CANDIDATE PATHWAY ASSOCIATION STUDY IN COCAINE DEPENDENCE: THE CONTROL OF NEUROTRANSMITTER RELEASE

Noèlia Fernàndez-Castillo[1,2,3], Bru Cormand[1,2,3], Carlos Roncero[4,5,6], Cristina Sánchez-Mora[4,7], Lara Grau-Lopez[6], Begoña Gonzalvo[6], Laia Miquel[6], Roser Corominas[1,2], Josep Antoni Ramos-Quiroga[4,5], Miquel Casas[4,5], Marta Ribasés[4, 7]

[1]Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Catalonia, Spain

[2] CIBER Enfermedades Raras, Barcelona, Catalonia, Spain

[3] Institut de Biomedicina de la Universitat de Barcelona (IBUB), Catalonia, Spain

[4]Department of Psychiatry, Hospital Universitari Vall d'Hebron, Barcelona, Catalonia, Spain

[5]Department of Psychiatry and Legal Medicine, Universitat Autònoma de Barcelona, Catalonia, Spain

[6] Outpatient Drug Clinic Vall Hebron. Psychiatry Services. Hospital Universitari Vall d'Hebron, Barcelona, Catalonia, Spain

[7] Psychiatric Genetics Unit, Hospital Universitari Vall d'Hebron, Barcelona, Catalonia, Spain

Short title: Association of *NSF* with cocaine dependence

Corresponding author:

Marta Ribasés. Department of Psychiatry, Hospital Universitari Vall d'Hebron, Barcelona, Catalonia, Spain, Passeig Vall d'Hebron 119-129, 08003, Barcelona, Catalonia, Spain. Tel.: (+34) 93 2746734. Fax: (+34) 93 4894587; Email: mribases@ir.vhebron.net

Number of words in the abstract: 210

Number of words in the text: 3189

Number of Figures: 2

Number of Tables: 3

Supplementary material: 2

Keywords: cocaine dependence, *NSF,* SNARE complex, case-control association study, synaptic exocytosis.

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 15 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 (*SYT, SYT2*, *CPLX1*, *CPLX2*, *CPLX3* and *CPLX4)* and regulatory elements (*STXBP1*, *SYP, SNPH*, *NSF,* 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=5.1e-04, 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 (≤ 2 years; P =2.98e-06, 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.

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 two 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 et al. 2000; Kendler and Prescott 1998; Tsuang et al. 1998), 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 (Kalivas 2007; Kuhar et al. 1991). 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. 1999; Volkow et al. 2002; Volkow et al. 1996). Other neurotransmitters such as serotonin or glutamate also play an important role in cocaine effects (Filip 2005; Filip et al. 2004; Filip et al. 2001; Spealman 1993; Walsh and Cunningham 1997). 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*(+/–) doble knock-out mice (Hall et al. 2002; Hall et al. 2004; Sora et al. 2001; Uhl et al. 2002). Interestingly, *NET*(-/-), *SERT*(-/-) and *NET*(-/-)/*SERT*(-/-) knock-outs showed an even increased rewarding cocaine effect (Hall et al. 2002; Sora et al. 1998; Xu et al. 2000). 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 (Di Ciano et al. 2003; Vorel et al. 2002). Glutamate agonists reduce the euphoric effects of cocaine and withdrawal symptoms (Dackis and O'Brien 2003; Dackis et al. 2003; Hart et al. 2008; Malcolm et al. 2006). 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 (De Vries and Schoffelmeer 2005; De Vries et al. 2001). In addition some promising medications that may prevent cocaine relapse (such as gamma-vinyl GABA "GVG", tiagabine and topiramate) are related to GABA neurotransmission (Cornish and Kalivas 2000; Dewey et al. 1997; Gonzalez et al. 2003; Morgan and Dewey 1998).

Finally, positive associations have been described between cocaine dependence and polymorphisms in genes of the dopaminergic, (Ballon et al. 2007; Comings et al. 1999; Fernàndez-Castillo et al. 2010; Guindalini et al. 2006; Noble et al. 1993),

serotoninergic (Mannelli et al. 2005; Patkar et al. 2001), noradrenergic (Cubells et al. 2000; Guindalini et al. 2008), endocannabinoid (Ballon et al. 2006; Zuo et al. 2009) and cholinergic neurotransmitter systems (Grucza 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 (Ahmed et al. 2005; Freeman et al. 2002; Yuferov et al. 2003).

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 (*SYT, SYT2*, *CPLX1*, *CPLX2*, *CPLX3* and *CPLX4)* and the regulatory elements Munc18.1

(*STXBP1*)*,* synaptophysin (*SYP*), syntaphilin (*SNPH*), *NSF*, αSNARE (*NAPA*) and

RAB3A.

METHODS AND MATERIALS

Subjects

The patient sample consisted of 360 cocaine dependent patients (mean age 34.6 ± 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, Fourth Edition 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 mentamphetamine dependence in 2.1% (n = 7). Seventy-six per cent 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 ± 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 $P \leq 0.00051$. 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, 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 (≤ 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 S1). 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.

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 1, Table S2). However, after correcting for multiple comparisons applying a FDR of 10% (P ≤ 0.00051), 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 1).

The analysis of all the possible SNP combinations within *NSF* revealed a two-marker haplotype (rs183211- rs1769817), that includes the SNP identified in the single-marker analysis, associated with cocaine dependence (global P = 0.031; Fig. 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 2b). 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 3).

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 3), and remained significant after the Bonferroni correction (Table 3).

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 (Fig. 2). When patients were subdivided in two subgroups, early (≤ 2 years) and late (>2 years) dependence onset, we identified a higher frequency of carriers of the 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 = 5.77 e-05, OR = 3.90 (2.01-7.57); Table 3), and was still significant after the Bonferroni

correction. No significant differences were observed when we compared the average age at the first cocaine consumption 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 = 6.9 e-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 (≤ 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 (Littleton et al. 2001; Malsam et al. 2008; Schweizer et al. 1998). 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 (AMPAR), the beta-2 adrenergic receptor (β2-AR), the DA receptors (more strongly D1 and D5), the adrenomedulin (AM) receptor and the γ-amino-butyric acid (GABA) receptor (Bomberger et al. 2005; Cong et al. 2001; Heydorn et al. 2004; Nishimune et al. 1998; Osten et al. 1998; Pontier et al. 2006; Song et al. 1998).

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 don't 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.

ACKNOWLEDGEMENTS

STATEMENT OF INTEREST

None to declare.

FIGURE LEGENDS

Figure 1. A. Diagram of the *NSF* gene (NM_006178). Black boxes indicate exons. The four tagSNPs 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 4 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.

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.

REFERENCES

Ahmed SH, Lutjens R, van der Stap LD, Lekic D, Romano-Spica V, Morales M et al. 2005. Gene expression evidence for remodeling of lateral hypothalamic circuitry in cocaine addiction. Proc Natl Acad Sci U S A 102(32):11533-8.

Ballon N, Leroy S, Roy C, Bourdel MC, Charles-Nicolas A, Krebs MO et al. 2006. (AAT)n repeat in the cannabinoid receptor gene (CNR1): association with cocaine addiction in an African-Caribbean population. Pharmacogenomics J 6(2):126-30.

Ballon N, Leroy S, Roy C, Bourdel MC, Olie JP, Charles-Nicolas A et al. 2007. Polymorphisms TaqI A of the DRD2, BalI of the DRD3, exon III repeat of the DRD4, and 3' UTR VNTR of the DAT: association with childhood ADHD in male African-Caribbean cocaine dependents? Am J Med Genet B Neuropsychiatr Genet 144B(8):1034-41.

Barszczewski M, Chua JJ, Stein A, Winter U, Heintzmann R, Zilly FE et al. 2008. A novel site of action for alpha-SNAP in the SNARE conformational cycle controlling membrane fusion. Mol Biol Cell 19(3):776-84.

Bomberger JM, Parameswaran N, Hall CS, Aiyar N, Spielman WS. 2005. Novel function for receptor activity-modifying proteins (RAMPs) in post-endocytic receptor trafficking. J Biol Chem 280(10):9297-307.

Brookes KJ, Knight J, Xu X, Asherson P. 2005. DNA pooling analysis of ADHD and genes regulating vesicle release of neurotransmitters. Am J Med Genet B Neuropsychiatr Genet 139B(1):33-7.

Comings DE, Blum K. 2000. Reward deficiency syndrome: genetic aspects of behavioral disorders. Prog Brain Res 126:325-41.

Comings DE, Gonzalez N, Wu S, Saucier G, Johnson P, Verde R et al. 1999. Homozygosity at the dopamine DRD3 receptor gene in cocaine dependence. Mol Psychiatry 4(5):484-7.

Cong M, Perry SJ, Hu LA, Hanson PI, Claing A, Lefkowitz RJ. 2001. Binding of the beta2 adrenergic receptor to N-ethylmaleimide-sensitive factor regulates receptor recycling. J Biol Chem 276(48):45145-52.

Cornish JL, Kalivas PW. 2000. Glutamate transmission in the nucleus accumbens mediates relapse in cocaine addiction. J Neurosci 20(15):RC89.

Cubells JF, Kranzler HR, McCance-Katz E, Anderson GM, Malison RT, Price LH et al. 2000. A haplotype at the DBH locus, associated with low plasma dopamine beta-hydroxylase activity, also associates with cocaine-induced paranoia. Mol Psychiatry 5(1):56-63.

Dackis C, O'Brien C. 2003. Glutamatergic agents for cocaine dependence. Ann N Y Acad Sci 1003:328-45.

Dackis CA, Lynch KG, Yu E, Samaha FF, Kampman KM, Cornish JW et al. 2003. Modafinil and cocaine: a double-blind, placebo-controlled drug interaction study. Drug Alcohol Depend 70(1):29-37.

De Vries TJ, Schoffelmeer AN. 2005. Cannabinoid CB1 receptors control conditioned drug seeking. Trends Pharmacol Sci 26(8):420-6.

De Vries TJ, Shaham Y, Homberg JR, Crombag H, Schuurman K, Dieben J et al. 2001. A cannabinoid mechanism in relapse to cocaine seeking. Nat Med 7(10):1151-4.

Dewey SL, Chaurasia CS, Chen CE, Volkow ND, Clarkson FA, Porter SP et al. 1997. GABAergic attenuation of cocaine-induced dopamine release and locomotor activity. Synapse 25(4):393-8.

Di Ciano P, Underwood RJ, Hagan JJ, Everitt BJ. 2003. Attenuation of cue-controlled cocaine-seeking by a selective D3 dopamine receptor antagonist SB-277011-A. Neuropsychopharmacology 28(2):329-38.

Dudbridge F. 2003. Pedigree disequilibrium tests for multilocus haplotypes. Genet Epidemiol 25(2):115-21.

Fernàndez-Castillo N, Ribasés M, Roncero C, Casas M, Gonzalvo B, Cormand B. 2010. Association study between the DAT1, DBH and DRD2 genes and cocaine dependence in a Spanish sample. Psychiatr Genet. E pub ahead of print. DOI: 10.1097/YPG.0b013e32833b6320.

Filip M. 2005. Role of serotonin (5-HT)2 receptors in cocaine self-administration and seeking behavior in rats. Pharmacol Rep 57(1):35-46.

Filip M, Bubar MJ, Cunningham KA. 2004. Contribution of serotonin (5-hydroxytryptamine; 5-HT) 5-HT2 receptor subtypes to the hyperlocomotor effects of cocaine: acute and chronic pharmacological analyses. J Pharmacol Exp Ther 310(3):1246-54.

Filip M, Nowak E, Papla I. 2001. On the role of serotonin2A/2C receptors in the sensitization to cocaine. J Physiol Pharmacol 52(3):471-81.

First MB, Spitzer RL, Gibbon M, Williams JBW (1997). Structured Clinical Interview for DSM-IV disorders (SCID-IV). Washington, DC: American Psychiatric Press.

Freeman WM, Brebner K, Patel KM, Lynch WJ, Roberts DC, Vrana KE. 2002. Repeated cocaine self-administration causes multiple changes in rat frontal cortex gene expression. Neurochem Res 27(10):1181-92.

Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B et al. 2002. The structure of haplotype blocks in the human genome. Science 296(5576):2225-9.

Gass JT, Olive MF. 2008. Glutamatergic substrates of drug addiction and alcoholism. Biochem Pharmacol 75(1):218-65.

Gonzalez G, Sevarino K, Sofuoglu M, Poling J, Oliveto A, Gonsai K et al. 2003. Tiagabine increases cocaine-free urines in cocaine-dependent methadone-treated patients: results of a randomized pilot study. Addiction 98(11):1625-32.

Gonzalez JR, Armengol L, Sole X, Guino E, Mercader JM, Estivill X et al. 2007. SNPassoc: an R package to perform whole genome association studies. Bioinformatics 23(5):644-5.

Grucza RA, Wang JC, Stitzel JA, Hinrichs AL, Saccone SF, Saccone NL et al. 2008. A risk allele for nicotine dependence in CHRNA5 is a protective allele for cocaine dependence. Biol Psychiatry 64(11):922-9.

Guindalini C, Howard M, Haddley K, Laranjeira R, Collier D, Ammar N et al. 2006. A dopamine transporter gene functional variant associated with cocaine abuse in a Brazilian sample. Proc Natl Acad Sci U S A 103(12):4552-7.

Guindalini C, Laranjeira R, Collier D, Messas G, Vallada H, Breen G. 2008. Dopamine-beta hydroxylase polymorphism and cocaine addiction. Behav Brain Funct 4:1.

Hall FS, Li XF, Sora I, Xu F, Caron M, Lesch KP et al. 2002. Cocaine mechanisms: enhanced cocaine, fluoxetine and nisoxetine place preferences following monoamine transporter deletions. Neuroscience 115(1):153-61.

Hall FS, Sora I, Drgonova J, Li XF, Goeb M, Uhl GR. 2004. Molecular mechanisms underlying the rewarding effects of cocaine. Ann N Y Acad Sci 1025:47-56.

Hart CL, Haney M, Vosburg SK, Rubin E, Foltin RW. 2008. Smoked cocaine self-administration is decreased by modafinil. Neuropsychopharmacology 33(4):761-8.

Heydorn A, Sondergaard BP, Hadrup N, Holst B, Haft CR, Schwartz TW. 2004. Distinct in vitro interaction pattern of dopamine receptor subtypes with adaptor proteins involved in post-endocytotic receptor targeting. FEBS Lett 556(1-3):276-80.

Kalivas PW. 2007. Neurobiology of cocaine addiction: implications for new pharmacotherapy. Am J Addict 16(2):71-8.

Kalivas PW, O'Brien C. 2008. Drug addiction as a pathology of staged neuroplasticity. Neuropsychopharmacology 33(1):166-80.

Kendler KS, Karkowski LM, Neale MC, Prescott CA. 2000. Illicit psychoactive substance use, heavy use, abuse, and dependence in a US population-based sample of male twins. Arch Gen Psychiatry 57(3):261-9.

Kendler KS, Prescott CA. 1998. Cocaine use, abuse and dependence in a population-based sample of female twins. Br J Psychiatry 173:345-50.

Kuhar MJ, Ritz MC, Boja JW. 1991. The dopamine hypothesis of the reinforcing properties of cocaine. Trends Neurosci 14(7):299-302.

Littleton JT, Barnard RJ, Titus SA, Slind J, Chapman ER, Ganetzky B. 2001. SNARE-complex disassembly by NSF follows synaptic-vesicle fusion. Proc Natl Acad Sci U S A 98(21):12233-8.

Malcolm R, Swayngim K, Donovan JL, DeVane CL, Elkashef A, Chiang N et al. 2006. Modafinil and cocaine interactions. Am J Drug Alcohol Abuse 32(4):577-87.

Malsam J, Kreye S, Sollner TH. 2008. Membrane fusion: SNAREs and regulation. Cell Mol Life Sci 65(18):2814-32.

Mannelli P, Patkar AA, Murray HW, Certa K, Peindl K, Mattila-Evenden M et al. 2005. Polymorphism in the serotonin transporter gene and response to treatment in

African American cocaine and alcohol-abusing individuals. Addict Biol 10(3):261-8.

Miller SA, Dykes DD, Polesky HF. 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16(3):1215.

Morgan AE, Dewey SL. 1998. Effects of pharmacologic increases in brain GABA levels on cocaine-induced changes in extracellular dopamine. Synapse 28(1):60-5.

Nishimune A, Isaac JT, Molnar E, Noel J, Nash SR, Tagaya M et al. 1998. NSF binding to GluR2 regulates synaptic transmission. Neuron 21(1):87-97.

Noble EP, Blum K, Khalsa ME, Ritchie T, Montgomery A, Wood RC et al. 1993. Allelic association of the D2 dopamine receptor gene with cocaine dependence. Drug Alcohol Depend 33(3):271-85.

O'Brien MS, Anthony JC. 2005. Risk of becoming cocaine dependent: epidemiological estimates for the United States, 2000-2001. Neuropsychopharmacology 30(5):1006-18.

Osten P, Srivastava S, Inman GJ, Vilim FS, Khatri L, Lee LM et al. 1998. The AMPA receptor GluR2 C terminus can mediate a reversible, ATP-dependent interaction with NSF and alpha- and beta-SNAPs. Neuron 21(1):99-110.

Patkar AA, Berrettini WH, Hoehe M, Hill KP, Sterling RC, Gottheil E et al. 2001. Serotonin transporter (5-HTT) gene polymorphisms and susceptibility to cocaine dependence among African-American individuals. Addict Biol 6(4):337-345.

Pontier SM, Lahaie N, Ginham R, St-Gelais F, Bonin H, Bell DJ et al. 2006. Coordinated action of NSF and PKC regulates GABAB receptor signaling efficacy. Embo J 25(12):2698-709.

Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira M, Bender D et al. 2007. PLINK: a toolset for whole-genome association and population-based linkage analysis. Am J Hum Genet 81(3): 559-75.

Rizo J, Rosenmund C. 2008. Synaptic vesicle fusion. Nat Struct Mol Biol 15(7):665-74.

Schweizer FE, Dresbach T, DeBello WM, O'Connor V, Augustine GJ, Betz H. 1998. Regulation of neurotransmitter release kinetics by NSF. Science 279(5354):1203-6.

Song I, Kamboj S, Xia J, Dong H, Liao D, Huganir RL. 1998. Interaction of the N-ethylmaleimide-sensitive factor with AMPA receptors. Neuron 21(2):393-400.

Sora I, Hall FS, Andrews AM, Itokawa M, Li XF, Wei HB et al. 2001. Molecular mechanisms of cocaine reward: combined dopamine and serotonin transporter knockouts eliminate cocaine place preference. Proc Natl Acad Sci U S A 98(9):5300-5.

Sora I, Wichems C, Takahashi N, Li XF, Zeng Z, Revay R et al. 1998. Cocaine reward models: conditioned place preference can be established in dopamine- and in serotonin-transporter knockout mice. Proc Natl Acad Sci U S A 95(13):7699-704.

Soria G, Mendizabal V, Tourino C, Robledo P, Ledent C, Parmentier M et al. 2005. Lack of CB1 cannabinoid receptor impairs cocaine self-administration. Neuropsychopharmacology 30(9):1670-80.

Spealman RD. 1993. Modification of behavioral effects of cocaine by selective serotonin and dopamine uptake inhibitors in squirrel monkeys. Psychopharmacology (Berl) 112(1):93-9.

Stephens M, Smith NJ, Donnelly P. 2001. A new statistical method for haplotype reconstruction from population data. Am J Hum Genet 68(4):978-89.

Storey J. 2002. A direct approach to false discovery Rates. J R Stat Soc Ser B 64:479-498.

Thomas MJ, Kalivas PW, Shaham Y. 2008. Neuroplasticity in the mesolimbic dopamine system and cocaine addiction. Br J Pharmacol 154(2):327-42.

Thorisson GA, Smith AV, Krishnan L, Stein LD. 2005. The International HapMap Project Web site. Genome Res 15(11):1592-3.

Tsuang MT, Lyons MJ, Meyer JM, Doyle T, Eisen SA, Goldberg J et al. 1998. Co-occurrence of abuse of different drugs in men: the role of drug-specific and shared vulnerabilities. Arch Gen Psychiatry 55(11):967-72.

Uhl GR, Hall FS, Sora I. 2002. Cocaine, reward, movement and monoamine transporters. Mol Psychiatry 7(1):21-6.

Uys JD, LaLumiere RT. 2008. Glutamate: the new frontier in pharmacotherapy for cocaine addiction. CNS Neurol Disord Drug Targets 7(5):482-91.

Volkow ND, Fowler JS, Wang GJ. 1999. Imaging studies on the role of dopamine in cocaine reinforcement and addiction in humans. J Psychopharmacol 13(4):337-45.

Volkow ND, Fowler JS, Wang GJ, Goldstein RZ. 2002. Role of dopamine, the frontal cortex and memory circuits in drug addiction: insight from imaging studies. Neurobiol Learn Mem 78(3):610-24.

Volkow ND, Wang GJ, Fowler JS, Gatley SJ, Ding YS, Logan J et al. 1996. Relationship between psychostimulant-induced "high" and dopamine transporter occupancy. Proc Natl Acad Sci U S A 93(19):10388-92.

Vorel SR, Ashby CR, Jr., Paul M, Liu X, Hayes R, Hagan JJ et al. 2002. Dopamine D3 receptor antagonism inhibits cocaine-seeking and cocaine-enhanced brain reward in rats. J Neurosci 22(21):9595-603.

Wagner FA, Anthony JC. 2002. From first drug use to drug dependence; developmental periods of risk for dependence upon marijuana, cocaine, and alcohol. Neuropsychopharmacology 26(4):479-88.

Walsh SL, Cunningham KA. 1997. Serotonergic mechanisms involved in the discriminative stimulus, reinforcing and subjective effects of cocaine. Psychopharmacology (Berl) 130(1):41-58.

Woolverton WL, Johnson KM. 1992. Neurobiology of cocaine abuse. Trends Pharmacol Sci 13(5):193-200.

Xu F, Gainetdinov RR, Wetsel WC, Jones SR, Bohn LM, Miller GW et al. 2000. Mice lacking the norepinephrine transporter are supersensitive to psychostimulants. Nat Neurosci 3(5):465-71.

Yuferov V, Kroslak T, Laforge KS, Zhou Y, Ho A, Kreek MJ. 2003. Differential gene expression in the rat caudate putamen after "binge" cocaine administration: advantage of triplicate microarray analysis. Synapse 48(4):157-69.

Zuo L, Kranzler HR, Luo X, Yang BZ, Weiss R, Brady K et al. 2009. Interaction between two independent CNR1 variants increases risk for cocaine dependence in European Americans: a replication study in family-based sample and population-based sample. Neuropsychopharmacology 34(6):1504-13.
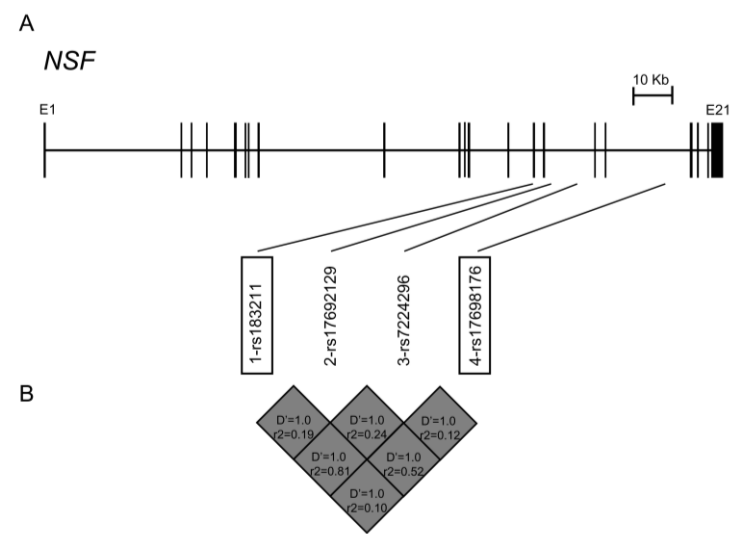
FIGURES

Figure 1. A. Diagram of the *NSF* gene (NM_006178). Black boxes indicate exons. The four tagSNPs 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 4 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.
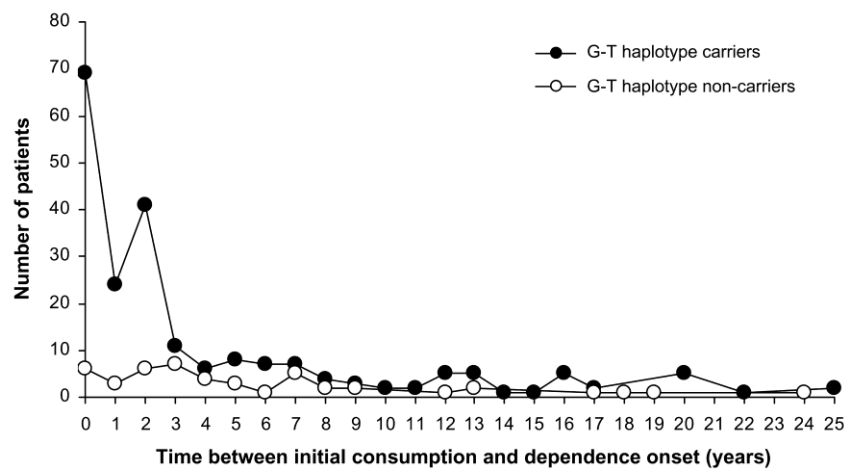

Figure 2. Time between initial consumption and cocaine dependence onset (years) in carriers and non-carriers of the *NSF* G-T (rs183211-rs1769817) risk haplotype in cocaine dependent patients.

TABLES

Table 1. Single-marker analysis: Nominal associations identified in 360 cocaine dependent patients and 360 controls

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

* When odds ratio < 1, the inverted score is shown
& Statistically significant P-value after applying a False Discovery Rate of 10% (P<0.00051), P-value adjusted for age = 7 e-04, P-value corrected by a permutation test= 0.00224)
NS: Not significant

Table 2. (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)

NSF

Marker * haplotype	Global P-value	Best haplotype-specific P-value (Adjusted P-value)	Haplotype-specific OR
1 4	0.031	0.013 (0.039)	1.3 (1.06-1.60)

(b)

Marker * haplotype	Cases	Controls	Haplotype specific P-value; OR (CI)
1 4			
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)**

* 1-rs183211; 4-rs17698176
**inverted Odds Ratio score is shown
NS: Not significant

Table 3. 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)	**0.001**	2.16 (1.38-3.83)	**0.001**
Cocaine dependence with Psychotic Symptoms	126 (84.6)	23 (15.4)	2.20 (1.33-3.62)	**0.0015**	2.51 (1.40-4.50)	**0.002**
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)	**0.0055**
Early cocaine dependence (≤2 years*)	134 (89.9)	15 (10.1)	3.58 (2.00-6.41)	**2.98 e-06**	3.90 (2.01-7.57)	**5.77 e-05**
Late cocaine dependence (>2 years*)	77 (71.3)	31 (28.7)	-	NS	-	NS
Controls	257 (71.4)	103 (28.6)				

* Time between initial consumption and dependence onset
In bold, significant P-values after Bonferroni correction (*P* < 0.01)
NS: Not significant