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Evaluation of common variants in 16 genes involved in the regulation of neurotransmitter release in ADHD

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STX1A

Abstract
Attention-deficit hyperactivity disorder (ADHD) is a neurobehavioral disorder characterized by inappropriate difficulties to sustain attention, control impulses and modulate activity level. Although ADHD is one of the most prevalent childhood psychiatric disorders, it also persists into adulthood in around 30–50% of the cases. Based on the effect of psychostimulants used in the pharmacological treatment of ADHD, dysfunctions in neuroplasticity mechanisms and synapses have been postulated to be involved in the pathophysiology of ADHD. With this background, we evaluated, both in childhood and adulthood ADHD, the role of several genes involved in the control of neurotransmitter release through synaptic vesicle docking, fusion and recycling processes by means of a population-based association study. We analyzed single nucleotide polymorphisms across 16 genes in a clinical sample of 950 ADHD patients (506 adults and 444

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children) and 905 controls. Single and multiple-marker analyses identified several significant associations after correcting for multiple testing with a false discovery rate (FDR) of 15%: (i) the *SYT2* gene was strongly associated with both adulthood and childhood ADHD (p=0.001, OR=1.49 (1.18–1.89) and p=0.007, OR=1.37 (1.09–1.72), respectively) and (ii) *STX1A* was found associated with ADHD only in adults (p=0.0041; OR=1.28 (1.08–1.51)). These data provide preliminary evidence for the involvement of genes that participate in the control of neurotransmitter release in the genetic predisposition to ADHD through a gene-system association study. Further follow-up studies in larger cohorts and deep-sequencing of the associated genomic regions are required to identify sequence variants directly involved in ADHD.

1. Introduction

Attention-deficit hyperactivity disorder (ADHD) is a neurobehavioral disorder characterized by difficulties to sustain attention, control impulses and modulate activity level. Although ADHD is one of the most prevalent childhood psychiatric disorders with an estimated worldwide prevalence around 7% in children (Polanczyk et al., 2007; Spencer et al., 2007), it also persists into adulthood in around 30–50% of patients with deleterious effects on educational, social and occupational outcomes as well as higher risk of developing substance abuse (Faraone et al., 2006; Kessler et al., 2006). Based on twin studies, a heritability of 77% has been estimated for ADHD (Biederman, 2005).

Based on the effect of psychostimulants used in the pharmacological treatment of ADHD, such as methylphenidate or amphetamines, dysfunctions in neuroplasticity mechanisms and synapses have been postulated to be involved in the pathophysiology of ADHD. In addition, animal models of hyperactivity have shed new light on the biological mechanisms underlying ADHD. Thus, the *Coloboma* mouse has been proposed as an animal model for ADHD since it exhibits spontaneous locomotor hyperactivity (Wilson 2000) and reductions in dopamine release in dorsal striatum, a brain region implicated in ADHD (Raber et al., 1997). This mouse model carries a ~2 cM deletion that encompasses a chromosomal region including the *Synaptosomal-Associated Protein of 25 kDa* (*Snap-25*) gene that encodes a nerve terminal protein involved in neurotransmitter release. Interestingly, replacement of the deleted *Snap-25* gene rescues the hyperactivity in the *Coloboma* mouse, which suggests that the reduction in the expression of this gene is directly involved in the hyperactivity observed in this mouse model (Bruno et al., 2007; Steffensen et al., 1999). In addition, the *Coloboma* mouse showed opposite responses to different psychostimulants. Thus, administration of amphetamine dramatically reduced the locomotor activity in a similar way as hyperkinetic children respond to psychostimulants, while methylphenidate increased locomotor activity in a dose-dependent manner in the *Coloboma* mouse (Hess et al., 1996). Because both psychostimulants act at the presynaptic terminals, these results suggest that abnormal presynaptic mechanisms that might involve Snap-25 could be responsible for the opposite effects of these two drugs. All these findings point to *SNAP-25* as a strong candidate gene in the pathophysiology of ADHD (Steffensen et al., 1999).

SNAP-25 encodes a presynaptic protein that is a core member of the SNARE complex (soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor) and participates in synaptic vesicle fusion and neurotransmitter release. The superfamily of the SNARE complex proteins are membrane-anchored proteins essential for intracellular trafficking and neurotransmitter release from vesicles into the synaptic cleft (Brookes et al., 2005) (Figure 1). The predominant neural SNARE complex is composed of three membrane-associated proteins, SNAP-25, Syntaxin 1A (STX1A) and the vesicular membrane-associated synaptobrevin (VAMP2), that form a bridge between the synaptic vesicle and the plasma membrane, driving the membrane fusion required for neurotransmitter release. There are two SNARE groups that form a complex during exocytosis: the target membrane SNARE (t-SNARE) formed by SNAP-25 and STX1A, and the vesicle membrane SNARE (v-SNARE) made of VAMP2. In addition, the SNARE core complex interacts with other proteins that mediate the fusion process, such as synaptotagmins (SYT) and complexins (CPLX), or regulate the release of synaptic vesicles, such as Munc18-1 (STXBP1), synaptophysin (SYP), syntaphilin (SNPH), NSF, αSNARE (NAPA) and Ras-associated protein (RAB3A).

Based on the *Coloboma* mouse model, association studies in ADHD have mainly focused on the *SNAP-25* gene. In this regard, two single nucleotide polymorphisms (SNPs) in *SNAP-25*, rs3746544 and rs1051312, located at the 3′UTR region involved in mRNA stability and translational efficiency, have been evaluated for their possible involvement in the susceptibility to ADHD in different cohorts, showing inconsistent results (Barr et al., 2000; Brophy et al., 2002; Choi et al., 2007; Faraone et al., 2005; Kustanovich et al., 2003; Mill et al., 2004). Interestingly, a meta-analysis identified association between ADHD and rs3746544 (OR=1.15 (1.01–1.31) p=0.028) (Forero et al., 2009) while the analysis of 61 tagSNPs covering, in terms of linkage disequilibrium (LD), a genomic region containing *SNAP-25* showed nominal association between ADHD and rs3787283, that is in strong LD with the two *SNAP-25* SNPs that have been more studied in ADHD, rs3746544 and rs1051312 (Kim et al., 2007). In addition, 10 novel variants were identified across *SNAP-25* and evidence for association with ADHD was detected for the −2015A/T SNP located in the promoter region, a microsatellite in intron 1 and the 80609G/A SNP located in intron 7 (Mill et al., 2002; Mill et al., 2004). However, these findings were not seen in other studies (Feng et al., 2005; Hess et al., 1995; Renner et al., 2008).

Apart from genetic studies focused on *SNAP-25*, Brookes et al. (2005) evaluated the involvement in ADHD of other genes encoding proteins that interact directly or indirectly with SNAP-25 in the neurotransmission release at the synapse (*STX1A*, *VAMP2*, *SYT1* and *SYP*) and found nominal

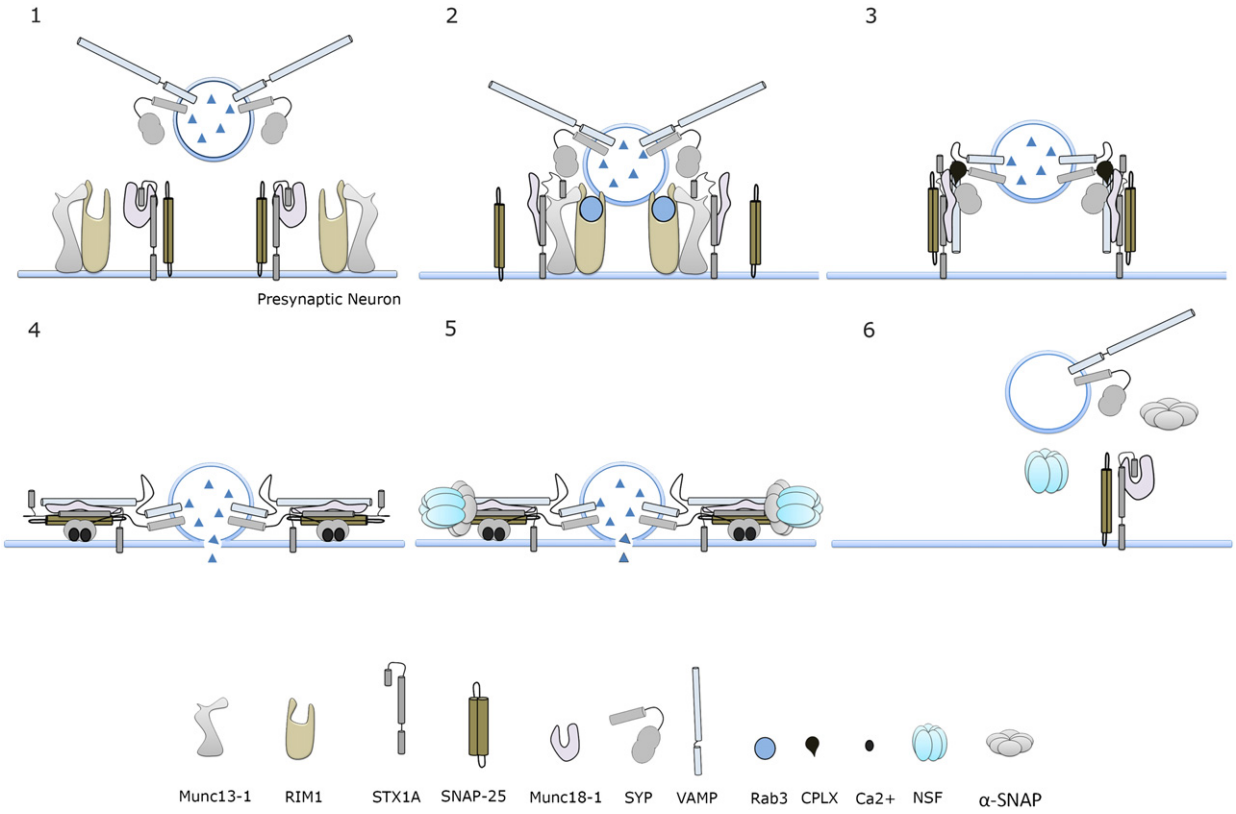

Figure 1 Schematic representation of regulatory components of the SNARE complex in the control of the neurotransmitter release through synaptic vesicle docking. (1) STX1A is in a close conformation due to its binding to Munc 18-1, anchored to the presynaptic membrane. (2) Rab3 catalyzes Munc 18-1/STX1A complex segregation that favors a STX1A open conformation and as a consequence the Rab3/RIM1/Munc13-1 complex is formed to contribute to synaptic vesicle fusion. (3) STX1A and SNAP-25 form the t-SNARE complex that interacts with the v-SNARE complex (SYP, VAMP and synaptic vesicle). Complexin acts on the regulation of synaptic vesicle release. (4) Membrane-vesicle fusion and the neurotransmitter release to the synaptic cleft is triggered by the increase of local Ca^{2+} concentration. (5 and 6) NSF and α-SNAP contribute to the disintegration of the SNARE complex with ATP hydrolysis.

association between rs2293945 in *SYP* and childhood combined ADHD (Brookes et al., 2005).

We performed a case–control association study to evaluate the potential role of 16 genes encoding proteins involved in the control of neurotransmitter release through synaptic vesicle docking, fusion and recycling processes in both childhood and adulthood ADHD. We considered 144 SNPs within genes encoding proteins involved in the neurotransmitter release machinery (SNAP-25, STX1A, VAMP1 and VAMP2), synaptic vesicle fusion (SYT1, SYT2, CPLX1, CPLX2, CPLX3 and CPLX4) and regulatory elements (STXBP1, SYP, SNPH, NSF, NAPA and RAB3A) in 506 adults and 444 children with ADHD and 905 sex-matched controls.

2. Materials and methods

2.1. Patients and controls

A total of 506 adulthood ADHD (63.6% combined, 32.6% inattentive and 3.5% hyperactive–impulsive) and 444 childhood ADHD (70.3% combined, 24.7% inattentive and 4.3% hyperactive–impulsive) patients of Caucasian origin from Spain were recruited and evaluated at Hospital Universitari Vall d' Hebron and at Hospital Universitari Mútua de Terrassa, located in the Barcelona area (Spain). All subjects met DSM-IV criteria for 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 Parts I and II). The diagnosis of ADHD in children was evaluated with the present and lifetime version of the Schedule for Affective Disorders and Schizophrenia for School-age children (K-SADS-PL). Clinical information of children and adults with ADHD is included in Supplementary Table S1. The control sample consisted of 905 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 life-time 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 30.2 years (SD=12.1) for adult patients, 9.3 years (SD=2.6) for child patients, and 39.9 years (SD=17.0) for control subjects. The study was approved by the ethics committee of each participating institution and informed consent was obtained from all subjects or parents in accordance with the Helsinki Declaration.

2.2. 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 PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, Oregon).

2.3. Gene and SNP selection

Sixteen genes involved in synaptic vesicle fusion and neurotransmitter release were selected: genes encoding proteins that integrate the neuronal core complex (*SNAP-25*, *STX1A*, *VAMP1* and *VAMP2*), fusion control elements (*SYT1*, *SYT2*, *CPLX1*, *CPLX2*, *CPLX3* and *CPLX4*) and regulatory elements that interact with SNARE complex proteins (*STXBP1*, *SYP*, *SNPH*, *NSF*, *NAPA* and *RAB3A*). SNP selection was based on genetic coverage criteria using the CEU genotype data from the HapMap database (HapMap data release 22/phase II Apr07, dbSNPb126) (Thorisson et al., 2005) for each candidate gene plus 3–5 kb flanking sequences. To avoid redundancy and warrant complete genetic coverage, we evaluated LD patterns using the Haploview software (Barrett et al., 2005) and set a maximum r^2 threshold of 0.85 for all SNPs with minor allele frequency (MAF) >0.15 in those genes with less than 20 tagSNPs or >0.25 in those genes with more than 20 tagSNPs (*SNAP25*, *SYT2*, *CPLX2* and *SNPH*). Under these conditions, a total of 141 tagSNP (72 in multi-loci bins and 69 singletons) were selected. Three additional SNPs were included in the study design: two synonymous SNPs, rs2293485 in exon 3 of *STX1A* and rs1968583 in exon 2 of *SYT2*, and rs2293945 in intron 6 of *SYP* that had been previously considered in ADHD (Brookes et al., 2005). SNPs were genotyped using the Illumina BeadXpress plataform and the GoldenGate Genotyping Assay (Illumina, San Diego, CA, USA). Prediction of functional effects of SNPs of interest was performed with the SNPinfo software (Xu and Taylor, 2009).

2.4. Statistical analyses

We first analyzed adulthood and childhood ADHD subjects independently and subsequently we combined the two datasets when a potential common susceptibility factor was identified. The minimal statistical power was estimated post hoc using the 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, α, of 0.0045 (corresponding to 15% FDR), the lowest MAF observed in our control sample (0.126) and a codominant model of inheritance. Potential genetic stratification in our sample was previously discarded (Ribases et al., 2008, 2009a,b). Due to the limited sample size, the different ADHD clinical subtypes were not considered.

Single-marker analysis: The analysis of Hardy–Weinberg equilibrium in the control sample (threshold set at $p<0.01$) and the comparison of genotype and allele frequencies between cases and controls were performed using the SNPassoc R package (Gonzalez et al., 2007). SNPs displaying nominal association under a codominant model were further evaluated using dominant and recessive models of inheritance. All tests were adjusted by gender. Genotype frequencies of SNPs in genes located on chromosome X (*SYP*) were only considered in females. For the multiple testing correction we considered a false discovery rate (FDR) of 15% using the *Q*-value R package, which corresponds to a significance threshold of $p\leq0.0045$ (Jung and Jang 2006).

Multiple-marker analysis: To minimize multiple testing and type I errors (α), the haplotype-based association study was performed in the group of age of interest considering only those genes associated with ADHD in the single-marker analyses after multiple testing corrections. For each gene, the best two-marker haplotype from all possible combinations was identified. Likewise, additional markers (up to four) were added in a stepwise manner to the initial two-SNP haplotype. Significance was estimated by a permutation procedure using 10,000 permutations with the UNPHASED software. Since the expectation-maximization algorithm implemented in the UNPHASED software does not accurately estimate low haplotype frequencies, haplotypes with low frequencies (<0.05) were excluded from the study (Fallin and Schork 2000). Estimated haplotypes were assigned to individuals with the PHASE 2.0 software (Stephens et al., 2001). The frequency of risk haplotype carriers was compared between cases and controls using a χ^2 test with the SPSS 15.0 statistical package (SPSS Inc., Chicago, USA).

3. Results

We performed an association study with 16 genes encoding proteins involved in the regulation of neurotransmitter release in 950 ADHD patients (506 adults and 444 children) and 905 unrelated controls. Of the initial 144 SNPs selected for the study, 26 were discarded for the following reasons: 19 showed genotype call rates $<90\%$, two were redundant ($r^2=1$ and $D'=1$) and five SNPs had a significant departure from Hardy–Weinberg equilibrium in the control group (Supplementary Tables S2 and S3). Thus, a total of 118 SNPs were used for the final analysis. The minimal statistical power for adulthood and childhood case–control samples were 63.2% and 58.2%, respectively.

3.1. Adulthood ADHD

When adults with ADHD were considered, nominal differences were found for nine SNPs located in five genes: *STX1A* (rs941298, rs2293485, rs3793243 and rs4363087), *CPLX1* (rs6832751), *CPLX2* (rs2114968), *CPLX4* (rs10503024 and rs640401), and *SYT1* (rs2251214; Table 1 and Figure 2). After applying a FDR of 15%, only the four SNPs in *STX1A* remained associated with adulthood ADHD (rs941298, rs2293485, rs3793243 and rs4363087; Table 1 and Figure 2) and, thus, this gene was subsequently considered for haplotype analysis in this group of patients. Multiple-marker analysis identified a three-marker haplotype in *STX1A* associated with adulthood ADHD (rs941298/rs2293485/rs4363087; Global *p*-value=0.0025; Table 2a). The analysis of the contribution of individual allelic combinations to ADHD showed over-representation of the rs941298T/rs2293485T/rs4363087C haplotype in adult patients ($p=0.0041$; OR=1.28 (1.08–1.51); Table 2b). We then considered the frequency of carriers of the rs941298T/rs2293485T/rs4363087C risk haplotype and confirmed the association between *STX1A* and ADHD in the adult dataset (54% of patients and 47.7% of controls; $p=0.026$; OR=1.28 (1.03–1.59)). These differences were not observed in children with ADHD ($p>0.05$).

3.2. Childhood ADHD

Nine SNPs located in five genes displayed nominal associations in the comparison of ADHD children and controls: *SYT2* (rs12739678, rs907697, rs9633344 and rs6427957), *SNPH* (rs3764715 and rs6134520), *CPLX1* (rs3733358), *CPLX2* (rs11134942) and *SYT1* (rs6539445; Table 1 and Figure 2). None of them were identified in the single-marker analysis of adults with ADHD and differences only remained significant for two SNPs in *SYT2*, rs907697 and rs6427957 (Table 1), after correcting for multiple testing. The analysis of multiple markers showed a four-marker haplotype in *SYT2* associated with childhood ADHD (rs12564274/rs11585565/

Table 1 Single-marker association study in 506 adult ADHD patients, 444 childhood ADHD patients and 905 sex-matched unrelated non-ADHD controls.

Gene	SNP	Genotypes													Alleles	
		Controls *N* (%)				Cases *N* (%)					Genotype 12+22 vs 11		Genotypes 22 vs 11+12		Allele2 vs Allele 1	
		11	12	22	Sum	11	12	22	Sum	*p*-Value	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value
Adulthood ADHD																
STX1A	**rs941298**[b]	460 (51.3)	361 (40.2)	76 (8.5)	897	222 (44.3)	217 (43.3)	62 (12.4)	501	0.012	1.32 (1.06-1.65)	0.012	1.52 (1.07-2.17)	0.020	1.29 (1.09-1.52)	0.003
	rs2293485[b]	288 (32.4)	439 (49.3)	163 (18.3)	890	129 (25.6)	246 (48.9)	128 (25.4)	503	0.002	1.39 (1.09-1.77)	0.008	1.52 (1.17-1.98)	0.002	1.32 (1.13-1.54)	4.2e−04
	rs3793243[b]	317 (35.1)	434 (48.0)	153 (16.9)	904	141(27.9)	248 (49.1)	116 (23.0)	505	0.003	1.39 (1.10-1.77)	0.006	1.46 (1.12-1.92)	0.006	1.31(1.12-1.53)	7.1e−4
	rs4363087[b]	401 (44.5)	395 (43.8)	105 (11.7)	901	181 (35.8)	243 (48.1)	81 (16.0)	505	0.003	1.44 (1.15-1.80)	0.001	1.45 (1.06-1.98)	0.021	1.31 (1.12-1.56)[a]	5.6e−04
CPLX1	**rs6832751**	491 (54.3)	348 (38.5)	66 (7.3)	905	300 (59.6)	173(34.4)	30 (6.0)	503	0.138	1.25 (1.0-1.56)[a]	0.050	1.19 (0.95-1.49)[a]	0.339	1.2 (1.0-1.43)	0.049
CPLX2	**rs2114968**	242 (30.1)	417 (51.9)	144 (17.9)	803	170 (36.5)	219 (47.0)	77 (16.5)	466	0.058	1.35 (1.05-1.72)[a]	0.017	1.09 (0.81-1.493)[a]	0.528	1.17 (1-1.38)[a]	0.050
CPLX4	**rs10503024**	407 (45.0)	390 (43.1)	108 (11.9)	905	254 (50.2)	204 (40.3)	48 (9.5)	506	0.118	1.23 (0.99-1.53)[a]	0.059	1.29 (0.90-1.85)[a]	0.155	1.19 (1.01-1.41)	0.036
	rs640401	580 (64.4)	275 (30.6)	45 (5)	900	355 (70.2)	131 (25.9)	20 (4.0)	506	0.088	1.30 (1.03- 1.64)[a]	0.028	1.28 (0.75-2.17)[a]	0.360	1.25 (1.02-1.54)[a]	0.027
SYT1	**rs2251214**	510 (56.5)	324 (35.9)	68 (7.5)	902	308 (60.9)	172 (34.0)	26 (5.1)	506	0.116	1.19 (0.96-1.49)[a]	0.114	1.52 (0.94-2.38)[a]	0.077	1.20 (1-1.45)[a]	0.044
Chilhood ADHD																
SYT2	**rs12739678**	459 (52.2)	372 (42.3)	49 (5.6)	880	207 (47.3)	196 (44.7)	35 (8.0)	438	0.097	1.24 (0.98-1.56)	0.070	1.46 (0.93-2.29)	0.105	1.20 (1.01-1.45)[a]	0.040
	rs907697[b]	287 (31.7)	452 (50.0)	165 (18.3)	904	105 (24.2)	229 (52.8)	100 (23.0)	434	0.008	1.45 (1.12-1.89)	0.0045	1.34 (1.01-1.78)	0.040	1.28 (1.09-1.51)	0.003
	rs9633344	337 (37.2)	446 (49.3)	122 (13.5)	905	137 (30.9)	234 (52.8)	72 (16.3)	443	0.059	1.32 (1.04-1.69)	0.022	1.24 (0.90-1.70)	0.184	1.21 (1.02-1.42)	0.025
	rs6427957[b]	267 (29.5)	450 (49.8)	187 (20.7)	904	98 (22.1)	243 (54.7)	103 (23.2)	444	0.016	1.47 (1.13-1.92)	0.004	1.15 (0.88-1.52)	0.309	1.22 (1.04-1.43)	0.017
SNPH	**rs3764715**	407 (45.1)	414 (45.8)	82 (9.1)	903	227 (51.1)	184 (41.4)	33 (7.4)	444	0.110	1.26 (1.01-1.59)[a]	0.041	1.13 (0.82-1.92)[a]	0.290	1.20 (1-1.43)[a]	0.043
	rs6134520	403 (44.5)	417 (46.1)	85 (9.4)	905	224 (50.8)	186 (42.2)	31 (7.0)	441	0.069	1.28 (0.62-0.98)[a]	0.034	1.36 (0.89-2.12)[a]	0.135	1.22 (1.03-1.46)	0.024
CPLX1	**rs3733358**	415 (48.1)	345 (40.0)	102 (11.8)	862	192 (46.6)	191 (46.4)	29 (7.0)	412	0.009	1.06 (0.84-1.35)	0.609	1.78 (1.14-2.70)[a]	0.006	1.07 (0.90-1.30)[a]	0.403
CPLX2	**rs11134942**	317 (35.7)	428 (48.3)	142 (16.0)	887	182 (43.1)	173 (41.0)	67 (15.9)	422	0.019	1.37 (1.08-1.75)[a]	0.008	1.0 (0.73-1.38)	0.992	1.17 (0.99-1.39)	0.062
SYT1	**rs6539445**	671 (75.6)	206 (23.2)	10 (1.1)	887	309 (69.8)	120 (27.1)	14 (3.2)	443	0.008	1.35 (1.05-1.75)	0.020	2.89 (1.27-6.57)	0.010	1.37 (1.10-1.72)[a]	0.005

[a]When OR <1, inverted score is shown.
[b]FDR=0.15 ($p \leq 0.0045$).

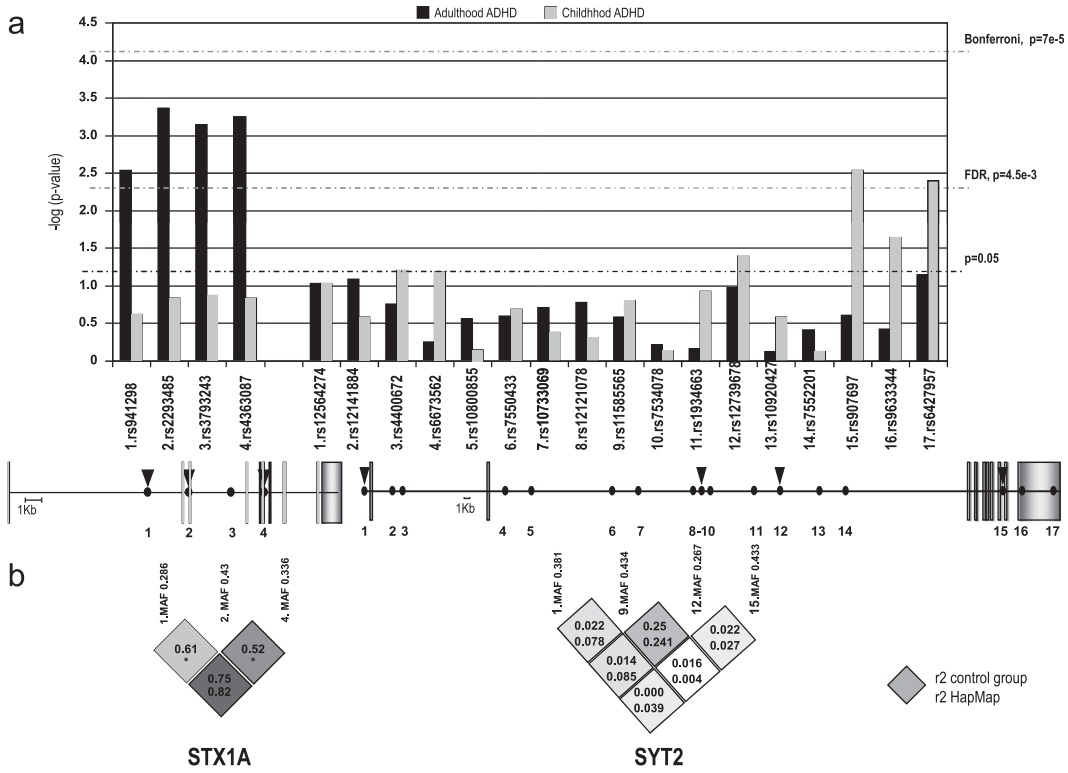

Figure 2 (a) Lowest level of significance, as a −log(*p*-value) found either in the comparison of genotypes under a codominant model or in allele frequencies, of individual SNPs within *STX1A* and *SYT2* genes in 506 adult ADHD patients (in black), 444 child ADHD patients (in gray) and 905 sex-matched unrelated controls. (b) Structure of the human genes *STX1A* (NM_004603; chr7: 72,751,477–72,771,924; HapMap, release 22) and *SYT2* (NM_177402; chr1: 200,831,511–200,946,168; HapMap, release 22) with the relative position of the SNPs considered in the case–control association study and the LD patterns of SNPs found associated with ADHD in the different datasets.

Table 2 (a) Haplotype analysis of four *STX1A* SNPs in a clinical sample of 506 adult ADHD patients and 905 control subjects using the UNPHASED software. (b) Haplotype distribution of the rs941298, rs2293485, rs3793243 and rs4363087 *STX1A* SNPs.

(a) *STX1A*—adult ADHD

Marker haplotype	Global *p*-value	Best haplotype-specific *p*-value (adjusted *p*-value)	Haplotype-specific OR (CI)
1 2	0.0010	2.0e−04 (0.0059)	1.26 (1.07–1.50)
1 2 4	**0.0025**	**1.9e−04 (7.9e-04)**	**1.28 (1.08-1.51)**
1 2 3 4	0.0053	4.8e−04 (0.0017)	1.26 (1.05–1.50)

(b) *STX1A*—adult ADHD

Marker haplotype	Cases (%)	Controls (%)	Haplotype-specific *p*-value; OR (CI)
1 2 4			
C-T-C	59 (6.13)	84 (4.9)	-
T-T-C	329 (34.20)	493 (28.9)	0.0041; 1.28 (1.08–1.51)
C-C-T	472 (49.06)	966 (56.6)	1.9e-04; 1.36 (1.16–1.58)[a]
C-T-T	102 (10.60)	165 (9.7)	-

1: rs941298, 2: rs2293485, 3: rs3793243 and 4: rs4363087.
[a]When OR<1, inverted score is shown.

rs12739678/rs907697; global *p*-value=0.0012; Table 3a), with over-representation of two different allelic combinations in this group of patients: rs12564274C/rs11585565G/rs12739678A/rs907697T (*p*=0.0064; OR=1.46 (1.09–1.96)) and rs12564274G/rs11585565A/rs12739678G/rs907697T (*p*=0.0071; OR=1.33 (1.02–1.73); Table 3b). We then considered the frequency of carriers of at least one of the risk haplotypes identified and confirmed the association between *SYT2* and ADHD in children (41.2% of patients and 31.9% of controls; *p*=0.001; OR=1.49 (1.18–1.89), Table 4).

Subsequently, we evaluated in the adulthood dataset the contribution to ADHD of the *SYT2* haplotype identified in

Table 3 (a) Haplotype analysis of four *SYT2* SNPs in a clinical sample of 444 children ADHD patients and 905 control subjects using the UNPHASED software. (b) Haplotype distribution of the rs12564274, rs11585565, rs12739678 and rs907697 *SYT2* SNPs.

(a) *SYT2*—children ADHD

Marker haplotype	Global *p*-value	Best haplotype-specific *p*-value (adjusted *p*-value)	Haplotype-specific OR (CI)
12 15	0.0012	8.47 e-5 (4.0e-04)	1.38 (1.04-1.80)
9 12 15	8.7e−04	6.87e−5 (9.0e−04)	1.41 (1.07-1.84)
1 9 12 15	**0.0012**	**8.15e−5 (0.0028)**	**1.46 (1.09-1.96)**

(b) *SYT2*—children ADHD

Marker haplotype	Cases (%)	Controls (%)	Haplotype-specific *p*-value; OR (CI)
1 9 12 15			
C-G-A-C	93 (11.36)	220 (12.88)	-
G-G-A-C	62 (7.57)	100 (5.85)	-
C-A-G-C	60 (7.33)	172 (10.07)	0.0098; 1.43 (1.04-1.92)[a]
G-A-G-C	95 (11.60)	183 (10.71)	-
C-G-G-C	64 (7.81)	206 (12.06)	8.15e−5; 1.61 (1.20-2.17)[a]
G-G-G-C	41 (5.01)	91 (5.33)	-
C-G-A-T	85 (10.38)	122 (7.14)	0.0065; 1.46 (1.09-1.96)
C-A-G-T	124 (15.14)	231 (13.52)	-
G-A-G-T	104 (12.70)	168 (9.84)	0.0071; 1.33 (1.02-1.73)
C-G-G-T	58 (7.08)	117 (6.85)	-
G-G-G-T	33 (4.03)	98 (5.74)	-

1: rs12564274, 9: rs11585565, 12: rs12739678 and 15: rs907697.
[a]When OR<1, inverted score is shown.

Table 4 Distribution of *SYT2* haplotype carriers (rs12564274C/rs11585565G/rs12739678A/rs907697T or rs12564274G/rs11585565A/rs12739678G/rs907697T) in 444 children and 506 adults with ADHD and 905 controls.

SYT2 Marker haplotype	Childhood ADHD			Adulthood ADHD			Chidlhood+adulthood ADHD		
	Cases (%)	Controls (%)	*p*-Value; OR (CI)	Cases (%)	Controls (%)	*p*-Value; OR (CI)	Cases (%)	Controls (%)	*p*-Value; OR (CI)
1 9 12 15									
C-G-A-T or G-A-G-T	183 (41.2)	289 (31.9)	0.001;1.49 (1.18-1.89)	198 (39.1)	289 (31.9)	0.007; 1.37 (1.09-1.72)	381 (40.1)	289 (31.9)	2.8e−4; 1.42 (1.18-1.72)
Others	261 (58.8)	616 (68.1)		308 (60.9)	616 (68.1)		569 (59.9)	616 (68.1)	

1: rs12564274, 9: rs11585565, 12: rs12739678 and 15: rs907697.

children and confirmed an increased frequency of carriers of one of the two *SYT2* risk allelic combinations in adults with ADHD (39.1% of patients and 31.9% of controls; p=0.007; OR=1.37 (1.09-1.72); Table 4). Consistently with these results, the joint analysis of children and adults with ADHD showed over-representation of *SYT2* risk haplotypes carriers in the overall clinical group (40.1% of patients and 31.9% of controls; p=2.8e−04; OR=1.42 (1.18-1.72); Table 4).

4. Discussion

The purpose of the present study was to examine the relationship between the SNARE complex and ADHD in two patients' cohorts, children and adults, through a case-control association study. This study design allowed identification of genetic risk factors potentially involved in the persistence of ADHD across lifespan. In this regard, we found association between ADHD and the *STX1A* and *SYT2* genes but, whereas *STX1A* is associated only with adult ADHD, *SYT2* showed association both in adults and in children.

The strong association between SYT2 and both childhood and adulthood ADHD supports the diagnostic continuity of ADHD throughout lifespan and the existence of common susceptibility factors involved in ADHD in children and adults. On the other hand, the association between *STX1A* and adult ADHD only, suggests, as previously described, the existence of age-specific risk factors (Ribases et al., 2008, 2009a,b). In this regard, longitudinal studies of patients diagnosed during childhood would allow discerning between

remitting and persistent ADHD subjects and may provide new insights into the participation of these genes in the persistence of the disorder.

STX1A is essential in the fusion of synaptic vesicles with the presynaptic membrane needed for neurotransmitter release to the extracellular space (Figure 1). In addition, STX1A interacts with the serotonin, dopamine and norepinephrin transporters, regulating their subcellular localization and expression (Arien et al., 2003; Condliffe et al., 2004; Dipace et al., 2007; Haase et al., 2001; Lee et al., 2004; Quick, 2006). Interestingly, STX1A directly interacts with the dopamine transporter (DAT) amino-terminus region and regulates the DAT-mediated amphetamine-induced efflux (Binda et al., 2008), which suggests that altered STX1A function may modulate the activity of neurotransmitter systems previously associated with the pathology of ADHD (Faraone and Khan, 2006). To date, only three studies have evaluated *STX1A* in ADHD, all of them in children, but only one identified nominal association between SNP rs1569061 and this psychiatric disorder (Brookes et al., 2005, 2006; Guan et al., 2009). However, this SNP was not considered in the present study and is in weak LD with those conforming the identified ADHD risk haplotype ($r^2 < 0.05$). In addition to ADHD, *STX1A* has been associated with other neurological or psychiatric disorders such as migraine, schizophrenia or autism (Corominas et al., 2009; Nakamura et al., 2008; Wong et al., 2004).

On the other hand, to our knowledge this is the first association study that evaluates the role of the *SYT2* gene in ADHD. SYT2 is an essential component of the calcium-triggering machinery for neurotransmitter release and its alteration enhances the rate of spontaneous synaptic vesicle exocytosis (Pang et al., 2006) (Figure 1). Interestingly, STY2 has similar functions as SYT1, which has been nominally associated with both combined and inattentive ADHD in a child dataset (Guan et al., 2009).

Previous association studies in ADHD that considered genes encoding proteins of the SNARE complex have mainly focused on *SNAP-25*, since this gene is included in the chromosomal region deleted in the hyperactive *Coloboma* mouse model. Although nominal associations between childhood ADHD and *SNAP-25*, mainly with rs3746544 and rs1051312, have been documented in previous studies, rs1051312 was not considered in our association analysis while the rs3746544 SNP was included in the rs4813925 tagSNP block that did not showed positive results in the present study (Barr et al., 2000; Brookes et al., 2006; Brophy et al., 2002; Feng et al., 2005; Forero et al., 2009; Guan et al., 2009; Kustanovich et al., 2003; Mill et al., 2004; Zhang et al., 2011). In addition to *SNAP-25*, other groups investigated the participation of polymorphisms within genes involved in the vesicular release of neurotransmitters at the synapse (*STX1A*, *VAMP2*, *SYT1* and *SYP*) (Brookes et al., 2005, 2006). Although in LD with other polymorphisms analyzed in the present study, most of the previously investigated SNPs were not considered here; in our SNP selection we prioritized systematic genetic coverage rather than forcing inclusion of genetic variants described in previous association studies.

The present case–control association study raises several methodological considerations. First, as strengths of the present work, cases and controls, recruited from the same restricted geographical area around Barcelona (Spain), were previously analyzed for potential confounding population stratification by genotyping a set of 45 non-linked anonymous SNPs (Ribases et al., 2008, 2009a,b). In addition, all tagSNPs considered in *STX1A* showed association with ADHD after correction for multiple testing (FDR 15%), which points at this gene as a strong candidate for replication efforts and further exploration in other cohorts. Even so, it is worth to mention that under the more conservative Bonferroni correction, taking into account 118 SNPs and both adult and children samples, none of the SNPs analyzed remained associated with ADHD. Finally, and contrary to all previous studies investigating the contribution of the SNARE complex or related proteins to ADHD, which considered only children cases, our study design includes both adult and childhood samples, which allowed us to test the possible participation of these genes in the persistence of the disorder.

On the other hand, our study has several limitations: the modest sample size (506 adults and 444 children with ADHD and 905 controls) may have prevented detection of susceptibility loci with low effect. Since statistical power decreased even further when patients were subdivided into clinical subtypes, the different ADHD subtypes were not considered separately in our analysis. In addition, although the study design pursued a full genetic coverage in terms of LD, the MAF threshold was set at 0.15 which may underestimate the contribution of less common sequence variants to the genetic susceptibility to ADHD. Finally, using a systematic approach to minimize multiple testing, only two of the 16 genes initially selected were further considered for haplotype analysis. In consequence, we cannot rule out that additional allelic combinations within other genes of the SNARE complex showing nominal association with ADHD in the single-marker analysis do contribute to the disease susceptibility.

Since we did not prioritize putative functional relevance in the SNP selection, most of the sequence variants within *STX1A* and *SYT2* associated with ADHD are located within introns. Only rs2293485 is a synonymous SNP located in exon 3 of *STX1A* (p.D68D) and lies within a putative exonic splice site enhancer (ESE) predicted to bind SRp55 ($p = 3.01$) and SFASF2 ($p = 2.29$; Xu and Taylor, 2009). These results suggest that the identified risk haplotypes may not have functional consequences by themselves, but are in LD with other yet unknown susceptibility variants that are directly involved in the genetic vulnerability to the disorder.

In conclusion, this study provides for the first time preliminary evidence for the involvement of *STX1A* and *SYT2* in adulthood ADHD through a comprehensive gene-system association study. Further follow-up studies in larger cohorts and deep-sequencing of the associated genomic regions are required to identify sequence variants directly involved in ADHD and to provide novel insights into the etiology of this psychiatric disorder.

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design, collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Contributors

Cristina Sánchez-Mora participated in the DNA isolation and genotyping assay design, undertook the statistical analyses and wrote the first draft of the manuscript.

Josep Antoni Ramos-Quiroga, Amaia Hervás, Rosa Bosch and Miquel Casas participated in the study design, clinical assessment and coordination of the clinical research.

Glòria Palomar, Mariana Nogueira, Núria Gómez-Barros, Vanesa Richarte, Montse Corrales, Silvina Guijarro and Aitana Bigorra participated in the clinical assessment and in the recruitment of patients.

Roser Corominas and Iris Garcia-Martinez participated in the genotyping assay and the statistical analyses.

Bru Cormand, Mònica Bayés and Marta Ribasés wrote the protocol, coordinated the genetic study design and statistical analysis and supervised the manuscript preparation.

All authors contributed to and have approved the final manuscript.

Conflict of interests

Any authors have conflict of interests or relevant financial interests or personal affiliations in connection with the content of this manuscript.

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Appendix A. Supporting information

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