 SNPs, two age groups, and the comparison of genotype and allele frequencies, corresponds to a significance threshold of $P \leq 2.0 \times 10^{-4}$.

Multiple-marker analysis. The haplotype-based association study was restricted to genes including genetic variants associated with ADHD in the single-marker analysis. All the genotyped variants within these genes were considered. The best two-marker haplotype from all possible pairwise combinations was identified. Likewise, additional markers (up to four) were added in a stepwise manner to the initial two-SNP haplotype. Significance was estimated using 10,000 permutations with the UNPHASED software (Dudbridge 2003). Since the expectation-maximization algorithm does not accurately estimate low haplotype frequencies (Fallin and Schork 2000), haplotypes with frequencies < 0.05 were excluded. We also tested the allelic combinations that showed positive association in the overall ADHD sample in the two diagnostic subgroups of combined and inattentive ADHD separately. The hyperactive/impulsive group was not considered due to its small sample size.

Family-based association study. HWE ($P > 0.01$) was confirmed for parental genotypes derived from the alleles not transmitted to the affected offspring. For the single and multiple-marker analyses, alleles or haplotypes transmitted and not transmitted from parents to the affected offspring were compared by the HRR strategy using the UNPHASED software (Dudbridge 2003). For the multiple-marker approach, we applied the same strategy described in the case-control study (see above). The best allelic combination was further considered in the Transmission Disequilibrium Test (TDT) using the PLINK software (Purcell et al. 2007).

Results

Case-control association study

TagSNPs in nine candidate genes of the dopaminergic system (*DRD1*, *DRD2*, *DRD3*, *DRD4*, *DRD5*, *DAT1*, *TH*, *DBH* and *COMT*) were analyzed in a Spanish sample of 322 children with ADHD and 322 sex-matched controls and 211 adults with ADHD and 211 sex-matched controls. Of the 64 SNPs selected for inclusion in the SNPlex assay, one was monomorphic and two had genotype calls $< 90\%$ and were excluded from the analysis (Supplementary Table I). The minimal statistical power calculated for the childhood sample was 36.4, 28.3 and 6.7% for the dominant, codominant and recessive model of inheritance, respectively, and for the adult population case-control samples it was 25.7, 19.7 and 6.1% considering a dominant, codominant and recessive model of inheritance, respectively.

Table I. Association study in 322 childhood ADHD patients (237 combined ADHD, 70 inattentive ADHD and 15 hyperactive-impulsive ADHD patients) and 322 sex-matched unrelated controls and 211 adult ADHD patients (140 combined ADHD, 61 inattentive ADHD and 10 hyperactive-impulsive ADHD patients) and 211 sex-matched unrelated controls.

Genotypes													Alleles												
Gene	SNP	Cases N (%)					Controls N (%)					Genotype 11 vs. 12+22			Genotype 11+12 vs. 22			Cases N (%)			Controls N (%)			Allele 2 vs. allele 1	
		11	12	22	Sum		11	12	22	Sum	P	OR (95% CI)	P	OR (95% CI)	P	1	2	OR (95% CI)	1	2	OR (95% CI)	P			
		Children																							
DRD1	rs835616	142 (44.4)	141 (44.1)	37 (11.6)	320	177 (55.0)	117 (36.3)	28 (8.7)	322	0.026	1.53 (1.12–2.09)	0.0072	–	0.23	425 (66.4)	215 (33.6)	173 (26.9)	1.37 (1.09–1.75)&	0.0086						
	rs835541	123 (38.6)	139 (43.6)	57 (17.9)	319	90 (28.0)	155 (848.1)	77 (23.9)	322	0.011	1.61 (1.16–2.27)&	0.0043	–	0.060	385 (60.3)	253 (39.7)	309 (48.0)	1.40 (1.12–1.75)&	0.0026						
	rs863126	149 (46.3)	129 (40.1)	44 (13.7)	322	119 (37.0)	169 (52.5)	34 (10.6)	322	0.0066	1.47 (1.07–2.0)&	0.016	–	0.23	427 (66.3)	217 (33.7)	237 (36.8)	–	0.24						
	rs265977	244 (76.0)	75 (23.4)	2 (0.60)	321	209 (64.9)	98 (30.4)	15 (4.7)	322	2.0e-04*	1.72 (1.22–2.44)&	0.002	7.69 (1.75–33.3)&	7e-04	563 (87.7)	79 (12.3)	128 (19.9)	1.77 (1.30–2.40)	2.1e-04						
TH	rs2070762	107 (33.5)	153 (48.0)	59 (18.5)	319	125 (38.8)	158 (49.1)	39 (12.1)	322	0.062	–	0.16	1.65 (1.06–2.55)	0.024	367 (57.5)	271 (42.5)	236 (36.6)	1.28 (1.02–1.59)&	0.033						
		Adults																							
TH	rs2070762	71 (34.1)	110 (52.9)	27 (13.0)	208	89 (42.2)	86 (40.8)	36 (17.1)	211	0.044	0.090	0.24	252 (60.6)	164 (39.4)	158 (62.6)	–	0.55								

*Statistically significant *P* values after applying Bonferroni correction ($P \leq 2.0e-04$); &When odds ratio < 1 , the inverted score is shown.

In the single-marker analysis the comparison of genotype frequencies under a codominant model and allele frequencies showed nominal association between rs2070762 in the *TH* gene and both childhood and adulthood ADHD. In addition, four SNPs in *DRD1* displayed nominal associations with ADHD in the childhood dataset (rs835616, rs835541, rs863126 and rs265977; Table I and Supplementary Table III; Figure 1). After applying the Bonferroni correction, only rs265977 in *DRD1* remained associated with ADHD in children ($P_{\text{codominant}} = 2.0 \times 10^{-4}$; $P_{\text{allele}} = 2.1 \times 10^{-4}$, OR = 1.77 (1.30–2.40)). To minimize the probability of type I errors, we further compared the 322 childhood cases with the 211 controls previously used in the adulthood comparison and confirmed the association between *DRD1* and ADHD in children (Supplementary Table IV).

We then considered *DRD1* for the haplotype-based analysis only in the children dataset. The study of the eight *DRD1* SNPs revealed a two-marker

haplotype (rs863126–rs265977) associated with childhood ADHD (Global P value = 4.36×10^{-5} ; Table II) with an overrepresentation of the A–C allelic combination ($P_{\text{A–C}} = 1.7 \times 10^{-4}$; OR = 1.54 (1.23–1.92)) and a reduced frequency of the A–T haplotype ($P_{\text{A–T}} = 1.4 \times 10^{-4}$; 1.77 (1.30–2.40); Figure 1, Table III) in the patients group. These differences were specific to the combined-type ADHD subgroup (Global P -value = 4.4×10^{-4} ; $P_{\text{A–C}} = 8.8 \times 10^{-4}$, OR = 1.50 (1.18–1.90); $P_{\text{A–T}} = 0.0011$, OR = 1.74 (1.24–2.43); Tables II and III).

Family-based association study

The eight *DRD1* SNPs were further tested in two childhood combined-type ADHD family-based samples from Spain ($n = 196$) and Germany ($n = 353$). All patients from the Spanish trios were part of the previously studied case–control sample. The minimum statistical power calculated for the Spanish sample was 20.5 and 32.9%, respectively.

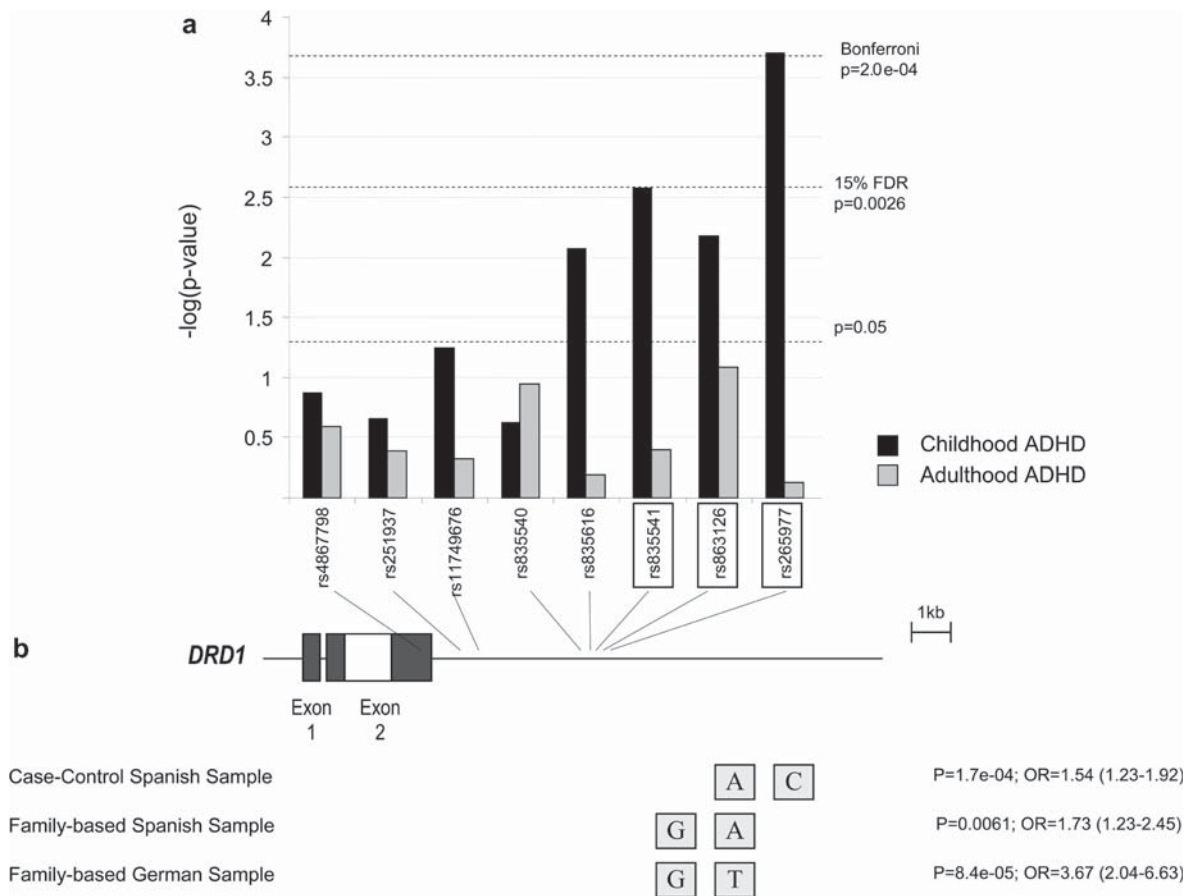


Figure 1. (a) Lowest level of significance, as $-\log(P$ value) found in either the codominant genotypes or the alleles comparisons, of individual SNPs within the *DRD1* gene when 322 child ADHD patients (in black) and 211 adult ADHD patients (in gray) were compared to controls. SNPs in ADHD risk haplotypes associated with ADHD are boxed. (b) Diagram of the *DRD1* gene with the coding region in white and 5' and 3' untranslated regions in gray. Allelic combinations associated with combined ADHD in children in the Spanish and German samples through case-control or family-based association studies are shown. FDR, false discovery rate.

Table II. Haplotype analysis of eight *DRD1* SNPs in a clinical sample of 322 child ADHD patients and 322 controls using the UNPHASED software.

Marker ^a haplotype	All ADHD (<i>n</i> = 322)			Combined ADHD (<i>n</i> = 237)			Inattentive ADHD (<i>n</i> = 70)		
	Global <i>P</i> value	Best haplotype <i>P</i> value (adjusted <i>P</i> value)	Risk haplotype-OR	Global <i>P</i> value	Best haplotype <i>P</i> value (adjusted <i>P</i> value)	Risk haplotype-OR	Global <i>P</i> value	Best haplotype <i>P</i> value (adjusted <i>P</i> value)	Risk haplotype-OR
7 8	4.36e-05	1.4e-04 (8.9e-04)	1.54 (1.23-1.92)	4.4e-04	8.8e-04 (0.0044)	1.50 (1.18-1.90)	0.044	0.027 (0.065)	-
4 7 8	9.19e-05	1.7e-04 (0.0012)	1.54 (1.24-1.93)	4.9e-04	9.0e-04 (0.0055)	1.50 (1.18-1.91)	0.096	-	-
1 4 7 8	1.3e-04	0.0019 (0.0142)	1.37 (1.08-1.74)	6.4e-04	0.011 (0.065)	-	0.11	-	-

^a1-rs4867798; 4-rs835540; 7-rs863126; 8-rs265977. Numbering of markers correlates with their position on the gene in the 5' to 3' direction (see Figure 1 for the detailed gene location of the SNPs). In bold the best allelic combination (highest OR).

Two SNPs, rs835616 and rs835540, had genotype call rates < 60% in both populations and were discarded from the family-based study.

Spanish sample. The haplotype relative risk (HRR) analysis showed no significant differences when individual *DRD1* markers were considered. However, the multiple-marker analysis showed evidence for association between combined ADHD and the two-marker haplotype rs835541-rs863126 (Global *P* value = 0.0029; Table IV). We observed an over-transmission of the G-A allelic combination ($P_{G-A} = 0.0061$; OR = 1.73 (1.23-2.45)) and marginally significant evidence for nontransmission of the G-T haplotype to the affected offspring ($P_{G-T} = 0.021$; OR = 2.27 (1.17-4.39); Table V, Figure 1). Although only 24% of parents were informative, the TDT analysis confirmed the nominal association of *DRD1* with combined ADHD in children from Spain ($P_{G-A} = 0.042$; OR = 2.31 (1.20-4.43); data not shown).

German sample. No significant differences in transmission were observed in the single-marker analysis of *DRD1*. However, the multiallelic version of the HRR test confirmed the strong association between combined ADHD and the rs835541-rs863126 haplotype identified in the Spanish sample (Global *P* value = 1.2e-04; Table IV). Interestingly, the analysis of individual haplotypes showed an excess of transmission of the G-T allelic combination to the ADHD probands ($P_{G-T} = 8.4e-05$; OR = 3.67 (2.04-6.63)) and a reduced transmission of the A-T haplotype ($P_{A-T} = 0.031$; OR = 1.46 (1.11-1.93); Table V, Figure 1). Consistently with the HRR results, the TDT analysis considering the 306 informative parents, also highlighted the *DRD1* association with combined-type ADHD in the German dataset ($P_{G-T} = 0.0024$; OR = 2.82 (1.46-5.45); data not shown).

Discussion

We followed a hypothesis-driven approach based on presumed ADHD neurobiology and investigated the main components of the dopaminergic system for their involvement in the genetic susceptibility to ADHD through a two-step population and family-based association study design. The analysis of nine dopaminergic candidate genes showed strong evidence for the contribution of *DRD1* to childhood combined ADHD in two independent datasets from Spain and Germany. Our study raises several methodological considerations that we discuss below:

Table III. Haplotype distributions of the rs863126 and rs265977 *DRD1* SNPs.

Marker* haplotype	All ADHD (<i>n</i> = 322)			Combined ADHD (<i>n</i> = 237)		
	Cases	Controls	Haplotype-specific <i>P</i> value; OR (CI)	Cases	Controls	Haplotype-specific <i>P</i> value; OR (CI)
7 8						
A T	79 (12.3)	128 (19.9)	1.4e-04; 1.77 (1.30–2.40)&	59 (12.5)	128 (19.9)	0.0011; 1.74 (1.24–2.43)&
A C	347 (54.1)	279 (43.3)	1.7e-04; 1.54 (1.23–1.92)	252 (53.4)	279 (43.3)	8.8e-04 ; 1.50 (1.18–1.90)
T C	216 (33.6)	237 (36.8)	–	161 (34.1)	237 (36.8)	–

*7-rs863126; 8-rs265977. Numbering of markers correlates with their position on the gene in the 5' to 3' direction (see Figure 1 for the detailed gene location of the SNPs).

&Underepresented in ADHD cases.

- (i) Standardized assessment of ADHD by structured interviews and rating scales were considered across the different population samples.
- (ii) Rather than focus on single SNPs in dopaminergic genes previously associated with ADHD, the study design ensured a full coverage of the genes in terms of linkage disequilibrium (LD).
- (iii) Since population-based association studies are particularly susceptible to stratification, cases and controls were previously tested for confounding population substructures by genotyping a set of 45 non-linked SNPs located in different chromosomes outside of any known gene (Ribasés et al. 2008, 2009a,b). Also, a follow-up family-based approach using case–parent triads was performed to confirm the initial association finding. The family-based study included a subset of the child patients used in the original case–control analysis and an independent cohort from Germany.
- (iv) To minimize the probability of type I errors, we applied a robust and stringent approach for dealing with multiple comparisons across all statistical tests performed and focused only on the single association that remained

statistically significant after the Bonferroni correction.

- (v) The identification of the best allelic combination conferring susceptibility to ADHD through family-based association studies in two independent datasets from Spain and Germany converges in the same two-marker haplotype, rs835541–rs863126, of the *DRD1* gene. This risk haplotype was not identical but overlapped with the associated two-marker haplotype identified by the population-based case–control approach in the Spanish cohort.
- (vi) The relationship between *DRD1* and combined ADHD in childhood is less straightforward than expected since we detected a “flip-flop” phenomenon, with opposite allelic risk variants of the same haplotype associated with ADHD in the two populations under study (Spanish cohort: rs835541G–rs863126A; German cohort: rs835541G–rs863126T). Rather than statistical artifacts, they may be attributable to the presence of non-causal SNPs in LD with the genetic variant directly involved in the vulnerability to ADHD. The fact that SNPs displaying association in both the single and multiple-marker analyses are located in the 3' region of the gene is in agreement with

Table IV. Haplotype analysis of six *DRD1* SNPs in a clinical sample of 196 childhood combined ADHD trios from Spain and 353 childhood combined ADHD trios from Germany using the UNPHASED software.

Marker* haplotype	Spain (<i>n</i> = 196)			Marker haplotype	Germany (<i>n</i> = 353)		
	Global <i>P</i> value	Best haplotype <i>P</i> value (Adjusted <i>P</i> value)	Risk Haplotype-OR		Global <i>P</i> value	Best haplotype <i>P</i> value (Adjusted <i>P</i> value)	Risk Haplotype-OR
6	0.11	0.11	–	6	0.056	0.057	–
6 7	0.0029	0.0061 (0.024)	1.73 (1.23–2.45)	6 7	1.2e-04	8.4e-05 (2.2e-04)	3.67 (2.04–6.63)
3 6 7	0.029	0.016 (0.076)	–	6 7 8	0.0044	0.0011 (0.0031)	2.47 (1.40–4.37)

Markers rs835616 and rs835540 showed genotype call rates < 60% and were excluded from the family-based study.

In bold the best allelic combination (highest OR).

*3-rs11749676; 6-rs835541; 7-rs863126; 8-rs265977. Numbering of markers correlates with their position on the gene in the 5' to 3' direction (see Figure 1 for the detailed gene location of the SNPs).

Table V. Haplotype distributions of the rs835541 and rs863126 *DRD1* SNPs considering a Haplotype Relative Risk analysis.

Marker* haplotype	Spain (<i>n</i> = 196)			Germany (<i>n</i> = 353)		
	Cases	Controls	Haplotype-specific <i>P</i> value; OR (CI)	Cases	Controls	Haplotype-specific <i>P</i> value; OR (CI)
6 7						
G A	138 (52.4)	103 (38.8)	0.0061; 1.73 (1.23–2.45)	223 (45.0)	229 (45.0)	–
A T	73 (27.8)	72.3 (27.2)	–	121 (24.4)	159 (32.0)	0.031; 1.46 (1.11–1.93)&
G T	14 (5.2)	30 (22.2)	0.021; 2.27 (1.17–4.39)&	51 (10.3)	15 (3.0)	8.4e-05; 3.67 (2.04–6.63)
A A	39 (14.6)	61 (22.8)	–	101 (20.3)	93 (18.8)	–

*6-rs835541; 7-rs863126. Numbering of markers correlates with their position on the gene in the 5' to 3' direction (see Figure 1 for the detailed gene location of the SNPs).

&Untransmitted to the affected ADHD offspring.

this hypothesis. Although the reason for such “flip-flop” results are unknown, they may be due to differences in the genetic background of the two studied populations, either in terms of differential LD architectures and/or the presence of specific ADHD risk loci and environmental factors interacting with *DRD1* (Lin et al. 2007).

- (vii) Despite the relatively limited sample size resulting in low power to detect genetic risk factors of small effect and the fact that parents included in the family-based approach were unscreened for ADHD, which may contribute to generate type II errors (false negatives), we identified *DRD1* as a risk factor for combined ADHD in two independent cohorts. However, since reduced sample sizes can provide imprecise estimates of the magnitude of the observed effects, further studies in larger samples would improve our knowledge about the role of *DRD1* in the disorder.

Previous studies support the connection of *DRD1* with ADHD. Dopamine D1 receptor mutant mice exhibit locomotor hyperactivity and two animal models of ADHD, the spontaneously hypertensive rat (SHR) and the Naples high excitability (NHE) rat, show alterations in the expression and/or function of *DRD1* (Sagvolden et al. 1992; Xu et al. 1994a,b; Clifford et al. 1998; Russell 2002; Viggiano et al. 2002). In addition, *DRD1* antagonists reverse the methylphenidate effects on prefrontal cortex cognitive function in rats (Arnsten and Dudley 2005; Arnsten 2006) and several association studies and a genome-wide association scan (GWAS) in juvenile but not in adult ADHD (Lesch et al. 2008) also emphasized the potential impact of *DRD1* in ADHD as well as in inattentive and impulsive symptoms (Taylor et al. 1997; Misener et al. 2004; Bobb et al. 2005; Brookes et al. 2006; Luca et al. 2007; Shaw et al. 2007; Lasky-Su et al. 2008). Mutational screen-

ing of the gene, however, did not identify any potential functional variant directly involved in the disorder and suggests that the causal sequence variant may reside outside the *DRD1* coding region (Feng et al. 1998; Thompson et al. 1998; Misener et al. 2004). Finally, because *DRD1* was associated with childhood but not with adulthood ADHD, we reasoned that it may be implicated in those specific symptoms, such as hyperactivity or impulsivity, that decline with increasing age (Hart et al. 1995; Biederman et al. 2000; Rietveld et al. 2004). This putative *DRD1* influence on hyperactivity-impulsivity may explain the specific association detected only in the combined but not in the inattentive clinical subtype. These results suggest differential genetic influences contributing to stability versus remission of ADHD symptoms across the lifespan and support previous studies pointing to different genetic factors emerging at distinct developmental periods (Kuntsi et al. 2005; Franke et al. 2010). In this regard, we have previously identified several genetic risk factors in our Spanish cohort that are specifically associated with an age group or disease subtype (*BAIAP2* and *MAOB* in adults, *NT3* and *NTRK2* in children and *HTR2A* in combined ADHD) (Ribases et al. 2008, 2009a,b). Alternatively, discrepancy between the combined and inattentive groups and between the age groups could also be attributed to limited statistical power, distinct environmental influences, additional genetic risk factors, clinical heterogeneity and comorbid disorders co-occurring with ADHD.

In agreement with the involvement of the dopaminergic neurotransmission in ADHD, we have previously identified association between ADHD and two genes encoding enzymes involved in dopamine and serotonin synthesis (monoamine oxidase B; *MAOB*) and degradation (Dopa decarboxylase; *DDC*) (Ribases et al. 2009b).

In summary, although the functional sequence variants in *DRD1* directly involved in the disorder remain to be uncovered, we have identified a

significant childhood-specific association between the gene and combined ADHD in two independent samples. Follow-up studies may shed light on the possible involvement of *DRD1* in changes of ADHD symptoms across life span.

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Statement of Interest

None to declare.

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Supplementary material available online

Supplementary Table I. Description of the SNPlex assay within 9 dopamine-related candidate genes for ADHD.

Supplementary Table II. Description of the 2426 subjects included in the association study.

Supplementary Table III. Nominal *P* values observed when genotype frequencies (under a codominant model) and allele frequencies of 61 SNPs within nine candidate genes were considered in 322 children with ADHD and 322 controls and 211 adults with ADHD and 211 controls.

Supplementary Table IV. Association study in 322 childhood ADHD patients (237 combined ADHD, 70 inattentive ADHD and 15 hyperactive-impulsive ADHD patients) and 211 unrelated controls.