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ORIGINAL PAPER

Association of *CHRNA5* Gene Variants with Crack Cocaine Addiction

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Abstract

Genome-wide studies provide increasing evidence of association of genetic variants with different behaviors. However, there is a growing need for replication and subsequent characterization of specific findings. In this sense, the *CHRNA5* gene has been associated with nicotine (with genome-wide significance), alcohol and cocaine addictions. So far, this gene has not been evaluated in smoked (crack) cocaine. We aimed to analyze the influence of *CHRNA5* variants in crack addiction susceptibility and severity. The sample includes 300 crack-addicted patients and 769 non-addicted individuals. The *CHRNA5* SNPs evaluated were rs588765, rs16969968, and rs514743. Homozygosity for rs16969968 and rs588765 major alleles was nominally associated with a risk to crack addiction (GG, $P = 0.032$; CC, $P = 0.036$, respectively). Haplotype analyses reveal significant associations (rs588765|rs16969968|rs514743 $p_{global\text{-}corrected} = 7.66 \times 10^{-5}$) and suggest a substantial role for rs16969968. These findings corroborate previous reports in cocaine addiction—in line with the expected effects of cocaine in the cholinergic system—and in the opposite direction of significant GWAS findings for nicotine addiction susceptibility. These results are strengthened by the first report of an association of rs588765 with crack addiction and by the haplotype findings. In summary, our study highlights the relevance of the α5 subunit on crack cocaine addiction, replicating previous results relating *CHRNA5* with the genetics and pathophysiology of addiction of different drugs.

Keywords Crack · Cocaine · Dependence · Nicotinic receptor · nAChR α5 · Substance use disorder

Introduction

The gene encoding the nicotinic acetylcholine receptor (nAChR) α5 subunit, also known as *CHRNA5*, has been associated with addiction of different substances, including nicotine (Bühler et al. 2015; Hancock et al. 2018; Saccone et al. 2018), alcohol (Haller et al. 2014; Hällfors et al. 2013; Wang et al. 2009b), and cocaine (Bühler et al. 2015; Grucza et al. 2008; Saccone et al. 2008). Indeed, the gene cluster encompassing *CHRNA5* is one of the most consistent findings in addiction behaviors in the catalog of genome-wide association studies (GWAS catalog) (Horwitz et al. 2018). This growing evidence of association in GWASes highlights the need for replication in other samples with similar profile and subsequent characterization of specific findings.

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The α5 is an auxiliary subunit that modulates the functionality of nAChRs and dopamine release (Albuquerque et al. 2009). The α4β2* nAChRs (the * means the possibility of an accessory subunit, as α5) are highly expressed on dopaminergic neurons (McGranahan et al. 2011). The presence of α5, forming α4β2α5, increases calcium permeability and turns the receptor more responsive to its ligand (Brown et al. 2007; Tapia et al. 2007). An important genetic variant in the coding region of α5 subunit, rs16969968 (G>A), promotes a change of the amino acid aspartic acid to asparagine, decreasing the calcium permeability and turning the receptor less responsive (Bierut et al. 2008; Kuryatov et al. 2011). This variant has been frequently associated with smoking behavior (Bühler et al. 2015; The Tobacco and Genetics Consortium et al. 2010; Saccone et al. 2018). Moreover, the same allele presents opposite effects on the susceptibility to nicotine or cocaine addiction (Sherva et al. 2010; Bühler et al. 2015).

Unlike the direct effect of nicotine in the cholinergic receptors, cocaine exerts its effects through a different mechanism. Repetitive cocaine administration induces neuroplasticity in cholinergic neurons in brain regions related to reward processes. This plasticity disappears after cocaine use is ceased, although this effect may persist depending on the administration process (Chen et al. 2008). Moreover, cocaine-treated cholinergic neurons exhibit reduced inhibitory synaptic transmission via noradrenaline and stimulation of adrenoreceptors in response to stress-exposure. Both cocaine-induced mechanisms of neuroadaptation could be linked to addiction behaviors and cocaine abuse (Kaneda 2018).

The administration route of cocaine (intranasal, intravenous and smoked) has been suggested to present distinct pharmacokinetic effects in the brain, where smoking cocaine (crack) would produce a higher reinforcement effect (Verebey and Gold 1988; Volkow et al. 2000). Nonetheless, no study has evaluated the influence of *CHRNA5* variants on crack addiction. Considering these findings, our aim is to evaluate *CHRNA5* polymorphisms on crack addiction and to investigate whether such associations corroborate previous findings on cocaine addiction, regardless of the route of drug administration.

Experimental Procedures

Sample and Diagnostic Procedures

This study enrolled 300 crack-addicted patients from public and voluntary detoxification units from Southern Brazil. Crack addiction was diagnosed after detailed interviews conducted by trained clinical psychologists and psychiatrists following the Diagnostic and Statistical Manual of Mental Disorders fourth edition (DSM-IV) criteria (American Psychiatric Association 1994). The control sample comprised 769 non-addicted individuals from either the general population or blood donors from the Hospital de Clínicas de Porto Alegre. All individuals are Brazilians of European descent at least 18 years old. The sample characteristics are shown in Table 1.

In cases, addiction and other problems were evaluated by the Addiction Severity Index-6 (ASI-6), a multidimensional semi-structured interview that evaluates patient's lifetime as well as recent status in nine functional areas of life including medical and drug use (Cacciola et al. 2011; Kessler et al. 2012). This instrument also evaluates other variables, such as age of first crack and cocaine use and years of regular crack and cocaine use. Cases were excluded if they present

Table 1 Sample characterization

	Cases *N* = 300 *N* (%)	Controls *N* = 769 *N* (%)	*P* value
Men	165 (55.0)	440 (57.2)	0.217
Presence of any mood disorder[a]	228 (82.0)	222 (28.9)	< 0.001
	Mean (SD)	Mean (SD)	
Age	30.73 (8.3)	29.21 (8.63)	0.124
ASI Drugs	50.83 (8.97)	–	–
ASI Psychiatric	47.60 (9.94)	–	–
Age of first cocaine use	18.41 (5.63)	–	–
Age of first crack use	22.98 (8.33)	–	–
Years of cocaine use	5.73 (6.18)	–	–
Years of crack use	5.46 (4.76)	–	–

[a]Major depression disorder, bipolar disorder and dysthymia

schizophrenia, psychotic symptoms, severe cognitive impairment resulting in alterations of consciousness and psychomotor agitation, diagnosis of a neurological, infectious or metabolic disease and Mini-Mental State Examination (MMSE) score < 18.

The control group was evaluated in regards to psychiatric disorders and substance use by DSM-IV screening module—SCID-I (First et al. 2002), or using the Alcohol, Smoking and Substance Involvement Screening test (ASSIST) for the first screening of drug use. Individuals were excluded if they showed any confusion status as well as presence of psychotic disorders or severe intellectual disability that could interfere in the correct use of research instruments. A more extensive sample description can be assessed in previous publications (Rovaris et al. 2017; Stolf et al. 2014). This study is in accordance with the Declaration of Helsinki. All participants signed the consent form approved by the participants institutional review boards (IRB).

SNP Selection and Genotyping

The SNP selection criteria for this study were minor allele frequency higher than 20%, possible functional effect and previous associations with substance use disorders (SUD). The rs588765 (12564C>T) modifies *CHRNA5* mRNA levels (Wang et al. 2009a) and rs16969968 (30064G>A, D398N) alters the receptor responsivity (Bierut et al. 2008). Although rs514743 (31366A>T) does not present known functional effect, its frequent association reports involving SUD led to its inclusion (Lubke et al. 2012; Polina et al. 2014). DNA was extracted from peripheral blood by the salting out method (Lahiri and Nurnberger 1991) or from saliva by Oragene DNA kit according to the manufacturer's instructions (Oragene DNA kit, DNA genotec, Canada). DNA concentration was determined using Nanodrop® Lite (Thermo Fisher Scientific). All SNPs were genotyped using predesigned and validated TaqMan SNP genotyping assays (StepOnePlus, Applied Biosystems, Foster City, CA, USA). We re-genotyped 20% of the sample randomly, and no divergence in the genotyping data was detected. All SNPs are in Hardy Weinberg Equilibrium in cases and controls.

Statistical Analyses

Logistic regression analyses were performed to evaluate the effect of each individual SNP on the susceptibility to crack cocaine addiction. To assess the effect of each SNP on severity of crack cocaine addiction as well as age of first use and years of regular crack and cocaine use through ASI-6, we conducted linear regression models. We performed the dominant (carrier of minor allele versus homozygous for the major allele) and genotypic models for each SNP. Potential covariates (i.e., age, sex and mood disorders) were tested, but no variable was associated at a level of $P < 0.05$ with both the outcome and the SNP tested and then included in the regression models. Analyses were performed using Statistical Package for the Social Sciences (SPSS) version 18 and PLINK program (Purcell et al. 2007). Multiple testing correction was performed using FDR.

In Silico Prediction Analyses

PROVEAN V1.1.3 (protein variation effect analyzer, https://provean.jcvi.org/index.php) (Choi and Chan 2015), SIFT (Kumar et al. 2009) and PolyPhen-2 (Polymorphism Phenotyping v2, https://genetics.bwh.harvard.edu/pph2/) (Adzhubei et al. 2010) tools were used to predict the impact of the amino acid substitution covering the rs16969968 on the structure and function of the CHRNA5 protein.

Furthermore, potential regulatory mechanisms involving all three *CHRNA5* SNPs were investigated using the HaploReg v4.1 (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php) and RegulomeDB (https://www.regulomedb.org/) databases. HaploReg explore annotations of non-coding variants, such as candidate regulatory SNPs at disease-associated loci, gathering information from 1000 Genomes, Roadmap Epigenomics and ENCODE projects. This tool was designed to evaluate the possible impact of non-coding variants on clinical phenotypes considering gene expression regulatory mechanisms (Ward and Kellis 2012). RegulomeDB annotates SNPs with known and predicted regulatory elements in the intergenic regions of the human genome and generates a score ranging from 1 to 7 linked to the regulatory evidence of each locus (Boyle et al. 2012). The Genotype-Tissue Expression (GTEx) project facilitated the identification of genome regions that influence whether and how much a gene is expressed (https://gtexportal.org/home/). GTEx provide data concerning expression quantitative trait locus (eQTLs) covering multiple human tissues. The data used for the analyses described in this manuscript were obtained from the GTEx Portal on September 2019.

Results

Case–Control Analyses

In the case–control analyses, homozygosity for the rs16969968 and rs588765 major alleles was nominally associated with risk to crack addiction in the genotypic model (GG, $P = 0.032/P_{corrected} > 0.05$, OR = 1.670; CC, $P = 0.036/P_{corrected} > 0.05$, OR = 1.707, respectively). rs514743 was not associated with crack addiction (Table 2).

All polymorphisms analyzed were in strong LD (rs588765 and rs16969968 $D' = 0.994$, $R = 0.252$; rs16969968 and rs514743 $D' = 0.992$, $R = 0.206$; rs588765 and rs514743

Table 2 Case–control analyses

		Genotypic and dominant model				
		Genotypes	Cases ($n=300$)	Controls ($n=769$)	OR (95% CI)	P value
rs588765	Genotypic model	TT (reference)	23 (7.6)	88 (11.4)		
		CT	132 (44.0)	356 (46.3)	1.419 (0.860–2.340)	0.171
		CC	145 (48.3)	325 (42.3)	1.707 (1.036–2.812)	0.036
	Dominant model	TT + CT (reference)				
		CC			1.278 (0.978–1.671)	0.073
rs16969968	Genotypic model	AA (reference)	27 (9.0)	100 (13.0)		
		AG	126 (42.0)	343 (44.6)	1.361 (0.849–2.180)	0.201
		GG	147 (49.0)	326 (42.4)	1.670 (1.046–2.666)	0.032
	Dominant model	AG + AA (reference)				
		GG			1.306 (0.999–1.706)	0.051
rs514743	Genotypic model	TT (reference)	16 (5.3)	63 (8.2)		
		AT	123 (41.0)	340 (44.2)	1.424 (0.793–2.560)	0.237
		AA	161 (53.6)	366 (47.6)	1.732 (0.971–3.091)	0.063
	Dominant model	AT + TT (reference)				
		AA			1.275 (0.976–1.666)	0.075

P value corrected > 0.05 (multiple testing correction—FDR)

$D' = 0.997$, $R = 0.819$). Global haplotype analyses revealed significant results after multiple test correction, among the three SNPs and crack addiction: rs588765|rs1696996 $P = 8.42 \times 10^{-5}$; rs16969968|rs514743 $P = 2.76 \times 10^{-4}$ and rs588765|rs16969968|rs514743 $P = 7.66 \times 10^{-5}$. Individual analyses of each haplotype block revealed different significant results. In particularly, the A allele (protection) of rs16969968 in the presence of any allele of rs588765 and rs514743 was associated with protection against crack addiction, while the G allele (risk) of rs16969968 was associated with risk to crack addiction only in the presence of C allele (risk) of rs588765 or/and in the presence of A allele of rs514743 (Table 3).

Regarding addiction severity, none of *CHRNA5* SNPs and haplotypes were associated with the outcomes analyzed (ASI drug score, ASI psychiatric score, age of first crack and cocaine use, and years of regular crack and cocaine use).

In Silico Analyses

We investigated possible SNPs effects on the *CHRNA5* regulation, structure and function of the CHRNA5 protein with in silico prediction analyses. PROVEAN, SIFT and Polyphen-2 tools predicted the potential effect of rs16969968 as neutral, tolerated and benign, respectively (Supplementary Table 1). There was no evidence for modifications in histone and chromatin state in brain tissues in HaploReg (Supplementary Table 2). Furthermore, prediction of potential functionality of *CHRNA5* SNPs in the RegulomeDB database reveal minimal evidence of transcription factors binding on the selected polymorphisms (scores ≥ 5).

The GTEx database showed that all SNPs studied here could be identified as eQTLs for the *CHRNA5* and associated with their expression levels. This effect covers different brain tissues including nucleus accumbens and caudate regions. In addition, the *CHRNA5* SNPs were identified as eQTLs for *CHRNA3* and CHRNA5 antisense RNA, RP11-650L12.2 (Supplementary Table 3).

Discussion

Nicotinic receptor genes, including *CHRNA5*, may be included in a relatively small list of genes with consistent and replicated effects in candidate gene and genome-wide association studies, especially in this case for nicotine addiction. The present study is the first analysis of *CHRNA5* nAChR auxiliary subunit variants in the susceptibility to crack addiction. We report the association of an additional *CHRNA5* variant (rs588765), as well as corresponding haplotype findings. The findings also confirm the influence of rs16969968 G allele, previously associated with snorted or intravenous cocaine addiction (Bühler et al. 2015; Grucza et al. 2008; Saccone et al. 2008), suggesting the same alleles are associated with different routes of cocaine administration. The associated allele is linked to a more responsive receptor, and previous studies have demonstrated that stimulation of nicotinic AChRs activate dopaminergic neurons in the ventral tegmental area (Yin and French 2000; Zhang et al. 2005). Also, during cocaine self-administration, increased acetylcholine levels are observed in this brain area

Table 3 *CHRNA5* haplotype analyses

				CHRNA5 individual haplotypes							
	NHAP	STAT	*P* value (gl corr*)	Window	rs588765	rs16969968	rs514743	Frequency	b	STAT	*P* value (ind/ind corr*)
CHRNA5											
rs588765lrs16969968	3	18.8	8.42e−005	1	C	A		0.337	0.791	5.19	0.0228/0.0300*
				1	T	G		0.331	0.795	4.71	0.03/0.0300*
				1	C	G		0.33	1.53	18.8	1.48e−005/4.44e−005*
rs16969968lrs514743	3	16.4	0.000276	2		G	T	0.29	0.795	4.25	0.0391/0.0391*
				2		A	A	0.337	0.790	5.19	0.0228/0.0342*
				2		G	A	0.372	1.48	16.4	5.2e−005/16.60 e−005*
rs588765lrs16969968lrs514743	3	19.0	7.66e−005	3	T	G	T	0.289	0.797	4.13	0.0422/0.0422*
				3	C	A	A	0.337	0.791	5.16	0.0231/0.0346*
				3	C	G	A	0.33	1.54	18.9	1.34e−005/4.02 e−005*

NHAP Number of individual haplotypes in that window

**P* global (gl) corrected (corr). The frequency threshold of 5% was determined using the '--mhf 0.05' command

(You et al. 2008), suggesting a potential activation of dopaminergic neurons via AChRs.

Interestingly, the consistently replicated effect of rs16969968 on nicotine addiction (Bühler et al. 2015; Pandey et al. 2017) is in the opposite direction, where the A allele confers risk. These distinct effects related to the same *CHRNA5* SNP may represent an opportunity to understand apparently contradictory genetic findings, including a GWAS hit, in light of the different impacts of agonist and antagonist drugs. Grucza et al. (2008) suggested that this contradictory effect is based on direct and indirect dopaminergic mechanisms. In the presence of a less responsive receptor (rs16969968 A allele), the indirect dopaminergic stimulation promoted by nicotine on GABA neurons would lead to a disinhibited dopamine signaling, enhancing dopamine response to nicotine. On the other hand, the same less responsive receptor in the context of dopaminergic neurons (direct effect) would be protective for cocaine addiction since it could promote a diminished dopamine effect (Grucza et al. 2008).

In relation to rs588765, we demonstrated for the first time an association with crack addiction, reinforcing previous associations of this SNP with SUD, especially in nicotine dependence. Functional studies demonstrated that presence of C allele is linked to lower mRNA levels. Nonetheless, more studies are needed to understand the impact of mRNA levels on SUD. Additionally, our haplotype findings corroborate the main effects observed in individual SNP analyses. Although other studies did not investigate these haplotype blocks in crack or cocaine addictions, results from Wang et al. (2009a) might support our associations. The haplotype block encompassing rs588765 and rs16969968 was associated with nicotine addiction in patients with lung cancer. While the C–G haplotype was associated with risk to crack addiction in our study, Wang et al. (2009a) showed that this same haplotype was related to protection against nicotine addiction. Similar results were observed with the C-A haplotype. In both studies, haplotype findings appear to be carried by the effect of rs16969968.

Taking into account all candidate gene and GWAS findings previously reported and the results presented here, the relationship between nAChRs and addiction is strong and corroborated by several studies. The functionality of these receptors seems to be very important to elucidate the mechanism of addiction. In particular, the role of the α5 subunit on crack cocaine addiction is supported by evidence directly linked to the nAChRs responsiveness. Furthermore, in silico analyses revealed that all SNPs analyzed in this study are eQTLs and modulate the expression of either *CHRNA5* mRNA or *CHRNA5* antisense RNA RP11-650L12.2. The co-expression of both genes occurs in multiple tissues (Barrie et al. 2017) including the nucleus accumbens, which is a brain region crucial to drug addiction. The eQTLs analyses are an important approach to

understand the putative influence of genetic variants and to presume their contribution to phenotypic variability. Diverse identified eQTLs in the same chromosomal region suggest the involvement of many variants with regulatory effects, which collectively could infer major impact on gene expression. The overlap between eQTLs and significant GWAS SNPs, as occurs in the *CHRNA5* gene, can be considered a vital cluster in the genome for addiction susceptibility.

This study has some limitations that should be addressed. The co-administration of nicotine and crack cocaine is very common among addicted individuals, hindering the individual assessment of these substances in the context of molecular mechanisms. Nonetheless, previous studies have also not considered the use of multiple substances in their analyses (Grucza et al. 2008; Saccone et al. 2008). Our sample is similar to the other samples of association studies between rs16969968 and cocaine addiction, where the vast majority of individuals had also a history of smoking. Much larger sample sizes would be needed in order to study "pure" crack or cocaine addiction, without smoking.

In conclusion, our study corroborates previous findings, showing that the same allele of rs16969968 is associated with crack and cocaine addiction regardless of the route of administration. Overall, our results reinforce the important role of *CHRNA5*—an auxiliary subunit—on addiction of different drugs.

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Compliance with Ethical Standards

Conflict of interest EHG was on the speaker's bureau for Novartis and Shire for the last 3 years. He also received travel awards (air tickets and hotel accommodations) for participating in two psychiatric meetings from Shire and Novartis. The remaining authors declare no conflict of interest.

Ethical Approval This study is in accordance with the Declaration of Helsinki. All participants signed the consent form approved by the participants institutional review boards (IRB).

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