RESEARCH ARTICLE

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Dopamine Receptor DRD4 Gene and Stressful Life Events in Persistent Attention Deficit Hyperactivity Disorder

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We performed a case-control association study in persistent ADHD considering eight candidate genes (*DRD4*, *DAT1/SLC6A3*, *COMT*, *ADRA2A*, *CES1*, *CYP2D6*, *LPHN3*, and *OPRM1*) and found additional evidence for the involvement of the Dup 120bp and VNTR 48bp functional variants within the dopamine receptor *DRD4* gene in the etiology of adult ADHD. We subsequently investigated the interaction of stressful life events with these two *DRD4* polymorphisms, and the impact of such events on the severity of ADHD symptomatology. The gene-by-environment analysis revealed an independent effect of stressful experiences on the severity of persistent ADHD, and a gene-by-environment interaction on the inattentive dimension

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of the disorder, where non carriers of the Dup 120bp (L) - VNTR 48bp (7R) haplotype were more sensitive to environmental adversity than carriers. These results are in agreement with previous works reporting a relationship between *DRD4* and the effect of adverse experiences, which may explain the discordant findings in previous genetic studies and strengthen the importance of gene-by-environment interactions on the severity of ADHD.

Key words: Attention deficit hyperactivity disorder; ADHD; DRD4; Stressful life events; GxE interaction

INTRODUCTION

Attention deficit and hyperactivity disorder (ADHD) is one of the most extensively studied neurodevelopmental disorders in childhood according to DSM-V criteria, with a high prevalence of 5.2%, among children and adolescents [Polanczyk et al., 2007]. In the past, ADHD was considered a childhood disorder that resolved with maturation. However, recent longitudinal follow-up studies have shown that at least 60% of patients diagnosed during childhood continue to suffer ADHD thereafter, with an estimated prevalence in adulthood in the range of 2.5–4.9% [Kessler et al., 2005; Brookes et al., 2006a; Simon et al., 2009; Faraone and Mick, 2010]. Family and twin studies have shown that ADHD is a highly heritable and multifactorial disorder, with approximately 76% of the phenotypic variance explained by genetic factors [Faraone and Mick, 2010]. In this context, the Cross-Disorder Group of the Psychiatric Genomics Consortium (http://www.med.unc.edu/pgc/for-investigators/cross-disorder-analysis) also demonstrated that single nucleotide polymorphisms (SNPs) explain about 28% of the genetic variance of ADHD [Lee and Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013].

Gene-based studies on ADHD have mainly focused on genes involved in dopaminergic neurotransmission, such as those encoding the dopamine receptor D4 (*DRD4*) or the dopamine transporter (*DAT1/SLC6A3*). Researchers have mainly studied a functional 48bp variable number of tandem repeats (VNTR) in exon 3 of the *DRD4* gene, and have found consistent associations between ADHD and the 7 repeat allele (7R), which is related to decreased functional activity [Asghari et al., 1995], in both children and adults [Johansson et al., 2008; Gizer et al., 2009; Nikolaidis and Gray, 2010; Sánchez-Mora et al., 2011; Tovo-Rodrigues et al., 2012, 2013]. A second polymorphism in *DRD4*, a 120bp duplication located 1.2 kb upstream from the translation start codon that may play a role in transcriptional activity [Paredes et al., 2013], has also been studied in ADHD with controversial results [Barr et al., 2001; D'Souza et al., 2004; Brookes et al., 2005; Bidwell et al., 2011, Hasler et al., 2015]

Since stimulant medication blocks the dopamine transporter, the *DAT1/SLC6A3* gene has also been considered a good candidate for ADHD. Two polymorphisms in this gene, a VNTR 40bp in the 3'untranslated region (3′ UTR) and a VNTR 30bp in intron 8, have been studied extensively [Franke et al., 2008; Johansson et al., 2008; da Silva et al., 2011; Kotte et al., 2013; de Azeredo et al., 2014; Tong et al., 2015]. Although the results are inconsistent, and each polymorphism individually may not explain the association between *DAT1/SLC6A3* and ADHD, different haplotype combinations including these two VNTRs have been associated with ADHD in children and in adults [Laucht et al., 2007; Franke et al., 2010]. A non-synonymous SNP, rs4680 (Val158Met), in the catechol-O-methyltransferase gene (*COMT*) encoding an enzyme involved in catecholamine turnover that regulates the levels of dopamine, norepinephrine and epinephrine in the synaptic cleft, has also been associated with persistent ADHD and with alterations in brain white matter in subjects with ADHD [Reuter et al., 2006; Gothelf et al., 2007; Halleland et al., 2009; Hong et al., 2014; Lee and Song, 2015]. Other candidate genes have been investigated in adult ADHD and include *ADRA2A*, which encodes the alpha-2A adrenergic receptor involved in adrenaline release and the pharmacological response to methylphenidate [Froehlich et al., 2010; Contini et al., 2011; Park et al., 2013; Castro et al., 2013; McCracken et al., 2014], *LPHN3*, which encodes a member of the latrophilin subfamily of G protein-coupled receptors that may function in both cell adhesion and signal transduction [Arcos-Burgos et al., 2010; Ribases et al., 2011] and genes involved in the metabolism of ADHD medication, such as *CYP2D6* or *CES1*. The *CYP2D6* gene encodes a member of the cytochrome P450 superfamily of monooxygenases that catalyses many reactions involved in the metabolism of drugs such as antidepressants or atomoxetine, a selective norepinephrine reuptake inhibitor used in the treatment of ADHD. Genetic variants of this highly polymorphic gene allow classification of individuals on the basis of their decreased or increased ability to metabolize the enzyme's substrates, and have been associated with the response to atomoxetine in individuals with ADHD [Bonnet et al., 2003; Michelson et al., 2007; Trzepacz et al., 2008; Choi et al., 2014]. Polymorphisms in *CES1*, which encodes a member of the carboxylesterase large family, have also been associated with methylphenidate response and side effects [Nemoda et al., 2009; Johnson et al., 2013].

To date, genome-wide association studies have been performed in nine ADHD datasets, two of which on adult samples [Lasky-Su J et al., 2008a,b; Lesh et al., 2008; Mick et al., 2008; Neale et al., 2008, 2010; Franke et al., 2009; Hinney et al., 2011; Lesch et al., 2011; Stergiakouli et al., 2012; Yang et al., 2013; Sánchez-Mora et al., 2015]. No single marker achieved genome-wide significance, and there was little overlap between the studies or with previous candidate gene association studies. These inconsistent results in genetic studies may be explained in part by gene-by-environment interactions (GxE) [Buitelaar, 2005], where genetic factors may moderate the influence of environmental factors in ADHD. In contrast to the high heritability estimated in ADHD, the contribution of the environment seems to be lower, with around 22% of ADHD variance explained by environmental factors [Hudziak et al., 2005]. A number of environmental risk factors for ADHD have been described, including maternal smoking during pregnancy [Langley et al., 2012; Kovess et al., 2014; Han et al., 2015], institutional deprivation [Kumsta et al., 2010], inter-parental conflict, parenting styles and childhood maltreatment [De Sanctiset al., 2012; Prayez et al., 2012]. In addition, some studies have supported GxE interactions in ADHD, mainly between *DRD4* and exposure to prenatal smoking, *DAT1/SLC6A3* and maternal use of alcohol during pregnancy, institutional deprivation or

psychosocial adversity [Brookes et al., 2006b; Laucht et al., 2007; Stevens et al., 2009], *5HTT* and psychosocial stress [Muller et al., 2008; Retz et al., 2008] and *MAOA* and negative parenting behavior [Li and Lee, 2012].

In the present study we have performed a case-control association study of eight candidate genes encoding proteins involved in the dopaminergic and noradrenergic neurotransmitter systems (*DRD4, DAT1/SLC6A3, COMT*, and *ADRA2A*), enzymes involved in the pharmacokinetics of methylphenidate or atomoxetine (*CES1* and *CYP2D6*, respectively), Latrophilin 3 (*LPHN3*) and the Opioid Receptor Mu 1 (*OPRM1*) in 604 adults with ADHD and 611 controls. Subsequently, based on previous findings suggesting that environmental factors may be particularly harmful in combination with susceptibility genes [Todd and Neuman, 2007], we investigated the impact of gene-by-environment interactions on the severity of adult ADHD symptoms by considering exposure to adverse life events and the studied ADHD genetic risk variants.

MATERIALS AND METHODS

Patients and Controls

A total of 604 adult ADHD (60% combined, 36.3% inattentive, and 3.7% hyperactive-impulsive) patients of Caucasian origin from Spain were recruited and evaluated at the Hospital Universitari Vall d'Hebron (Barcelona, Spain). All subjects met DSM-IV criteria for ADHD. The diagnosis of ADHD in adulthood was evaluated with the Conners' Adult ADHD Diagnostic Interview for DSM-IV (CAADID Parts I and II) and the DSM-IV Axis I and II Disorders (SCID-I and SCID-II). More detailed information on the clinical assessment was provided previously [Sánchez-Mora et al., 2013]. To investigate ADHD severity, a four-point Likert-type scale (from 0 = not at all/never to 3 = very much/very frequently) was used from the inattention and hyperactivity/impulsivity subscales of the ADHD Rating Scale-IV (ADHD-RS), each with nine items [DuPaul, 1998]). The severity of inattention and hyperactivity/impulsivity was measured by counting the number of occurrences in which the informant rated items from each scale at the three different levels of symptom severity. Total ADHD severity was calculated by considering the scores for both the inattention and hyperactivity/impulsivity domains. Childhood stressful life events were assessed retrospectively with the CAADID Part I in 425 subjects with persistent ADHD. A total of 12 life events were assessed for each participant and included: separation or loss, sexual abuse, physical abuse, emotional abuse, domestic violence, emotional and physical neglect, extreme family stress, financial stress and poverty, malnutrition, exposure to heavy metals, other trauma in childhood or adolescence, and prenatal exposure to nicotine.

The control sample consisted of 611 unrelated Caucasoid blood donors matched for gender with the ADHD group in which DSM-IV ADHD symptoms were excluded under the following criteria: (1) no prior ADHD diagnosis and (2) negative answers to the lifetime presence of the following DSM-IV ADHD symptoms: (1) often has trouble keeping attention on tasks, (2) often loses things needed for tasks, (3) often fidgets with hands or feet or squirms in seat, and (4) often gets up from seat when remaining in seat is expected. The average age at assessment was 33 years (SD = 10.6) for ADHD and 60 years (SD = 14.4) for control subjects. The study was approved by the ethics committees of the participating institutions, and informed consent was obtained from all subjects or parents, in accordance with the Helsinki Declaration.

DNA Isolation and Quantification

Genomic DNA samples were obtained either from peripheral blood lymphocytes by the salting out procedure or from saliva using the Oragene DNA Self-Collection Kit (DNA Genotek, Kanata, Ontario Canada). DNA concentrations were determined using the Pico-Green dsDNA Quantitation Kit (Molecular Probes, Eugene, OR).

Selection of Genes and SNPs

Eight potentially functional biallelic polymorphisms in six candidate genes for ADHD were selected for the association study and included: *COMT* (rs4680), *ADRA2A* (rs1800544), *CES1* (rs121912777), *CYP2D6* (rs3892097), *OPRM1* (rs1799971), *LPHN3* (rs6813183, rs12503398 and rs1868790) and a 120bp insertion/deletion in the promoter region of DRD4. Three additional variable number of tandem repeats (VNTR), a VNTR 48bp in exon 3 of *DRD4* and a VNTR 30bp and VNTR 40bp in intron 8 and in the 3' UTR region of *DAT1/SLC6A3*, respectively, were also included in the analysis. All the SNP genotype assays were performed using commercial TaqMan® assays in a 7,500 Real-Time PCR System (Thermo Fisher Scientific Inc.). The analyses of 48bp VNTRs and the Dup 120bp polymorphism in *DRD4* were performed using a standard PCR method, as previously described [Sánchez-Mora et al., 2011; Franke et al., 2010], visualized on an ABI 3130XL sequencer, and automatically called using GeneMapper software (Thermo Fisher Scientific Inc., Waltham, MA).

Statistical Analysis

The minimal statistical power was estimated post hoc using Genetic Power Calculator software (http://pngu.mgh.harvard.edu/~purcell/gpc/), assuming an odds ratio (OR) of 1.5, disease prevalence of 0.05, significance level of 0.05, the lowest minimum allele frequency (MAF) observed in our control sample (0.166) and a codominant model of inheritance. The presence of population substructures was previously discarded by means of genetic stratification testing using a panel of 45 unlinked non-genic SNPs [Ribasés et al., 2009].

The analysis of Hardy–Weinberg equilibrium (HWE; threshold set at $P < 0.01$) in the control sample and the comparison of genotype and allele frequencies under a codominant genetic model were performed with the SNPassoc R package [Gonzalez et al., 2007] for biallelic markers and with OTT software [Ott J et al., 1985] and the statistical package SPSS 22.0 for VNTRs (SPSS Inc., Chicago, IL). For multiallelic markers, rare genotypes or alleles (MAF < 0.05) were grouped in a single class. For the multiple-marker analysis, haplotype frequencies were estimated using PHASE 2.0 software [Stephens et al., 2001] and values below 5% were grouped as "others" in the association analysis. The frequency of carriers of the risk haplotypes was compared between ADHD subjects and controls using SPSS 22.0.

To assess the contribution of environmental risk factors to ADHD symptom severity, the overall ADHD and the inattentive and hyperactive-impulsive symptom scores were considered. The relationship between symptom severity and the number of stressful life events was evaluated with Pearson's Correlation tests. Gene-by-environment (GxE) interactions were assessed by linear regression models to estimate the association between symptom severity and (i) the Dup 120bp polymorphism, the VNTR 48bp or the Dup 120bp - VNTR 48bp haplotype in *DRD4*, (ii) the number of stressful life events and (iii) their interaction using SPSS 22.0 (SPSS Inc.).

RESULTS

We studied 12 polymorphisms in eight candidate genes for ADHD that encode proteins involved in dopaminergic and noradrenergic neurotransmission (*DRD4*, *DAT1*/*SLC6A3*, *COMT*, and *ADRA2A*), enzymes involved in the pharmacokinetics of methylphenidate or atomoxetine (*CES1* and *CYP2D6*, respectively), Latrophilin 3 (*LPHN3*) and the Opioid receptor Mu 1 (*OPRM1*). Of the 12 polymorphisms initially selected for inclusion in the genotyping assay, one was monomorphic in our sample (rs121912777 in *CES1*), which resulted in 11 polymorphisms in seven candidate genes finally considered in a total sample of 604 adults with ADHD and 611 controls. No significant departures from HWE were identified in the control sample and, taking into account the sequence variant with the lowest MAF (0.166), the sample showed a minimum statistical power of 89%.

The case-control association study between ADHD and single markers revealed significant differences for the two *DRD4* polymorphisms, with the L allele of the Dup 120bp polymorphism ($P_{codominant} = 0.04$) (Supplementary Table S1A) and the 7R allele of the VNTR 48bp being nominally associated with adult ADHD ($P_{dominant} = 0.026$) (Table IA). A more detailed analysis of common alleles of the VNTR 48bp showed evidence for overrepresentation of the 7R allele in the clinical sample ($P_{7R} = 4.6e\text{-}03$; OR = 1.37 (1.11–1.69)) (Table IB). We subsequently considered *DRD4* for the haplotype-based analysis and detected an association between the Dup 120bp - VNTR 48bp haplotype and adult ADHD (Pglobal-haplogenotypes = 0.008 and Pglobal-haplotype = 0.046, Table IIA), with overrepresentation of the L-7R allelic combination and increased frequency of carriers of this risk haplotype in the ADHD group ($P_{carriersL\text{-}7R} = 0.017$; OR = 1.36 (1.06–1.75)) (Table IIB). Although not reaching significance, the analysis of an age-matched subsample of 122 ADHD adults and 122 unrelated controls also showed an overrepresentation of L-7R carriers among the cases (cases: 42%, controls: 34%; $P = 0.23$). No significant differences were observed for any of the other sequence variants considered in *DAT1/SLC6A3*, *COMT*, *ADRA2A*, *CYP2D6*, *LPHN3*, or *OPRM1* (Supplementary Table S1).

Under the hypothesis that *DRD4* is directly involved in ADHD, but may also modulate the dopaminergic response to stressful events, we subsequently tested potential gene-by-environment (GxE) interactions on ADHD severity. Participants were assessed for the severity of ADHD, as well as hyperactive and inattentive subscales, according to the ADHD-RS [DuPaul, 1998]. Sixty-five percent of participants had experienced stressful life events (23%

TABLE I. (A) Genotype and Allelic Distribution of the DRD4 VNTR 48bp Polymorphism and (B) Comparison of Carriers of Common Genotypes and Alleles of the DRD4 VNTR 48bp Polymorphism

(A)

	Genotype					Allele				
	Others[a]	2R4R	4R4R	4R7R	Sum	Others[b]	2R	4R	7R	Sum
Controls N (%)	87 (15.9%)	76 (13.9%)	259 (47.3%)	125 (22.9%)	547	55 (5.0)	102 (9.3)	759 (69.4)	178 (16.3)	1,094
Cases N (%)	114 (20.2%)	70 (12.4%)	232 (41.1%)	149 (26.4%)	565	56 (5.0)	113 (10)	724 (64.1)	237 (21)	1,130
Sum	202 (18.1%)	146 (13.1%)	491 (44.1%)	274 (24.6%)	1,112	111 (5)	215 (9.7)	1,483 (66.7)	415 (18.7)	2,224

(B)

	Cases N (%)		Controls N (%)			
	Carriers	Non Carriers	Carriers	Non Carriers	*P*-value	OR (IC 95%)
Genotype						
2R4R	70 (12.4)	495 (87.6)	76 (13.9)	471 (86.1)	—	—
4R4R	232 (41.1)	333 (58.9)	259 (47.3)	288 (52.7)	0.04	1.29 (1.01–1.31)
4R7R	149 (26.4)	416 (73.6)	125 (22.9)	422 (77.1)	—	—
Allele						
2R	113 (10)	1,017 (90)	102 (9.3)	992 (90.7)	—	—
4R	724 (64.1)	406 (35.9)	759 (69.4)	335 (30.6)	8.0e-03	1.27 (1.06–1.51)
7R	237 (21)	893 (79)	178 (16.3)	916 (83.7)	5.0e-05	1.37 (1.09–1.69)[c]

[a]Others: genotypes or alleles with frequency <5%, *P*-value = 0.06.
[b]Others: genotypes or alleles with frequency <5%, *P*-value = 0.026.
[c]When OR <1 inverted score is shown.

TABLE II. (A) Haplotype Distribution of the *DRD4* Gene Dup 120bp and the VNTR 48bp Polymorphisms in a Sample of 561 Adult ADHD Subjects and 547 Controls and (B) Comparison of Frequency of Carriers of Common Haplotypes Between Cases and Controls

(A)

	Haplogenotype distribution N [%]							Haplotype distribution N [%]					
	(L-2R)/(L-4R)	(S-2R)/(L-4R)	(L-4R)/(L-4R)	(L-4R)/(S-4R)	(L-4R)/(L-7R)	(S-4R)/(L-7R)	Others[a]	Sum	S-2R	S-4R	L-4R	L-7R	Others[b]	Sum
Controls	26 (4.8)	46 (8.4)	150 (27.4)	98 (17.9)	88 (16.1)	37 (6.8)	102 (18.6)	547	64 (5.9)	175 (16)	584 (53.4)	176 (16.1)	95 (8.7)	1,094
Cases	33 (5.9)	28 (5.0)	153 (27.3)	68 (12.1)	106 (18.9)	36 (6.4)	137 (24.4)	561	62 (5.5)	151 (13.5)	568 (50.6)	222 (19.8)	119 (10.6)	1,122

(B)

	Cases N [%]			Controls N [%]				
	Carriers	Non Carriers	Sum	Carriers	Non Carriers	Sum	*P*-value	OR (IC 95%)
S-2R	59 (10.5)	502 (89.5)	561	63 (11.5)	484 (88.5)	547	—	—
S-4R	141 (25.1)	420 (74.9)	561	164 (30)	383 (70)	547	—	—
L-4R	415 (74)	146 (26)	561	434 (74)	113 (20.7)	547	0.039	1.31 (1.02–1.79)
L-7R	200 (35.7)	361 (64.3)	561	111 (20.3)	436 (79.7)	547	0.017	1.36 (1.06– 1.76)

[a]Others: genotypes or alleles with frequency <5%, *P*-value = 0.008.
[b]Others: genotypes or alleles with frequency <5%, *P*-value = 0.046.

had experienced one event, 13% had experienced two events, and 29% had experienced three or more events). Positive correlations were identified between the number of stressful life events and the severity of ADHD ($r = 0.23$, $P = 1.4e\text{-}06$), hyperactive ($r = 0.22$, $P = 4.3e\text{-}06$) and inattentive ($r = 0.172$, $P = 3.7e\text{-}04$) symptoms. Subjects with higher severity scores had been exposed more frequently to stressful life events than those with lower scores (ADHD: $F = 16.8$, $P = 5.1e\text{-}05$; hyperactivity: $F = 13.0$, $P = 3.5e\text{-}04$; inattention: $F = 11.4$, $P = 1.0e\text{-}03$). When stressful life events were considered separately, significant differences in overall ADHD severity were observed for sexual abuse ($P = 6.0e\text{-}03$), physical abuse ($P = 2.2e\text{-}06$), emotional abuse ($P = 5.0e\text{-}07$), domestic violence ($P = 4.3e\text{-}05$), extreme family stress ($P = 4.2e\text{-}04$), financial stress and poverty ($P = 1.7e\text{-}03$), and exposure to heavy metals ($P = 0.018$) (Fig. 1).

Given the impact of an adverse environment on ADHD severity, we subsequently investigated whether the interaction of *DRD4* with stressful life events moderates the effects of environmental factors on the ADHD phenotype. No significant differences in the number of stressful life events were observed between carriers and non carriers of the Dup 120-bp (L) variant, the VNTR 48bp (7R) variant or the Dup 120bp (L) - VNTR 48bp (7R) haplotype. No GxE interactions on the overall ADHD severity or on the hyperactive symptoms were detected when we considered the number of stressful life events and the *DRD4* variants (Supplementary Tables S2A and B). Regarding inattention scores, no significance was detected when we evaluated the two *DRD4* variants separately, but it rendered significant results when we cosidered stressful life events ($P = 3.6e\text{-}05$) or the interaction between the Dup 120bp (L) - VNTR 48bp (7R) haplotype and the number of stressful life events ($P = 0.031$) (Table III). There were no significant main effects on inattention symptoms for the Dup 120bp (L) - VNTR 48bp (7R) haplotype ($P = 0.4$). The effect of the number of stressful life events on inattention scores was stronger among subjects who do not carry the L-7R haplotype. ADHD-affected subjects carrying the L-7R haplotype who experienced stressful life events had lower scores in inattentive symptoms than non carriers (Table III and Fig. 2A). This significant interaction showed that stress positively predicted inattention symptoms among non carriers ($b = 2.3$, $SE = 0.5$, $t = 4.6$, $P = 7.6e\text{-}06$) and L-7R heterozygotes ($b = 1.3$, $SE = 0.7$, $t = 2.0$, $P = 0.045$), but not in the group of L-7R homozygotes ($b = 1.2$, $SE = 1.9$, $t = 0.6$, $P = 0.55$).

DISCUSSION

We aimed to perform a case-control association study on adult ADHD by focusing on eight classical candidate genes for the disorder, and to further examine the impact of their interaction with stressful life events on the severity of ADHD symptoms. Our results provide evidence for (i) the contribution of *DRD4* to adult ADHD; (ii) a main effect of experienced stressful life events on the severity of ADHD symptomatology; and (iii) a gene-by-environment interaction on the severity of inattention symptoms, in which the individual response to adverse life events may be modulated by *DRD4* genotypes.

Based on previous genetic studies, we considered two functional polymorphisms in *DRD4*: a 120bp duplication in the promoter

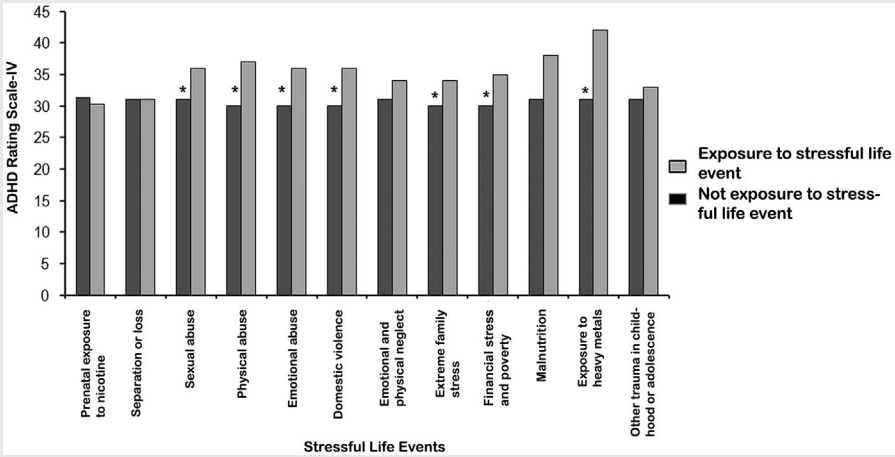

FIG. 1. ADHD Rating scale-IV scores in ADHD subjects according to the presence or absence of stressful life events; *significant difference (*P*-value <0.05).

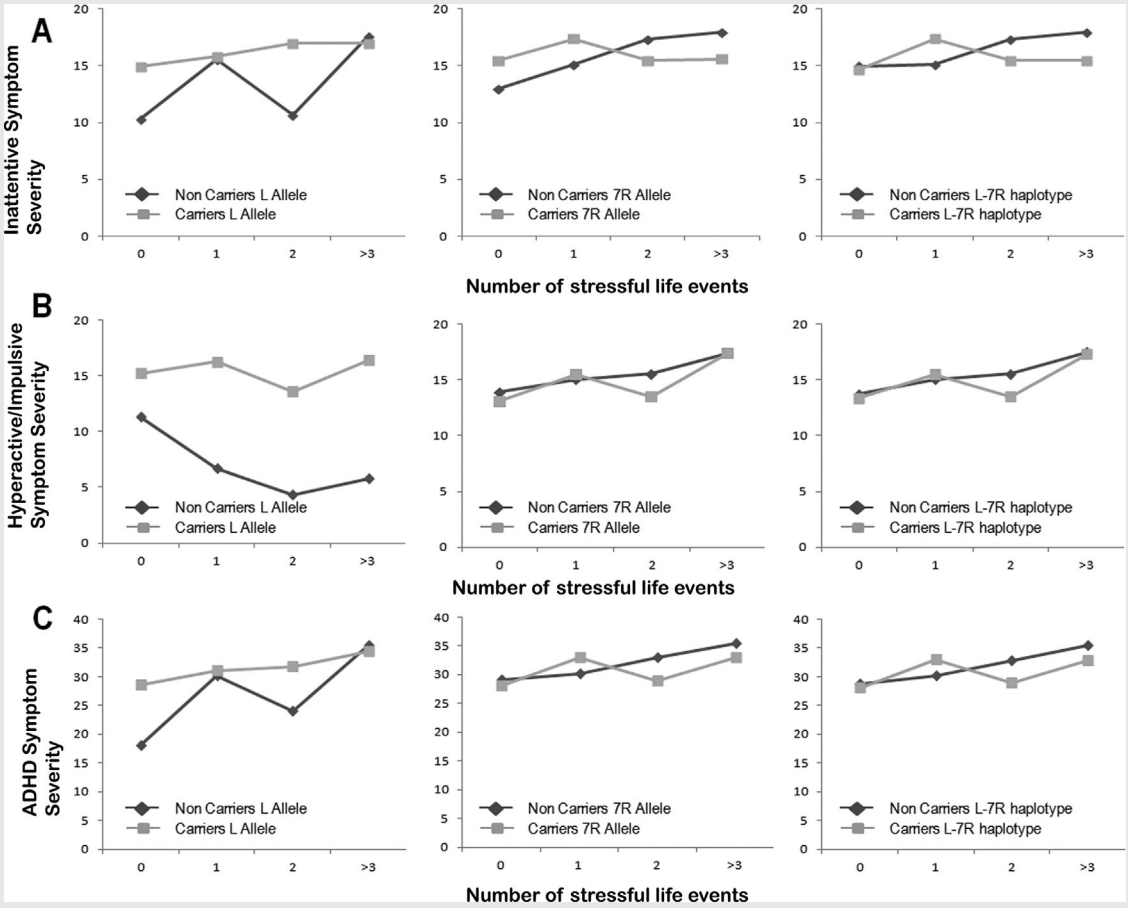

FIG. 2. Association between the number of stressful life events and ADHD symptom severity measured with the ADHD Rating Scale IV as a function of carriers of the Dup 120bp (L) allele, the VNTR 48bp (7R) allele or the L-7R haplotype in DRD4. (A) Inattentive symptom severity; (B) hyperactive-impulsive symptom severity; (C) ADHD symptom severity.

TABLE III. Results of Multiple Regression Analyses Estimating the GxE Interaction Between Carriers of the *DRD4* Risk Variants and Stressful Life Events in Inattentive Symptom Severity in 425 Adults With ADHD

	Predictor variables											
	Intercept	Stressful life events				DRD4				GxE interaction		
Inattentive symptoms	**β**	**β**	**SE**	**t**	***P*-value**	**β**	**SE**	**t**	***P*-value**	**β**	**SE**	**t**	***P*-value**
Dup 120bp polymorphism	11.967	1.592	1.139	1.398	0.163	3.069	2.18	1.407	0.16	0.877	1.158	0.758	0.449
L allele carriers vs. non carriers													
VNTR 48bp polymorphism	14.793	1.025	0.261	3.932	$9.86e^{-5}$	0.344	0.765	0.449	0.653	0.749	0.417	1.796	0.073
7R allele carriers vs. non carriers													
Dup 120bp - VNTR 48bp haplotype	14.689	1.078	0.258	4.174	$3.63e^{-5}$	0.634	0.768	0.825	0.41	0.906	0.419	2.162	0.031
L/7R carriers vs. non carriers													

region, and a VNTR 48bp in exon 3. We found evidence for nominal association between the L and 7R alleles, as well as the L-7R allelic combination and persistent ADHD. Many association studies have evaluated the role of *DRD4* in ADHD and show divergent results [Frank et al., 2004; Bellgrove et al., 2005; Bakker et al., 2005; Brookes et al., 2005; Bhaduri et al., 2006; Kieling et al., 2006; Gornik et al., 2007; Shaw et al., 2007; Johnson et al., 2008; Altink et al., 2008; Nikolaidis and Gray, 2010; Smith, 2010; Sánchez-Mora et al., 2011; Bidwell et al., 2011; Tovo-Rodrigues et al., 2012; Tovo-Rodrigues et al., 2013; Hasler et al., 2015]. In agreement with our findings, some of these studies reported an association between the L or 7R variants and childhood ADHD [Faraone et al., 2001; El-Faddagh et al., 2004; Gornick et al., 2007; Gabriela et al., 2009], which suggests common susceptibility factors for ADHD both in children and adults, and supports the continuity of the disorder across the lifespan. Other studies, however, identified opposite ADHD risk variants or found a lack of association between *DRD4* and ADHD, which indicates that the involvement of genetic variants in *DRD4* in this neuropsychiatric disorder is less straightforward than expected [Bakker et al., 2005; Sánchez-Mora et al., 2011]. In this regard, the previous meta-analysis performed by our research group highlighted the L-4R haplotype, instead of the L-7R, as a risk variant for combined ADHD [Sánchez-Mora et al., 2011]. These inconsistent results between studies might be related with the modulatory role that *DRD4* may exert in mediating the effects of life stress exposures on ADHD symptoms, with specific allele combinations being more vulnerable to environmental adversity. Additional explanations for divergent results could be the limited overlap between the two studies, the subtype-specific association observed in the previous meta-analysis, and differential LD distribution across populations.

Gene-by-environmental interactions might be particularly relevant in ADHD, where symptom severity is influenced by environmental risk factors, such as prenatal exposure to nicotine, family adversity, parental ADHD, inter-parental conflict, parenting styles or childhood maltreatment [Linnet et al., 2005; Schmitz et al., 2006; Banerjee et al., 2007; Ellis and Nigg 2009; Nigg et al., 2010; Freitag et al., 2012; Caye et al., 2013]. Therefore, we explored the potential impact of adverse life events on ADHD severity and found evidence of the effect of stressful life events, including sexual and physical abuse, emotional abuse, domestic violence, extreme family stress, financial stress and poverty or exposure to heavy metals, on higher severity of inattentive and hyperactive symptoms in a large clinical sample of adults with ADHD. We then hypothesized that the influence of stressful experiences across the lifespan on ADHD may be modulated by the genetic background. As previously described in other psychiatric conditions such as depression, the risk of ADHD after a stressful event may be higher among genetically susceptible subjects than among those at low genetic risk [Caspi et al., 2003]. Although no differences in the severity of ADHD symptoms were found between carriers and non carriers of the studied *DRD4* genetic variants, we found evidence for gene-by-environmental interactions. Our results suggest that *DRD4* may moderate the effect of adverse life events on ADHD severity, as non carriers of the L-7R haplotype who experienced stressful life events had more inattentive symptoms than those carrying this allelic combination. The protective effect of the 7R allele at *DRD4* agrees

with previous reports showing an association between this variant and lower scores of ADHD symptoms on the Conners' scales, a milder form of ADHD, and better neuropsychological function and clinical outcome [Bellgrove et al., 2005; Gornick et al., 2007; Shaw et al., 2007; Altink et al., 2008; Boonstra et al., 2008]. In addition, the 7R allele variant was previously related to the effect of adverse experience, such as stress exposure during prenatal life, or aggression in adulthood [Bakermans-Kranenburg and van Ljzendoorn, 2006; Berry et al., 2013; Dilalla et al., 2013; Buchmann et al., 2014].

Our results should be viewed in the context of some limitations. First, since statistical power is a critical issue in GxE studies, we minimized the number of comparisons performed by applying a conservative statistical approach and restricting the analysis of GxE only to genes that showed evidence of association with ADHD in the case-control study. Therefore, we cannot rule out having missed genes other than *DRD4* that contribute to mediating the effect of stressful life events on ADHD symptoms. In addition, since no corrections for multiple testing were applied, all results should be considered as nominal associations. Second, further studies in larger age-matched samples are required to elucidate the effect of age at assessment on ADHD symptomatology. However, age differences between cases and controls may not represent a major issue in the present study since, in addition to the assessment of ADHD in adulthood, DSM-IV ADHD symptoms in childhood were retrospectively excluded in the control group. Thirdly, the retrospective ascertainment of adverse life events should be considered particularly important in the interpretation of the results, since retrospective recall measures may undercount such events and, therefore, result in inaccurate reports [Moffit et al., 2010]. Further studies using prospective methods may be more representative of lifetime stressful exposure and will provide insight into *DRD4* involvement in the modulation of environmental influences.

Under the hypothesis that specific genes modulate the effect of environmental factors on ADHD and that these gene-environmental interactions may be responsible for some of the inconsistent findings in genetic association studies, other candidate genes, in addition to *DRD4*, have been evaluated in ADHD subjects by other authors. Interaction between the serotonin transporter *5HTT* and stress on ADHD severity has been described, where carriers of the short allele of the 5HTT-LPR polymorphism are more sensitive to stress and environment adversity than homozygotes for the long allele [Muller et al., 2008; Retz et al., 2008; van der Meer et al., 2014]. In addition, interactions between *MAOA* and negative parenting or *DAT1/SLC6A3* and psychosocial adversity have been identified that increase ADHD symptoms [Laucht et al., 2007; Stevens et al., 2009; Li and Lee 2012], which also supports the role of gene-by-environment interactions in the etiology of ADHD.

In summary, we have identified a tentative association between ADHD and *DRD4* and found preliminary evidence for the contribution of *DRD4* in the modulation of the effect of stressful life events on the inattentive dimension of the disorder. These results strengthen the hypothesis of gene-by-environmental interactions on the severity of ADHD, may explain discrepancies in previous genetic studies that investigate the relationship between ADHD and *DRD4*, and emphasize the need to include stressful life experiences in further genetic studies.

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