



## ORIGINAL INVESTIGATION

# Candidate pathway association study in cocaine dependence: The control of neurotransmitter release

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### Abstract

**Objectives.** Cocaine is the second most used illegal drug in Europe. The transition from use to dependence involves both genetic and environmental factors. Genetic variation in neurotransmitter systems is involved in the susceptibility to cocaine dependence. We examined the possible contribution to cocaine dependence of 16 genes involved in the cellular machinery that controls neurotransmitter release: genes encoding proteins of the SNARE complex (*STX1A*, *SNAP25*, *VAMP1* and *VAMP2*), fusion control elements (*SYT1*, *SYT2*, *CPLX1*, *CPLX2*, *CPLX3* and *CPLX4*) and regulatory elements (*STXBPI*, *SYP*, *SNPH*, *NSF*, *NAPA* and *RAB3A*). **Methods.** We genotyped 121 SNPs, selected according to genetic coverage criteria, in 360 cocaine-dependent patients and 360 controls from Spain. **Results.** Single and multiple-marker analyses revealed a strong association between cocaine dependence and the *NSF* gene, encoding the *N*-ethylmaleimide-sensitive factor ( $P = 5.1 \times 10^{-4}$ , OR = 2.44 (1.45–4.00) and  $P = 0.001$ , OR = 1.82 (1.28–2.59), respectively). The presence and absence of psychotic symptoms were also studied. Interestingly, when we considered the time between initial consumption and the onset of cocaine dependence, we observed that the association was mainly restricted to the group of patients that rapidly developed drug dependence ( $\leq 2$  years;  $P = 2.98 \times 10^{-6}$ , OR = 1.33 (1.20–1.47)). **Conclusions.** Our data show preliminary evidence that *NSF* may predispose not only to cocaine dependence, but also to an early onset of the dependence.

**Key words:** Cocaine dependence, *NSF*, SNARE complex, case-control association study, synaptic exocytosis

### Introduction

Cocaine is the second most used illegal drug in Europe, with around 13 million consumers (3.9% of adult Europeans) (EMCDDA Annual Report 2009; European Monitoring Centre for Drugs and Drug Addiction). Cocaine is a powerful addictive drug with almost 16% of cocaine users developing cocaine dependence within 10 years after the first cocaine use (Wagner and Anthony 2002), and 5–6% within the first 2 years (O'Brien and Anthony 2005). The transition from use to dependence is not a fixed pharmacological property of cocaine, since both

environmental and genetic factors influence cocaine dependence. Heritability studies have estimated that 60–70% of an individual's risk for developing cocaine dependence is due to genetic factors (Kendler and Prescott 1998; Tsuang et al. 1998; Kendler et al. 2000), although the underlying genetic susceptibility factors are poorly understood.

Cocaine binds to the dopamine, serotonin and norepinephrine transporters (DAT1, SERT and NET, respectively), inhibiting the reuptake of these neurotransmitters and increasing their levels at the neuronal synapses (Kuhar et al. 1991; Kalivas 2007).

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Interestingly, the dopamine (DA) neurotransmission and the indirect activation of DA receptors have been established as central mediators of cocaine response (Woolverton and Johnson 1992; Volkow et al. 1996, 1999, 2002). Other neurotransmitters such as serotonin or glutamate also play an important role in cocaine effects (Spealman 1993; Walsh and Cunningham 1997; Filip 2005; Filip et al. 2001, 2004). Thus, cocaine indirectly influences glutamate transmission in the limbic system producing persistent changes in neuronal function that alter the behavioral effects of cocaine (Gass and Olive 2008; Kalivas and O'Brien 2008; Thomas et al. 2008; Uys and LaLumiere 2008).

In addition, animal models and also pharmacological and association studies support an essential role of these neurotransmitter systems in cocaine dependence. The rewarding effects of cocaine were abolished in homozygous *DAT1(-/-)SERT(-/-)* and heterozygous *DAT1(-/-)SERT(+/-)* double knock-out mice (Sora et al. 2001; Hall et al. 2002, 2004; Uhl et al. 2002). Interestingly, *NET(-/-)*, *SERT(-/-)* and *NET(-/-)SERT(-/-)* knock-outs showed an even increased rewarding cocaine effect (Sora et al. 1998; Xu et al. 2000; Hall et al. 2002). Other knock-out studies also revealed an important role of the endocannabinoid system in cocaine self-administration and in the consolidation of the psychostimulant addictive process (Soria et al. 2005).

Pharmacological studies also provide insights into the role of neurotransmitter systems in cocaine dependence. Dopamine receptor D3 antagonists block cocaine acquisition and place preference and reduce cocaine induced reinstatement of self-administration (Vorel et al. 2002; Di Ciano et al. 2003). Glutamate agonists reduce the euphoric effects of cocaine and withdrawal symptoms (Dackis and O'Brien 2003; Dackis et al. 2003; Malcolm et al. 2006; Hart et al. 2008). Cannabinoid receptor agonists attenuate relapse induced by environmental cocaine-associated cues or cocaine re-exposure and antagonists induce relapse to cocaine seeking after a prolonged withdrawal period (DeVries et al. 2001; DeVries and Schoffelmeer 2005). In addition, some promising medications that may prevent cocaine relapse (such as gamma-vinyl GABA "GVG", tiagabine and topiramate) are related to GABA neurotransmission (Dewey et al. 1997; Morgan and Dewey 1998; Cornish and Kalivas 2000; Gonzalez et al. 2003).

Finally, positive associations have been described between cocaine dependence and polymorphisms in genes of the dopaminergic (Noble et al. 1993; Comings et al. 1999; Ballon et al. 2007; Guindalini et al. 2006; Fernández-Castillo et al. 2010), serotonergic (Patkar et al. 2001; Mannelli et al. 2005), noradrenergic (Cubells et al. 2000; Guindalini et al. 2008),

endocannabinoid (Ballon et al. 2006; Zuo et al. 2009) and cholinergic neurotransmitter systems (Gruza et al. 2008).

All these neurotransmitter systems are candidates for being involved in cocaine dependence and depend on mechanisms that control neurotransmitter release at the synapse, including synaptic vesicle docking, fusion and recycling. The process is complex and involves different proteins such as the *N*-ethylmaleimide sensitive factor (NSF), the soluble NSF attachment proteins (SNAPs), the SNAP receptors (SNAREs), synaptobrevins (VAMP1, VAMP2), syntaxin-1 and SNAP-25, the SM protein Munc18-1 (STXBP) and small GTPases from the RAB3 family (Rizo and Rosenmund 2008). Interestingly, cocaine induces expression changes of some genes encoding proteins involved in this neurotransmitter release machinery, such as synaptotagmin, synaptobrevin (VAMP1), syntaxin-1, synaptophysin and RAB3A (Freeman et al. 2002; Yuferov et al. 2003; Ahmed et al. 2005).

Based on previous data that link different neurotransmission systems with cocaine dependence, we hypothesized that alterations in the neurotransmitter release machinery may be involved in the genetic susceptibility to this disorder, as well as cocaine induced psychotic symptoms and time between initial consumption and the onset of cocaine dependence. We performed a case-control association study in 360 cocaine-dependent patients and 360 sex-matched controls, with SNPs covering 16 candidate genes that encode proteins of the neurotransmitter release machinery: SNARE complex formed by syntaxin 1A, SNAP-25 and synaptobrevins (*STX1A*, *SNAP25*, *VAMP1* and *VAMP2*), the fusion control elements synaptotagmins and complexins (*SYT1*, *SYT2*, *CPLX1*, *CPLX2*, *CPLX3* and *CPLX4*) and the regulatory elements Munc18.1 (*STXBP1*), synaptophysin (*SYP*), syntaxin-1 (*SNPH*), NSF,  $\alpha$ SNARE (*NAPA*) and *RAB3A*.

## Methods and materials

### Subjects

The patient sample consisted of 360 cocaine dependent patients (mean age  $34.6 \pm 7.6$  years and 83% males ( $n = 299$ )) recruited and evaluated at the Psychiatry Department of the Hospital Universitari Vall d'Hebron (Barcelona, Spain) according to DSM-IV TR criteria (Diagnostic and Statistical Manual of Mental Disorders, 4th ed., text revision). The Structured Clinical Interview (SCID) (First et al. 1997) was administered and volunteers with current DSM-IV diagnosis of cocaine dependence were included in the study. Other drug dependences were assessed in 334 patients (92.8%): alcohol dependence was present in 22.7% of the patients ( $n = 76$ ), cannabis

dependence in 26% ( $n = 87$ ), opiate dependence in 13.8% ( $n = 46$ ), benzodiazepine dependence in 5.1% ( $n = 17$ ), and amphetamine or methamphetamine dependence in 2.1% ( $n = 7$ ). Seventy-six percent of the patients were evaluated for the presence ( $n = 149$ ) or absence ( $n = 124$ ) of psychotic symptoms, and 71.4% ( $n = 257$ ) reported age at the initial consumption as well as age at dependence onset. Three hundred and sixty sex-matched unrelated controls (mean age  $54.9 \pm 16.6$  years) were recruited at the Blood and Tissues Bank of the Hospital Universitari Vall d'Hebron. None of them had injected drugs intravenously. Both patients and controls were Spanish and Caucasian. The study was approved by the Ethics Committee of Hospital Universitari Vall d'Hebron, and written informed consent was obtained from all the participating individuals.

#### DNA isolation and quantification

Genomic DNA samples were obtained either from peripheral blood lymphocytes by the salting-out procedure (Miller et al. 1988) or from saliva using the Oragene DNA Self-Collection Kit (DNA Genotek, Kanata, Ontario, Canada). The double-stranded concentrations of all samples were determined on a Gemini XPS fluorometer (Molecular Devices, Sunnyvale, CA, USA) using the PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, OR, USA), following the manufacturer's instructions.

#### Gene and SNP selection

Sixteen candidate genes involved in the synaptic vesicle fusion and neurotransmitter release at the synapse were selected for this study: *STX1A*, *SNAP25*, *VAMP1*, *VAMP2* (SNARE complex), *SYT1*, *SYT2*, *CPLX1*, *CPLX2*, *CPLX3* and *CPLX4* (synaptotagmins and complexins) and *STXBP1*, *SYP*, *SNPH*, *NSF*, *NAPA* and *RAB3A* (regulatory elements). SNP selection was based on genetic coverage parameters in terms of linkage disequilibrium (LD). Genotyping data of CEU population for each candidate gene plus 5-kb flanking sequences upstream and downstream were downloaded from the HapMap database (HapMap data release 22/phase II Apr07, dbSNPb126) (Thorisson et al. 2005). To minimize redundancy, LD was evaluated using the Haploview software (Barrett et al., 2005) setting a maximum  $r^2$  threshold at 0.85 for all SNPs with minor allele frequency (MAF) of 0.15 or 0.25 for those genes with more than 20 tagSNPs (*SNAP25*, *SYT2*, *CPLX2*, *SNPH*). A total of 141 tagSNP (72 in multi-loci bins and 69 singletons) were selected with these criteria. Three additional SNPs were included: rs2293485 in exon 3

of *STX1A*, rs1968583 in exon 2 of *SYT2* (both exonic and synonymous) and rs2293945 in intron 6 of *SYP* (previously studied in ADHD (Brookes et al. 2005)).

#### Plex design, genotyping and quality control

From the initial selection of 145 SNPs, a VeraCode assay of 141 SNPs was designed (four SNPs did not pass the pipeline). SNPs were genotyped using the Illumina BeadXpress platform and the GoldenGate Genotyping Assay (Illumina, San Diego, CA, USA). This technology is based on allele-specific primer extension and highly multiplexed PCR with universal primers. Raw hybridization intensity data processing, clustering and genotype calling were performed using the genotyping module in the Illumina GenomeStudio package. The genotype cluster plots generated by GenomeStudio were visually inspected for quality of calls and edited when necessary. A total of 21 HapMap individuals including 7 trios were genotyped and used to help in the clustering and as a control of the genotyping process.

#### Statistical analysis

The minimal statistical power was estimated *post hoc* using the Genetic Power Calculator software (<http://pngu.mgh.harvard.edu/~purcell/gpc/cc2.html>), assuming an odds ratio (OR) of 1.5, prevalence of 0.0062 (16% developing dependence of 3.9% consumers), significance level of 0.05 and the lowest MAF value of 0.126. The analysis of Hardy-Weinberg equilibrium (threshold set at  $P < 0.01$ ) and the comparison of genotype frequencies between cases and controls was performed using the SNPassoc R package (Gonzalez et al. 2007). Only when a SNP displayed nominal association under a codominant model, the dominant (11 vs. 12+22) and recessive (11+12 vs. 22) were considered. Genotype frequencies of SNPs within the genes located on chromosome X (*SYP*) were only considered in the female sample. For the multiple testing correction we used the Q-value R package (Storey 2002), considering all the tests performed and assuming a false discovery rate (FDR) of 10%, which corresponded to a significance threshold of  $P \leq 5.1e-04$ . Additionally, we also corrected the significant  $P$  values that overcame the 10% FDR threshold by performing a permutation test using 10,000 permutations with the PLINK software (Purcell 2007). Significant  $P$  values after multiple testing corrections were adjusted for age. The haplotype-based association study was restricted to the single gene that was found associated with cocaine dependence in the single-marker analysis

after correction for multiple comparisons. The best two-marker haplotype from all possible combinations was identified in the whole sample and additional markers (up to four) were added to the initial two-SNP haplotype in a stepwise manner. Significance was estimated by a permutation procedure using 10,000 permutations with the UNPHASED software (Dudbridge 2003). Haplotypes with frequencies <0.05 were excluded. Specific estimated haplotypes were assigned to individuals with the PHASE 2.0 software (Stephens et al. 2001). The comparison of the risk haplotype carriers in cases and controls as well as the effect of this risk haplotype in the presence of cocaine-induced psychotic symptoms, age at initial consumption and the time between initial and regular consumption were evaluated using the statistical package SPSS 15.0 (SPSS Inc., Chicago, IL, USA). For the age at the first consumption and the lapse between initial consumption and the onset of cocaine dependence, normality was rejected using a Kolmogorov–Smirnov test and the comparison of medians was performed using the non-parametric Mann–Whitney *U*-test. Additionally, time between initial consumption and onset of cocaine dependence was dichotomized into early ( $\leq 2$  years) or late ( $> 2$  years) dependence onset and the comparison of the risk haplotype carriers between the two groups was performed with two-tailed Fisher's exact test. In the multiple-marker analysis *P* values were also adjusted for age and, considering cocaine dependence as well as related phenotypes, the significance threshold was set at  $2P < 0.01$  after the multiple testing correction of Bonferroni considering five comparisons (cocaine dependence, presence or absence of psychotic symptoms, and early or late dependence versus controls).

**Results**

We examined tagSNPs in 16 candidate genes encoding proteins of the neurotransmitter release machinery in 360 cocaine dependent patients and 360 controls. Of the 145 SNPs initially selected, 23 were discarded (four did not pass the Veracode pipeline design, 19 had genotype calls <90% and one had a significant departure from Hardy–Weinberg equilibrium in the control group). A total of 121 SNPs within 15 genes (the two SNPs of the *NAPA* gene failed) with an average call rate of 99.2% (SD = 1.8) were considered for the analysis (Table SI). The minimal statistical power, considering the SNP with the lowest MAF (0.126), was 57.9% assuming a codominant model, 68.3% considering a dominant model and 8.4% under a recessive model of inheritance.

Table I. Single-marker analysis: nominal associations identified in 360 cocaine dependent patients and 360 controls.

Gene	SNP	Cases N (%)						Controls N (%)						Genotypes															
		11		12		22		Sum		11		12		22		Sum		P		OR (95% CI)		P							
		11	12	11	12	22	6.4	23	44.2	159	44.2	178	49.4	164	45.6	145	40.3	51	14.2	360	2.4e-03	–	–	NS	2.44	(1.45–4)	5.1e-04*		
<i>NSF</i>	rs183211*	178	(49.4)	159	(44.2)	23	(6.4)	360	360	164	(45.6)	145	(40.3)	51	(14.2)	360	2.4e-03	–	–	NS	2.44	(1.45–4)	5.1e-04*	–	–	NS	–	–	NS
<i>SYT1</i>	rs10861941	145	(42.9)	160	(47.3)	33	(9.8)	338	338	105	(32.9)	171	(53.6)	43	(13.5)	319	0.023	1.54	(1.11–2.08)	8.3e-03	1.54	(1.11–2.08)	8.3e-03	–	–	NS	–	–	NS
<i>SYT2</i>	rs10800855	162	(45.0)	141	(39.2)	57	(15.8)	360	360	124	(34.6)	175	(48.9)	59	(16.5)	358	0.012	1.54	(1.14–2.08)	4.5e-03	1.54	(1.14–2.08)	4.5e-03	–	–	NS	–	–	NS
	rs4400672	167	(46.4)	147	(40.8)	46	(12.8)	360	360	161	(45.0)	169	(47.2)	28	(7.8)	358	0.048	–	–	NS	1.73	(1.05–2.83)*	0.028	–	–	NS	–	–	NS
<i>CPLX1</i>	rs11722977	133	(38.0)	181	(51.7)	36	(10.3)	350	350	163	(48.8)	133	(39.8)	38	(11.4)	334	6.4e-03	1.56	(1.15–2.11)*	4.3e-03	1.56	(1.15–2.11)*	4.3e-03	–	–	NS	–	–	NS
<i>CPLX2</i>	rs4868539	133	(36.9)	182	(50.6)	45	(12.5)	360	360	140	(38.9)	153	(42.5)	67	(18.6)	360	0.029	–	–	NS	1.61	(1.06–2.44)	0.023	–	–	NS	–	–	NS
<i>CPLX4</i>	rs1914321	279	(77.9)	78	(21.8)	1	(0.3)	358	358	271	(77.0)	72	(20.5)	9	(2.6)	352	0.022	–	–	NS	9.09	(1.17–100)*	5.9e-03	–	–	NS	–	–	NS
	rs640401	245	(68.1)	107	(29.7)	8	(2.2)	360	360	233	(64.7)	106	(29.4)	21	(5.8)	360	0.042	–	–	NS	2.7	(1.19–6.25)	0.012	–	–	NS	–	–	NS

NS, not significant.

\*When odds ratio <1, the inverted score is shown.

&Statistically significant *P* value after applying a false discovery rate of 10% ( $P < 5.1e-04$ ), *P* value adjusted for age =  $7e-04$ , *P* value corrected by a permutation test = 0.00224).



The comparison of genotype frequencies between cocaine dependent patients and controls allowed identification of nominal differences for eight SNPs located in six genes: *NSF*, *SYT1*, *SYT2*, *CPLX1*, *CPLX2* and *CPLX4* (Table I, Table SII). However, after correcting for multiple comparisons applying a FDR of 10% ( $P \leq 5.1e-04$ ), only rs183211 in the *NSF* gene ( $P = 5.1e-04$ , OR = 2.44 (1.45–4.00)) remained associated with cocaine dependence, with a higher frequency of carriers of the common G allele in cases (93.6%) than in the control group (85.9%). Consistently, the G allele is present in 71.5% of cases and in 65.7% of controls. The rs183211 SNP in *NSF* remained significantly associated with cocaine dependence after adjusting for age and correcting by permutation (Table I).

The analysis of all the possible SNP combinations within *NSF* revealed a two-marker haplotype (rs183211–rs17698176), that includes the SNP identified in the single-marker analysis, associated with cocaine dependence (global  $P = 0.031$ ; Figure 1), which remained significant after correcting by permutation ( $P$ -adjusted = 0.039). The evaluation of the contribution of individual haplotypes to the phenotype showed an over-representation of the G-T allelic combination ( $P = 0.013$ , OR = 1.3 (1.06–1.60)) and an under-representation of the A-T haplotype ( $P = 0.017$ , OR = 1.3 (1.05–1.64)) in the cocaine dependence sample (Table IIb). Consistently, we also identified an increased frequency of individuals carrying the G-T risk haplotype in this clinical sample, result that remained significant after adjusting by age ( $P$ -adjusted = 0.001, OR = 2.16 (1.38–3.83; Table III).

When patients were subdivided based on the presence/absence of psychotic symptoms, we did not identify differences between these two subgroups ( $P = 0.627$ ) and observed an over-representation of G-T carriers in the two clinical samples when they were compared to the control sample (patients with psychotic symptoms:  $P$ -adjusted = 0.002, OR = 2.51 (1.4–4.5); patients without psychotic symptoms:  $P$ -adjusted = 0.0055, OR = 2.3 (1.28–4.14); Table III), and remained significant after the Bonferroni correction (Table III).

We then focused on the time between first consumption and onset of cocaine dependence and observed an earlier dependence onset among carriers of the G-T *NSF* risk haplotype than in non-carriers ( $Z = -3.15$ ,  $P = 0.0015$ ). Interestingly, the main differences were clearly observed in the group of patients whose dependence onset started within two years after the initial drug use (Figure 2). When patients were subdivided in two subgroups, early ( $\leq 2$  years) and late ( $>2$  years) dependence onset, we identified a higher frequency of carriers of the

Table II. (a) Haplotype analysis of four *NSF* SNPs in a clinical sample of 360 cocaine-dependent patients and 360 controls using the UNPHASED software; (b) haplotype distributions of the rsrs183211 and rs17698176 *NSF* SNPs.

(a)			
<i>NSF</i>			
Marker* haplotype	Global <i>P</i> value	Best haplotype- specific <i>P</i> value (Adjusted <i>P</i> -value)	Haplotype-specific OR
14	0.031	0.013 (0.039)	1.3 (1.06–1.60)
(b)			
Marker* haplotype	Cases	Controls	Haplotype specific <i>P</i> value; OR (CI)
<b>14</b>			
A T	205 (28.5)	247 (34.3)	0.017; 1.3 (1.05–1.64)**
G G	111 (15.4)	116 (16.1)	NS
G T	404 (56.1)	357 (49.6)	0.013; 1.3 (1.06–1.60)

NS, not significant.

\*1-rs183211; 4-rs17698176.

\*\*Inverted odds ratio score is shown.

G-T haplotype in the group of patients showing an early regular cocaine consumption ( $P = 2.2e-04$ , OR = 1.85 (1.4–2.4)). These differences were also observed when this group of patients, but not those showing late regular drug consumption, was compared with controls ( $P$ -adjusted =  $5.77e-05$ , OR = 3.90 (2.01–7.57); Table III), and was still significant after the Bonferroni correction. No significant differences were observed when we compared the average age at the first cocaine consumption

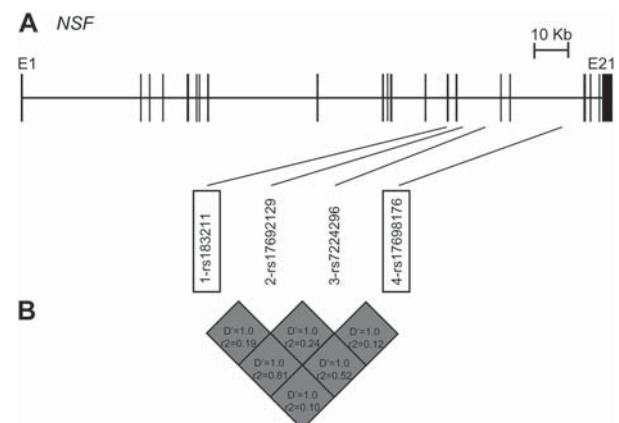


Figure 1. (A) Diagram of the *NSF* gene (NM\_006178). Black boxes indicate exons. The four tag SNPs included in the study are shown on top, with the two SNPs that conform the risk haplotype associated with cocaine dependence boxed. (B) Linkage disequilibrium plot of the four SNPs analyzed in the *NSF* gene, according to Haploview. Considering the Confidence Interval algorithm (Gabriel et al. 2002), the four SNPs are located in the same LD block in our control sample.

Table III. Distribution of carriers of the G-T (rs183211–rs1769817) allelic combination within the NSF gene.

	Haplotypes, n (%)		Adjusted for age			
	G-T carriers	Other haplotypes	OR (95%CI)	P	OR (95%CI)	P
Cocaine dependence	295 (81.9)	65 (18.1)	1.82 (1.28–2.59)	<b>0.001</b>	2.16 (1.38–3.83)	<b>0.001</b>
Cocaine dependence with psychotic symptoms	126 (84.6)	23 (15.4)	2.20 (1.33–3.62)	<b>0.0015</b>	2.51 (1.40–4.50)	<b>0.002</b>
Cocaine dependence without psychotic symptoms	102 (82.3)	22 (17.7)	1.86 (1.11–3.11)	0.017	2.30 (1.28–4.14)	<b>0.0055</b>
Early cocaine dependence ( $\leq 2$ years*)	134 (89.9)	15 (10.1)	3.58 (2.00–6.41)	<b>2.98e-06</b>	3.90 (2.01–7.57)	<b>5.77e-05</b>
Late cocaine dependence ( $> 2$ years*)	77 (71.3)	31 (28.7)	–	NS	–	NS
Controls	257 (71.4)	103 (28.6)				

NS, not significant.

In bold, significant  $P$  values after Bonferroni correction ( $P < 0.01$ ).

\*Time between initial consumption and dependence onset.

between carriers and non-carriers of the G-T haplotype ( $P$ -adjusted = 0.372). Interestingly, the individual analysis of rs183221, the only SNP displaying positive signals in the single-marker analysis, also showed association with cocaine dependence when those cases with early dependence were considered ( $P$ -adjusted =  $6.9e-04$ , OR = 2.42 (1.45–4.04)).

## Discussion

The present case–control association study aims at covering an entire candidate pathway or functional network rather than focusing on single candidate genes. To our knowledge, this is the first association study in cocaine dependence focused on genes coding for the main components of the neurotransmitter release machinery and have found a

significant association with NSF, mainly in the group of patients that rapidly develop drug dependence ( $\leq 2$  years from the initial cocaine consumption). These results suggest that genetic factors may contribute to the neurobiological mechanisms underlying not only cocaine dependence but also an early development of this dependence. No relationship was observed between NSF and age at the first cocaine use or the presence of cocaine-induced psychotic symptoms.

The NSF gene encodes the *N*-ethylmaleimide sensitive factor, which participates in the SNARE complex recycling, ensuring that sufficient amounts of free SNAREs are available for the maintenance of intracellular membrane trafficking (Barszczewski et al. 2008). NSF is essential for the synaptic vesicle turnover as it modulates the kinetics of neurotransmitters release and the integrative properties of synapses (Schweizer et al. 1998; Littleton et al. 2001;

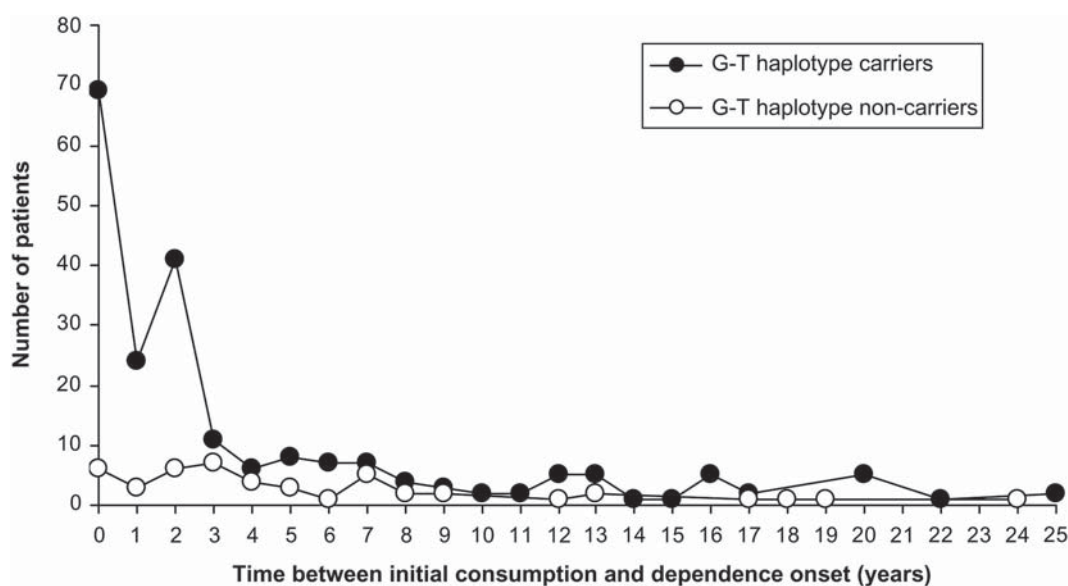


Figure 2. Time between initial consumption and cocaine dependence onset (years) in carriers and non-carriers of the NSF G-T (rs183211–rs1769817) risk haplotype in cocaine-dependent patients.

Malsam et al. 2008). It also has an essential role in the modulation of the trafficking between the plasma membrane and endosomes and in the binding of several cell-surface signalling receptors, such as the alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPA), the beta-2 adrenergic receptor ( $\beta$ 2-AR), the DA receptors (more strongly D1 and D5), the adrenomedullin (AM) receptor and the  $\gamma$ -amino-butyric acid (GABA) receptor (Nishimune et al. 1998; Osten et al. 1998; Song et al. 1998; Cong et al. 2001; Heydorn et al. 2004; Bomberger et al. 2005; Pontier et al. 2006).

Altered NSF function may modulate the activity of the neurotransmitter systems involved in cocaine's effect and dependence. Thus, in agreement with the "reward deficiency syndrome", hypothesis that postulates that hypodopaminergic activity predisposes to cocaine addiction (Comings and Blum 2000), malfunction of NSF could have an effect on the turnover and availability of DA vesicles, altering the DA release to the synaptic cleft.

Some methodological considerations, however, should be taken into account in the present case—control association study: (i) in order to avoid sample heterogeneity that may bias the results in association studies, our sample consisted of patients and controls recruited in the same geographical area around Barcelona (Spain), all of them were Spanish, Caucasian and sex-matched; (ii) although significant after 10% FDR corrections for multiple testing, *NSF* did not remain associated with cocaine addiction under the most restrictive Bonferroni correction, considering 121 SNPs; (iii) the *NSF* risk haplotype associated with cocaine dependence consists of two SNPs located within introns, so it is possible that they do not cause functional effects by themselves, but rather are in LD with other sequence variants directly involved in the genetic susceptibility to cocaine dependence; (iv) the modest sample size (360 patients versus 360 controls) may have prevented from detecting subtle phenotypic effects; (v) cocaine dependence could not be discarded in the control sample, which may potentially dilute positive findings in the association study; and (vi) although the SNP selection was designed to cover 16 genes, gaps still exist in eight genes due to experimental constraints. Specifically, *NAPA* could not be tested for association as the two tagSNPs covering this gene failed in the genotyping assay.

To sum up, our study suggests that *NSF* contributes to the genetic susceptibility to cocaine dependence and, more specifically, to an early development of dependence. These results, however, need to be replicated in other samples. Also, further genetic and functional studies of the *NSF* gene are necessary to

identify those functional variants directly involved in this psychiatric disorder.

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## Statement of Interest

None to declare.

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### Supplementary material available online

Table SI. Description of the VeraCode genotyping assay within 16 candidate genes encoding proteins involved in vesicle fusion for neurotransmitters release (data from HapMap Phase II\_April07\_dbSNP126\_NCBI B36).

Table SII. Nominal *P* values observed when genotype frequencies of 121 SNPs within 15 candidate genes were considered in 360 cocaine-dependent patients and 360 controls.