Association Study of 10 Genes Encoding Neurotrophic Factors and Their Receptors in Adult and Child Attention-Deficit/Hyperactivity Disorder

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Background: Attention-deficit/hyperactivity disorder (ADHD) is a common childhood-onset psychiatric disorder that often persists into adolescence and adulthood and is characterized by inappropriate levels of inattention, hyperactivity, and/or impulsivity. Genetic and environmental factors are believed to be involved in the continuity of the disorder as well as in changes in ADHD symptomatology throughout life. Neurotrophic factors (NTFs), which participate in neuronal survival and synaptic efficiency, are strong candidates to contribute to the neuroplasticity changes that take place in the human central nervous system during childhood, adolescence, and early adulthood and might be involved in the genetic predisposition to ADHD.

Methods: We performed a population-based association study in 546 ADHD patients (216 adults and 330 children) and 546 gender-matched unrelated control subjects with 183 single nucleotide polymorphisms covering 10 candidate genes that encode four neurotrophins (*NGF, BDNF, NTF3,* and *NTF4/5*), a member of the cytokine family of NTFs (*CNTF*), and their receptors (*NTRK1, NTRK2, NTRK3, NGFR, and CNTFR*).

Results: The single-marker and haplotype-based analyses provided evidence of association between *CNTFR* and both adulthood (p = .0077, odds ratio [OR] = 1.38) and childhood ADHD (p = 9.1e-04, OR = 1.40) and also suggested a childhood-specific contribution of *NTF3* (p = 3.0e−04, OR = 1.48) and *NTRK2* (p = .0084, OR = 1.52) to ADHD.

Conclusions: Our data suggest that variations in NTFs might be involved in the genetic susceptibility to ADHD, support the contribution of the *CNTFR* locus as a predisposition factor for the disorder, and suggest that *NTF3* and *NTRK2* might be involved in the molecular basis of the age-dependent changes in ADHD symptoms throughout life span.

Key Words: ADHD, association study, attention-deficit hyperactivity disorder, *CNTFR*, neurotrophic factors, neurotrophins, *NTF3*, *NTRK2*

Attention-deficit/hyperactivity disorder (ADHD) is a highly heterogeneous childhood-onset condition characterized by pervasive impairment of attention, hyperactivity, and/or impulsivity that can persist into adulthood with deleterious effects on educational, social, and occupational outcomes (1). Recent epidemiological studies report a worldwide ADHD prevalence of 8% to 12% for children and 1.2% to 7.3% for adults (2–8). Twin, family, and adoption studies suggest an essential role of genetic factors in the etiology of ADHD: 1) there are higher concordance rates in monozygotic than dizygotic ADHD twins with a mean estimated heritability of .76; 2) first-degree relatives of ADHD patients show a two- to eight-fold increased risk of developing ADHD, and 3) the adoptive relatives of ADHD patients show a lower risk of developing ADHD than the biological relatives (3,5,9).

Given that ADHD is a common neurodevelopmental disorder, neurotrophic factors (NTFs), which support neuronal survival and differentiation during development and participate in synaptic efficiency and neuronal plasticity in the adult nervous system, are strong candidates to be involved in the etiology of this complex disorder. Two different groups of NTFs are distinguished according to their actions and signal transduction pathways: 1) the nerve growth factor (NGF) family (also known as neurotrophins), which includes NGF, brain-derived NTF (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5), and whose effects are mediated through specific high-affinity neurotrophic tyrosine kinase receptors (NTRKs) and the nonselective low affinity receptor ($p75^{NGFR}$); and 2) a heterogeneous group of molecules that belong to the cytokine family that includes ciliary NTF (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), and interleukin 6 (IL6) (10–12).

Animal models, pharmacological evidence, and molecular genetic studies—mainly focused on *BDNF*—suggest that NTFs might be involved in the susceptibility to ADHD. Homozygous *BDNF* (−/−) knockout mice die during the second postnatal week (13), but heterozygous *BDNF* (−/+) knockout mice and *BDNF* (−/−) conditional knockout mice, in which the neurotrophin is eliminated in a tissue- or temporal-specific manner, display hippocampal-dependent learning deficiencies, aggressiveness, anxiety, and hyperactive locomotor behavior when compared with wild-type littermates (14–17). Interestingly, reduction of BDNF in the brain of adult mice results in impaired hippocampal function, whereas loss of the neurotrophin during

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Table 1. Statistically Significant Results of the Association Study in 330 Child Attention-Deficit/Hyperactivity Disorder (ADHD) Patients (242 Combined ADHD, 72 Inattentive ADHD, and 16 Hyperactive-Impulsive ADHD Patients), 216 Adult ADHD Patients (144 with Combined ADHD, 62 Inattentive ADHD, and 10 Hyperactive-Impulsive ADHD Patients), and 546 Control Subjects

		Genotypes								
		Cases *n* (%)				Control Subjects *n* (%)				
Gene	SNP	11	12	22	Sum	11	12	22	Sum	*p*
Adults										
BDNF	rs1491850	56 (26.3)	109 (51.2)	48 (22.5)	213	181 (33.6)	260 (48.2)	98 (18.2)	539	.11
NGF	rs6327	66 (30.8)	93 (43.5)	55 (25.7)	214	153 (28.0)	290 (53.1)	103 (18.9)	546	.036
CNTF	rs550942	164 (77.0)	41 (19.2)	8 (3.8)	213	365 (67.0)	157 (28.8)	23 (4.2)	545	.019
CNTFR	rs7036351	164 (76.6)	39 (18.2)	11 (5.1)	214	359 (66.0)	172 (31.6)	13 (2.4)	544	2.5e-04[b]
	rs3763613	147 (68.4)	55 (25.6)	13 (6.0)	215	319 (58.4)	206 (37.7)	21 (3.8)	546	.004
Children										
BDNF	rs1491850	130 (40.0)	148 (45.5)	47 (14.5)	325	181 (33.6)	260 (48.2)	98 (18.2)	539	.12
	rs11030096	77 (23.5)	155 (47.4)	95 (29.1)	327	151 (28.0)	261 (48.4)	127 (23.6)	539	.14
NGF	rs6537860	140 (43.1)	160 (49.2)	25 (7.7)	325	287 (52.6)	220 (40.3)	39 (7.1)	546	.023
	rs2856811	105 (32.3)	175 (53.8)	45 (13.8)	325	223 (41.0)	245 (45.0)	76 (14.0)	544	.025
	rs719452	164 (50.5)	141 (43.4)	20 (6.2)	325	316 (57.9)	203 (37.2)	27 (4.9)	546	.10
	rs2254404	133 (41.2)	165 (51.1)	25 (7.7)	323	281 (51.6)	217 (39.8)	47 (8.6)	545	.005
CNTFR	rs7036351	242 (73.3)	82 (24.8)	6 (1.8)	330	359 (66.0)	172 (31.6)	13 (2.4)	544	.073
	rs1080750	149 (45.4)	145 (44.2)	34 (10.4)	328	220 (40.4)	245 (45.0)	80 (14.7)	545	.12
	rs2381164	126 (38.5)	152 (46.5)	49 (15.0)	327	240 (44.5)	248 (46.0)	51 (9.5)	539	.030
NTF3	rs6332	59 (18.1)	173 (53.1)	94 (28.8)	326	151 (28.1)	281 (52.2)	106 (19.7)	538	3.7e-04[b]
NTRK2	rs1545285	125 (38.0)	155 (47.1)	49 (14.9)	329	180 (33.0)	253 (46.4)	112 (20.6)	545	.077
	rs11795386	262 (80.1)	62 (19.0)	3 (.9)	327	388 (72.1)	138 (25.7)	12 (2.2)	538	.018
	rs1387926	271 (82.4)	57 (17.3)	1 (.3)	329	397 (72.8)	136 (25.0)	12 (2.2)	545	8e-04[b]
	rs10780695	190 (57.6)	125 (37.9)	15 (4.5)	330	281 (51.6)	221 (40.6)	43 (7.9)	545	.067
	rs1073049	262 (79.4)	66 (20.0)	2 (.6)	330	394 (72.3)	136 (25.0)	15 (2.8)	545	.009

SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval; BDNF, brain derived neurotrophic factor; NGF, nerve growth factor; CNTFR, ciliary neurotrophic factor receptor; NTRK2, neurotrophic tyrosine kinase receptor 2.

[a]When odds ratio < 1, the inverted score is shown.

[b]Statistically significant *p* values after applying a false discovery rate of 10% ($p < 8.1e\text{-}04$).

[c]Statistically significant *p* values after applying Bonferroni correction ($p < 1.5e\text{-}04$).

early stages of development leads to more dramatic phenotypes with hyperactivity as well as more severe learning impairments (18). In addition, a recent study described gender differences in hyperactive and depression-like behaviors of *BDNF* (−/−) conditional knockout mice in which the *BDNF* gene was selectively inactivated in the forebrain (19). Finally, chronic infusion of BDNF or NTF3 into substantia nigra of animal models alters the rotational behavior and locomotion activity, whereas intracerebroventricular administration of NGF or BDNF induces motor activation and decreases locomotion, motility, and rearing, respectively (20–22).

Pharmacological evidence also emphasizes the potential involvement of NTFs in ADHD. Psychostimulant drugs, such as methylphenidate or amphetamine, and antidepressant drugs commonly used for ADHD treatment, including tricyclic antidepressant drugs and selective serotonin reuptake inhibitors, modulate the expression of *BDNF* and its specific receptor *NTRK2* (23–30). In addition, BDNF, NTF3, and CNTF mediate psychostimulant-induced neuroadaptations and locomotor activity through the dopaminergic, serotoninergic, and noradrenergic neurotransmitter systems that have been previously involved in ADHD (21,27,31–33).

Recent family and population-based association studies also support the involvement of NTFs in ADHD. Association between ADHD and rs6265 and −270C>T polymorphisms in *BDNF* and nominal association with rs6330 in *NGF* have been reported (34–36). Other studies, however, found no evidence of the participation of *BDNF*, glial cell line-derived neurotrophic factor (*GDNF*), or *NTF3* single nucleotide polymorphisms (SNPs) in the susceptibility to the disorder (36–39). In addition to association studies, one BDNF-haploinsufficient patient carrying a chromosomal inversion presented hyperactivity and impaired memory, language, attention, and numerical abilities, whereas a de novo missense mutation in *NTRK2* was reported to be involved in a more severe phenotype that includes obesity, developmental delay, and impairment of attention, memory, and learning (40,41).

On the basis of all these evidences, we suggest that alterations in the activity of NTFs might contribute to the genetic susceptibility to childhood and adulthood ADHD. To test this hypothesis, we performed a population-based association study in 546 ADHD patients (216 adults and 330 children) and 546 gender-matched unrelated control subjects, with 183 SNPs covering 10 candidate genes that encode five neurotrophins (*NGF*, *BDNF*, *NTF3*, *NTF4/5*, and *CNTF*) and their receptors (*NTRK1*,*NTRK2*, *NTRK3*, *NGFR*, and *CNTFR*).

Methods and Materials

Subjects

The clinical sample consisted of 546 Caucasoid patients with ADHD recruited from two centers in the Barcelona area (Spain) between 2004 and 2007. All subjects met DSM-IV criteria for ADHD and consisted of 216 adult cases (66.7% combined ADHD, 28.7% inattentive ADHD, and 4.6% hyperactive-impulsive ADHD patients) and 330 children (73.3%

Table 1. (continued from previous page)

Genotypes				Alleles	
Genotype 11 vs. 12+22		Genotype 22 vs. 11+12		Allele 2 vs. Allele 1	
OR (95% CI)[a]	*p*	OR (95% CI)[a]	*p*	OR (95% CI)[a]	*p*
1.42 (1.00–2.02)	.05	—	.18	1.26 (1.01–1.59)	.040
—	.44	1.49 (1.02–2.16)	.040	—	.48
1.64 (1.15–2.38)	.006	—	.77	1.48 (1.08–2.04)	.013
1.69 (1.18–2.44)	.0037	—	.058	—	.062
1.54 (1.10–2.13)	.010	—	.20	—	.094
—	.057	—	.15	1.24 (1.01–1.51)	.037
—	.15	—	.074	1.22 (1–1.49)	.044
1.46 (1.11–1.93)	.0067	—	.7	1.27 (1.03–1.56)	.026
1.46 (1.09–1.94)	.010	—	.96	—	.076
1.35 (1.02–1.78)	.033	—	.45	1.25 (1–1.56)	.046
1.52 (1.15–2.01)	.003	—	.65	1.25 (1.01–1.54)	.038
1.41 (1.05–1.92)	.022	—	.057	1.33 (1.02–1.75)	.030
—	.14	—	.063	1.23 (1–1.51)	.048
—	.083	1.69 (1.11–2.56)	.015	1.28 (1.05–1.59)	.015
1.77 (1.26–2.48)	7.7e-04[b]	1.65 (1.20–2.27)	.0022	1.47 (1.21–1.78)	1.2e-04[c]
—	.14	1.47 (1.02–2.13)	.034	1.25 (1.02–1.52)	.029
1.56 (1.12–2.17)	.0076	—	.13	1.12 (1.54–2.08)	.0049
1.75 (1.23–2.44)	.0011	7.14 (.96–50)	.012	1.75 (1.27–2.4)	3.6e-04[b]
—	.083	1.78 (1.02–1.61)	.048	1.28 (1.02–1.59)	.030
1.47 (1.06–2.04)	.018	4.5 (1.05–20)	.015	1.51 (1.12–2.04)	.0054

combined ADHD, 21.8% inattentive ADHD, and 4.9% hyperactive-impulsive ADHD patients). Because two child samples were sons of two adult patients, the children were excluded when all the samples were appraised together. Seventy-nine percent of patients were male (73.1% of adults and 82.4% of children). Diagnosis was blind to genotype. The control sample consisted of 546 unrelated Caucasoid blood donors recruited from the Blood and Tissue Bank at Hospital Universitari Vall d'Hebron for whom DSM-IV ADHD symptoms were retrospectively excluded. Control subjects matched for gender the ADHD clinical group. The average age at assessment was 9.3 years (SD = 2.6) for childhood ADHD patients, 29.6 years (SD = 12.06) for adulthood ADHD patients, and 39.9 years (SD = 17.0) for the control group. 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. 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 Part I and II) (42). 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) (43), the ADHD Rating Scale (ADHD-RS) (44), the ADHD Screening Checklist (45), and the Wender Utah Rating Scale (WURS) (46) 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). Additional tests used for patient assessment are available in Ribasés *et al.* (47).

Childhood ADHD. All children were evaluated with the present and lifetime version of the Schedule for Affective Disorders and Schizophrenia for School-age children (KSADS-PL) reported by parents. The ADHD symptoms were assessed with the Conners' Parent Rating Scale (CPRS-48) and the Conners' Teacher Rating Scale (CTRS-28). For additional information on patient assessment see Ribasés *et al.* (47).

Exclusion criteria for both children and adults were IQ < 70; pervasive developmental disorders; 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 (48) or with 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 with a Gemini XPS fluorometer (Molecular Devices, Sunnyvale, California) with the PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, Oregon), following the manufacturer's instructions. Subsequently, all DNA samples were normalized to 75 ng/μL.

SNP Selection

We selected 10 candidate genes that encode five NTFs (*NGF*, *BDNF*, *NTF3*, *NTF4/5*, and *CNTF*) and five neurotrophic receptors (*NTRK1*, *NTRK2*, *NTRK3*, *NGFR*, and *CNTFR*) (Supplement 1). We used information on the CEPH panel from the HapMap database (http://www.hapmap.org; release 20, January 2006) to select

SNPs (49). To minimize redundancy of the selected markers and ensure full genetic coverage of candidate genes, we used the LD-select software (50) to evaluate the linkage disequilibrium (LD) pattern of the region spanning each candidate gene plus 3 to 5 kb of flanking sequences. TagSNPs were selected at an r^2 threshold of .85 from all SNPs with a minor allele frequency (MAF) > .10. One hundred ninety-eight tagSNPs (79 in multi-loci bins and 119 singletons) were chosen with these criteria (Supplement 1). One additional SNP, rs1007211, located within the first exon of the *NTRK1* gene was included in the analysis.

Plex Design, Genotyping, and Quality Control

Of the 199 SNPs initially selected, 16 did not pass through the SNPlex design pipeline at http://ms.appliedbiosystems.com/snplex/snplexStart.jsp, resulting in a design rate of 92%. Four SNPlex genotyping assays of 45, 48, 46, and 44 SNPs were designed. To detect population admixture, 48 anonymous unlinked SNPs located at least 100 kb distant from known genes were also analyzed (51). All SNPs were genotyped with the SNPlex platform (Applied Biosystems, Foster City, California) as described by Tobler *et al.* (52). Finally, the specifically bound fluorescent probes were eluted and analyzed with an Applied Biosystems 3730xl DNA Analyzer. Two HapMap samples (NA11992 and NA11993) were included in all genotyping assays, and 99.96% concordance with HapMap data was obtained. In addition, no differences were found in the genotypes of four replicate samples.

Statistical Analyses

To better understand the genetic predisposition to adult and childhood ADHD, we first analyzed the two clinical samples independently. Then, and only when a potential common susceptibility factor was identified, the two datasets were analyzed together. The analysis of minimal statistical power was performed post hoc with the Genetic Power Calculator software (53), assuming an odds ratio (OR) of 1.75, disease prevalence of .05, significance level of .05, and MAF of .10. We tested potential genetic stratification in our sample by analyzing the SNPs in Hardy-Weinberg equilibrium (HWE) from the 48 anonymous SNP set with three different approaches: 1) STRUCTURE software (version 2.0) (54,55) 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; 2) the Fst coefficient calculated with the Weir and Cockerham approach with the FSTAT software and the 95% confidence interval (CI) determined by bootstrapping (56,57); and 3) the Pritchard and Rosenberg method (58) implemented to test whether the genotype distributions at each marker loci (under codominant, dominant, and recessive models) were the same in the case and control groups.

Single-Marker Analysis. The analysis of HWE (threshold set at $p < .01$) and the comparison of both genotype and allele frequencies between cases and control subjects were performed with the SNPassoc R library (59). 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. For the multiple comparison correction, we considered all tests performed and assumed a false discovery rate (FDR) of 10% with the Q-value R library (60), which corresponds to a significance threshold of $p < 8.1e\text{-}04$. The Bonferroni correction,

Table 2. Haplotype Analysis of 15 *CNTFR* SNPs in a Clinical Sample of 216 Adult ADHD Patients, 330 Child ADHD Patients, and 546 Control Subjects With the UNPHASED Software

	CNTFR									
	Adults			Children			Adults + Children			
Marker[a] Haplotype	Global *p*	Best Haplotype—*p* (Adjusted *p* Value)	Risk Haplotype—OR	Global *p*	Best Haplotype—*p* (Adjusted *p* Value)	Risk Haplotype—OR	Global *p*	Best Haplotype—*p* (Adjusted *p* Value)	Risk Haplotype—OR
1 5	.050	.026 (.070)	1.30 (1.04–1.62)	—	—	—	—	—	—
1 5 10[b]	.042	.0077 (.031)	1.38 (1.10–1.74)	.0069	9.1e-04 (.0052)	1.40 (1.15–1.72)	.0019	2.0e-04 (.0014)	1.39 (1.17–1.66)
1 5 10 14	.052	.034 (.15)	1.33 (1.03–1.72)	—	—	—	—	—	—

Abbreviations as in Table 1.
[a]1-rs7036351; 5-rs1080750; 10-rs1124882; 14-rs2274592.
[b]Best allelic combination (higher OR).

Table 3. Haplotype Distributions of the rs7036351, rs1080750, and rs1124882 *CNTFR* SNPs

	Adults			Children			Adults + Children		
Marker[a] Haplotype	Cases	Control Subjects	Haplotype-Specific *p*; OR (CI)	Cases	Control Subjects	Haplotype-Specific *p*; OR (CI)	Cases	Control Subjects	Haplotype-Specific *p*; OR (CI)
1 5 10									
C A C	140 (33.8)	396 (36.9)	—	206 (32.0)	396 (36.9)	—	346 (32.8)	396 (36.9)	—
C G C	175 (42.3)	372 (34.6)	.0077; 1.38 (1.10–1.74)	275 (42.7)	372 (34.6)	9.1e-04; 1.40 (1.15–1.72)	447 (42.4)	372 (34.6)	2.0e-04;1.39 (1.17–1.66)
T G C	58 (14.0)	191 (17.8)	—	94 (14.6)	191 (17.8)	—	151 (14.3)	191 (17.8)	—
C G G	41 (9.9)	115 (10.7)	—	69 (10.7)	115 (10.7)	—	110 (10.5)	115 (10.7)	—

Abbreviations as in Table 1.

[a] 1-rs7036351; 5-rs1080750; 10-rs1124882.

taking into account 166 SNPs and both adult and childhood samples, set the significance threshold at $p < 1.5e\text{-}04$.

Multiple-Marker Analysis. To avoid multiple testing and type I errors, we decided a priori to restrict the haplotype-based association study to those genes associated with ADHD in the single-marker analysis 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 relevant age group. Likewise, additional markers (up to four) were added in a stepwise manner to the initial two-SNP haplotype. The two-, three-, or four-marker haplotype showing the best OR within each gene was subsequently evaluated in the other age group, and only when a potential common susceptibility factor between childhood and adulthood ADHD was identified did we analyze the two datasets together. Significance was estimated by a permutation procedure with 5000 permutations with the UNPHASED software (61), with the exception of the 4-marker haplotype analysis where 1000 permutations were considered, owing to computing limitations. Because the expectation-maximization (EM) algorithm implemented in the UNPHASED software does not accurately estimate low haplotype frequencies (62), haplotypes with frequencies < .05 were excluded. Once the risk haplotypes were identified, they were further analyzed in the combined and inattentive ADHD subtypes. The hyperactive-impulsive sample was not considered, owing to its limited sample size. To evaluate potential additive and epistatic effects between the risk haplotypes identified, we first assigned specific estimated haplotypes to individuals considering cases and control subjects separately with the PHASE 2.0 software (63). Then we implemented a stepwise logistic regression procedure with the SPSS 12.0 statistical package. 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 10 candidate genes encoding different NTFs and their receptors in 546 ADHD cases (330 children and 216 adults) and 546 control subjects. Of the 183 SNPs included in the SNPlex assay, 9 were not successfully genotyped (genotype call rate < 90%) and 3 showed significant departures from HWE (Supplement 1). To avoid redundancies in genetic information, we discarded five additional SNPs that were in strong LD in the control group with other SNPs in the same candidate genes ($r^2 > .85$) (Supplement 1). Thus, a total of 166 SNPs were used for the final analysis. The minimal statistical power for the χ^2 test was 87.5% and 94% when the adult and childhood samples were considered, respectively.

Single-Marker Analysis

After excluding population admixture in our sample with the STRUCTURE software (Supplement 2), the Fst coefficient ($\Theta = 0$ with a 95% CI of .000–.001), and the Pritchard and Rosenberg method ($p = .301$), the comparison of genotype and allele frequencies between adulthood ADHD patients and control subjects showed nominal significant differences for five SNPs located in four genes: *BDNF*, *NGF*, *CNTF*, and *CNTFR* (Table 1 and Supplement 3). However, after correcting for multiple comparisons by applying an FDR of 10% ($p < 8.1e\text{-}04$), only

Table 4. Haplotype Analysis of 6 *NTF3* SNPs in a Clinical Sample of 330 Child ADHD Patients and 546 Control Subjects With the UNPHASED Software

	NTF3		
	Children		
Marker[a] Haplotype	Global *p*	Best Haplotype—*p* (Adjusted *p* Value)	Risk Haplotype—OR
2 3	2.37e-05	3.0e-06 (2.0e-04)	1.47 (1.20–1.79)
2 3 5	2.40e-04	1.1e-05 (2.0e-04)	1.43 (1.17–1.74)
2 3 4 5[b]	2.30e-04	2.1e-05 (1.0e-04)	1.48 (1.20–1.84)

Abbreviations as in Table 1.
[a]2- rs4074967; 3- rs6332; 4- rs6489630; 5- rs7956189.
[b]Best allelic combination (higher OR).

rs7036351 in *CNTFR* (p = 2.5e-04, under a codominant model) remained positively associated with ADHD in adults.

Single-marker analysis considering the childhood ADHD dataset identified 15 SNPs in five genes with uncorrected p values of < .05: *BDNF*, *NGF*, *CNTFR*, *NTF3*, and *NTRK2* (Table 1 and Supplement 3). However, only two SNPs, rs6332 in *NTF3* (p = 1.2e-04, OR = 1.47 [1.21–1.78]) and rs1387926 within the *NTRK2* gene (p = 3.6e-04, OR = 1.75 [1.27–2.40]), met the 10% FDR correction criterion. Furthermore, under the more conservative Bonferroni correction (p < 1.5e-04), rs6332 in *NTF3* was still associated with childhood ADHD.

Multiple-Marker Analysis

To minimize multiple testing, in the multiple-marker analysis we selected only those genes that showed evidence of association in the single-marker analysis after correction for multiple comparisons (*CNTFR* for adulthood ADHD and *NTF3* and *NTRK2* for childhood ADHD). All the associations described in the following sections remained significant after applying a multiple comparison correction by permutation (see adjusted p-values in Tables 2, 4, and 6).

CNTFR. The analysis of the 15 *CNTFR* SNPs showed evidence, in agreement with the single-marker study, of association between adulthood ADHD and a three-marker haplotype (rs7036351-rs1080750-rs1124882; global p-value = .042) (Table 2). An over-representation of the C-G-C haplotype class was observed in the adulthood ADHD group (OR = 1.38 [1.10–1.74]) (Table 3), accounting for 1.8% of the adulthood ADHD phenotype variance in our Spanish sample. Because children with ADHD form a heterogeneous group that includes persistent patients who will become adults with ADHD, we aimed to evaluate independently the contribution of the C-G-C haplotype to ADHD in children. Interestingly, the strong association between *CNTFR* and ADHD was also detected in the childhood subgroup (OR = 1.4 [1.15–1.72]) and when both clinical samples were considered together (OR = 1.39 [1.17–1.66]) (Table 3). We further subdivided patients according to ADHD clinical subtype and observed that the *CNTFR* association with ADHD was common for the combined (OR = 1.38 [1.14–1.67]) and inattentive ADHD groups (OR = 1.47 [1.12–1.94]) (Supplement 4).

NTF3. The analysis of all possible SNP combinations within the *NTF3* gene revealed a four-marker haplotype (rs4074967-rs6332-rs6489630-rs7956189) associated with childhood ADHD (global p value = 2.3e-04) (Table 4). The analysis of individual haplotypes showed that one of the five allelic combinations observed, the T-G-C-A haplotype, was significantly more frequent in ADHD children than in the control group (OR = 1.48 [1.20–1.84]), whereas the T-A-C-A combination was under-represented in the clinical sample (OR = 1.6 [1.28–2.00]) (Table 5). This association remained statistically significant when children were subdivided according to ADHD subtypes (combined: OR = 1.47 [1.16–1.85]; inattentive: OR = 1.57 [1.07–2.30]) (Supplement 5) but was not observed in the adulthood ADHD dataset. We further compared childhood and adulthood ADHD patients and confirmed the over-representation of the risk T-G-C-A haplotype in the child subset of patients (OR = 1.37 [1.05–1.79]).

NTRK2. A strong association between childhood ADHD and a four-marker haplotype of the *NTRK2* gene (rs7816-rs11795386-rs1387926-rs1586681; global p value = 2.1e-04) (Table 6) was detected, owing to over-representation of the A-C-G-A haplotype (OR = 1.52 [1.17–1.98]) and an under-representation of the A-T-A-A allelic combination (OR = 3.92 [1.85–8.32]) in children with ADHD (Table 7). When we subdivided child patients according to ADHD clinical subtypes, significant results were only obtained for the combined ADHD sample (A-C-G-A: OR = 1.48 [1.11–1.99]; A-T-A-A: OR = 2.54 [1.24–5.21]) (Supplement 6). We also evaluated the contribution of the rs7816-rs11795386-

Table 5. Haplotype Distributions of the rs4074967, rs6332, rs6489630, and rs7956189 *NTF3* SNPs

	Children		
Marker[a] Haplotype	Cases	Control Subjects	Haplotype-Specific *p*; OR (CI)
2 3 4 5			
G A C A	71 (12.5)	102 (10.7)	—
T A C A	167 (29.2)	381 (39.9)	2.1e-05; 1.6 (1.28–2.00)[b]
T A T G	26 (4.6)	56 (5.8)	—
T G C A	249 (43.8)	328 (34.3)	3.0e-04; 1.48 (1.20–1.84)
T G T G	57 (9.9)	89 (9.3)	—

Abbreviations as in Table 1.
[a]2- rs4074967; 3- rs6332; 4- rs6489630; 5- rs7956189.
[b]Down-represented in ADHD patients in comparison with control subjects.

Table 6. Haplotype Analysis of 42 *NTRK2* SNPs in a Clinical Sample of 330 Child ADHD Patients and 546 Control Subjects With the UNPHASED Software

	NTRK2		
	Children		
Marker[a] Haplotype	Global *p*	Best Haplotype—*p* (Adjusted *p* Value)	Risk Haplotype—OR
15 37	3.1e-04	.0021 (.010)	1.42 (1.11–1.82)
15 32 37	2.0e-04	3.2e-04 (.0040)	1.48 (1.14–1.91)
15 32 37 42[b]	2.1e-04	1.5e-04 (.0030)	1.52 (1.16–1.98)

Abbreviations as in Table 1.
[a]15- rs7816; 32- rs11795386; 37- rs1387926; 42- rs1586681.
[b]Best allelic combination (higher OR).

rs1387926-rs1586681 *NTRK2* haplotype to adulthood ADHD, but no effect was detected when either the complete dataset or the combined subtype was considered. Finally, when we compared children and adults with ADHD, we observed the under-representation of the A-T-A-A haplotype in the childhood dataset (OR = 3.11 [1.43–7.24]), but no association was identified when the A-C-G-A risk haplotype was considered.

In summary, haplotype analysis showed a strong association between *CNTFR* and both adult and childhood ADHD and suggested a childhood-specific contribution of *NTF3* and *NTRK2* to ADHD (Figure 1).

Analysis of Additive and Epistatic Effects

We evaluated potential additive effects of the C-G-C (rs7036351-rs1080750-rs1124882), T-G-C-A (rs4074967-rs6332-rs6489630-rs7956189), and A-C-G-A (rs7816-rs11795386-rs1387926-rs1586681) risk haplotypes in the *CNTFR*, *NTF3*, and *NTRK2* genes, respectively. We estimated that the combined effect of these three risk haplotypes contributes 6.7% of the childhood ADHD phenotype variance in our Spanish sample under an additive model (affectation status versus *NTF3*+*NTRK2*+*CNTFR*), with a sensitivity of 26.2% and a specificity of 87% (Table 8). We further evaluated possible interactions between these risk haplotypes identified in the childhood dataset but found no evidence for the existence of epistatic effects between them in the risk to develop ADHD (data not shown).

Discussion

To our knowledge, this is the first comprehensive study that investigates SNPs across genes coding for different NTFs and their receptors to identify genetic factors that confer susceptibility to adulthood and childhood ADHD. Our data provide evidence of association between *CNTFR* and both adult and childhood ADHD and suggest a childhood-specific contribution of *NTF3* and *NTRK2* to ADHD. The probability that these statistical associations are genuine is high for several reasons: 1) appropriately stringent corrections for multiple comparisons have been applied; 2) cases and gender-matched control subjects have been carefully selected from the same geographical area; 3) genetic stratification has been discarded with a different set of markers and several statistical approaches; 4) for the *CNTFR* gene, results were independently replicated in adults and children; and 5) stringent laboratory quality control procedures have been applied.

Although several follow-up studies reported that ADHD symptoms persist into adolescence or adulthood in the majority of children with ADHD (64–66), little is known about common genes involved in the etiology of childhood and adulthood ADHD. The detection of common susceptibility factors in the *CNTFR* gene in our ADHD child and adult datasets supports the diagnostic continuity of ADHD throughout life. Interestingly, CNTF promotes survival and maintenance of hippocampal neurons, which are implicated in the pathophysiology of ADHD (67,68), and modulates the serotoninergic and cholinergic neurotransmitter systems that might also be involved in the etiology of the disorder (69–73).

We also identified a childhood-specific association between ADHD and the *NTF3* and *NTRK2* genes. Even though the observed results might be interpreted with caution until replication data in other ADHD populations are available, they suggest a differential genetic component between childhood ADHD with and without symptomatic remission through life span. In this sense, recent studies point out that the susceptibility to ADHD is in fact a dynamic process in which new genes and environmental factors are involved at different developmental periods, as additional contributions to the etiological influences that emerge at earlier ages (74–76). The NTFs are strong candidates for participating in these neuroplasticity changes that take place in the human central nervous system (CNS) during childhood, adolescence, and early adulthood. Age-related changes in

Table 7. Haplotype Distributions of the rs7816, rs11795386, rs1387926, and rs1586681 *NTRK2* SNPs

	Children		
Marker[a] Haplotype	Cases	Control Subjects	Haplotype-Specific *p*; OR (CI)
15 32 37 42			
A C G A	122 (22.1)	150 (15.7)	.0084; 1.52 (1.17–1.98)
A T A A	8 (1.5)	52 (5.5)	1.5e-04; 3.92 (1.85–8.32)[b]
T C G A	315 (57.4)	561 (59.1)	—
T C G C	72 (13.1)	112 (11.8)	—
T T A A	33 (5.9)	75 (7.9)	—

Abbreviations as in Table 1.
[a]15- rs7816; 32- rs11795386; 37- rs1387926; 42- rs1586681.
[b]Down-represented in ADHD patients in comparison with control subjects.

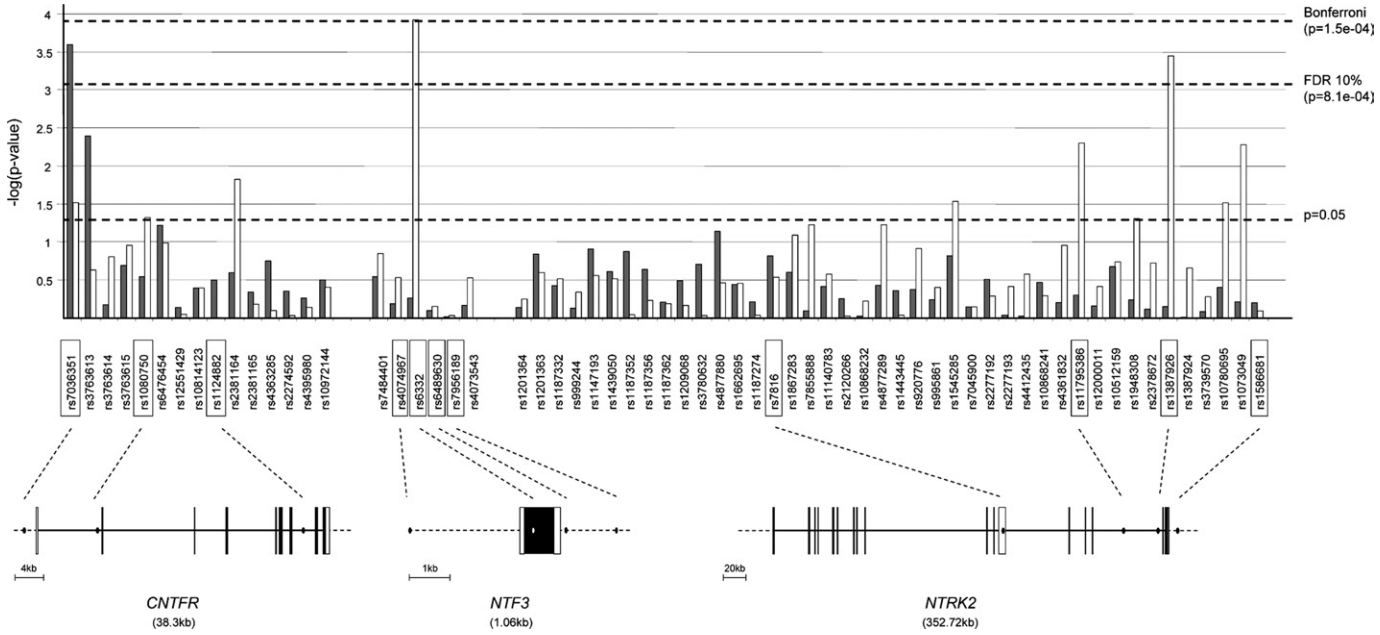

Figure 1. Diagram of the ciliary neurotrophic factor receptor (CNTFR), neurotrophic factor receptor 3 (NTF3), and neurotrophic tyrosine kinase receptor 2 (NTRK2) genes with all tagSNPs included in the study. Above, the lowest significance level, as -log (*p* value), observed when genotype and allele frequencies of individual single nucleotide polymorphisms (SNPs) were compared between 216 adult attention-deficit/hyperactivity disorder (ADHD) patients (in grey) or 330 child ADHD patients (in white) and 546 control subjects. The three genes, with their exon/intron structures, are drawn to different scales. The SNPs in haplotypes significantly associated with ADHD are boxed. FDR = false discovery rate.

NTRK2 and *NTRK3* expression have been described in different regions of the CNS, which suggests that impaired expression of these neurotrophic receptors or their ligands could influence changes of the ADHD symptomatology across life span, as pointed out for the *Monoamine oxidase A* (*MAOA*) gene (77–83). Because it is not possible to discern between remitting and persistent ADHD in childhood, only follow-up studies will reveal the role of *NTF3* and *NTRK2* as susceptibility factors in the subgroup of ADHD children who will not meet the diagnostic criteria in adolescence or young adulthood.

Most of the SNPs that we found associated with ADHD have no obvious functional consequences, because they were selected according to LD criteria, with the exception of rs6332 and rs7816 located in the coding region of *NTF3* and in the 3'untranslated region of *NTRK2*, respectively. Although rs6332 is a synonymous SNP, recent studies reported that differential codon usage could affect cotranslational folding and protein function, suggesting that silent SNPs should not be neglected in association studies (84). The SNP rs7816 is located in the 3'untranslated region sequence of an *NTRK2* truncated messenger RNA isoform (TRKB.T1) that lacks the kinase domain and exhibits dominant inhibitory effects (85). Interestingly, this region might participate in the cis-regulation of gene expression at the translational level (86), as previously described for *NTRK3*. The rest of the variants that we found associated with ADHD are located within introns, upstream or downstream of the candidate genes, which suggests that the identified risk haplotypes might not have functional consequences by themselves, but they are in LD with other unknown susceptibility variants directly involved in the genetic susceptibility to ADHD. Further sequencing of the three ADHD-associated genes might allow the identification of uncommon rare genetic variants (MAF < .10) that could contribute to the predisposition to this complex phenotype.

One previous study investigated four SNPs (including rs6332) across the *NTF3* gene with both family-based and case-control designs with 120 family trios and 120 ADHD cases versus 120 control subjects, respectively, but found no association with childhood ADHD (36). This discrepancy with our findings could be attributed to the different statistical power of the studies, the existence of genetic differences among the populations under study, or the presence of clinical heterogeneity due to different frequencies of ADHD subtypes or possible comorbid disorders that co-occur with ADHD, such as mood, anxiety, or antisocial personality disorders. Finally, several studies attempted to ana-

Table 8. Evaluation of Additive Effects Between *NTF3*, *NTRK2,* and *CNTFR* in Childhood ADHD Through Logistic Regression Analyses in 330 Children With ADHD and 546 Control Subjects

Variable[a]	Log Likelihhod	χ^2 (*df*)	*p*	R^2	OR (95% CI)
NTF3	—	—	—		1.54 (1.15–2.06)
NTRK2	—	—	—		2.28 (1.62–3.21)
CNTFR	—	—	—		2.04 (1.35–3.08)
	516.68	41.43 (3)	5.3e-09	6.7	

Abbreviations as in Table 1.

[a]Risk alleles: *NT3:* T-G-C-A (rs4074967/rs6332/rs6489630/rs7956189); *NTRK2*: A-C-G-A (rs7816/rs11795386/ rs1387926/ rs1586681); and *CNTFR*: C-G-C (rs1124882/rs1080750/rs7036351).

lyze the role of *BDNF* in ADHD and yielded controversial results. Although we found nominal association between *BDNF* and both adulthood and childhood ADHD, these results were not significant after correcting for multiple testing and therefore are in agreement with other studies (37–39) that do not support the *BDNF* participation in ADHD described in previous reports (34,35). Unfortunately, the preferential transmission of paternal *BDNF* alleles to ADHD offspring previously described (34,87) could not be assessed in the present case-control study.

In conclusion, we identified a *CNTFR* risk haplotype involved in both adulthood and childhood ADHD. Additionally, our results provide evidence for a childhood-specific association between ADHD and the *NTF3* and *NTRK2* genes, suggesting their potential influence on changes in ADHD symptomatology throughout life span. Further genetic analyses in other large population datasets are required to confirm these results, disclose the functional variants involved, and elucidate the genetic component underlying predisposition to ADHD.

Drs. Ribasés and Hervás contributed equally to this article.

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Supplementary material cited in this article is available online.

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