Association analysis of *GRK3* gene promoter variants in cocaine abuse

Camila Guindalini[a,c], David Collier[a,b], Ronaldo Laranjeira[d], Tom B. Barrett[e,f], John Kelsoe[e,f], Adauto Castelo[d], Homero Vallada[b,c] and Gerome Breen[a,b]

The G protein-coupled receptor kinase 3 gene (*GRK3*) is a candidate gene for cocaine adiction because it is involved in the regulation of several neurotransmitter receptors, including the response to dopaminergic agonists such as methamphetamine and cocaine. We hypothesized that genetic variants in the *GRK3* gene might be associated with an increased risk of cocaine addiction. To test this, we genotyped three variants located in 5′ untranslated and promoter regions of the gene in a sample of 711 cocaine users and 862 healthy control individuals from Sao Paulo, Brazil. Genotypic, allelic and haplotypic analyses provided no evidence for an association between alleles at these polymorphisms and cocaine abuse in this sample. Population stratification was tested for and its effect corrected for, but this did not affect the association test results. In conclusion, our results do not support a major role for *GRK3* gene promoter variants in cocaine addiction. *Psychiatr Genet* 17:239–242 © 2007 Lippincott Williams & Wilkins.

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[a]MRC Social Genetic and Developmental Psychiatry Research Centre, [b]Division of Psychological Medicine, Institute of Psychiatry, King's College London, UK, [c]Department of Psychiatry, University of São Paulo Medical School, [d]UNIAD (Unit of Drug and Alcohol Research), Department of Psychiatry, Federal University of São Paulo, Brazil, [e]Department of Psychiatry, University of California San Diego, San Diego and [f]Department of Psychiatry, San Diego VA Healthcare System, La Jolla, California, USA

Correspondence to Gerome Breen, Section of Genetics, Room 222 SGDP Centre, PO 81, MRC Social Genetic and Developmental Psychiatry Research Centre, Institute of Psychiatry, Kings College London SE5 8AF, UK
Tel: +44 20 7848 0409; fax: +44 20 7848 0575;
e-mail: g.breen@iop.kcl.ac.uk

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Introduction

Over 13 million people worldwide are thought to be regular users of cocaine, placing it in the top three most used illegal drugs (United Nations Office on Drugs and Crimes, 2005). Epidemiological studies suggest a substantial genetic component in the vulnerability of individuals to become addicted once exposed to cocaine (Kendler and Prescott, 1998; Kendler *et al.*, 2000). Specific genetic factors involved in the development of drug dependence, however, have yet to be identified.

Cocaine influences dopamine, norepinephrine and serotonin neurotransmission by inhibiting their reuptake after they have been released in the synapse (Ritz *et al.*, 1990). The postsynaptic receptors of these neurotransmitters belong to the G-protein-coupled receptor (GPCR) super-family (Gainetdinov *et al.*, 2004). In the presence of the appropriate activating ligand or agonist (neurotransmitters), the G-protein will bind to the receptor and activate different signalling cascades, or second messenger pathways, inside the cell. When exposed to persistent and continuous stimuli, GPCR signalling demonstrates diminished responsiveness, a process refered to as 'homologous desensitization', an important regulatory feature that prevents overstimulation and permits the linear response range to vary near the ambient stimulation level (Gainetdinov *et al.*, 2004). This process is facilitated by another protein family termed GPCR kinases (GRKs). The GRKs will phosphorylate the agonist-occupied receptor, permitting the subsequent binding of a protein called β-arrestin. Arrestin binding will then prevent further G-protein activation regardless of the continued presence of the agonist (Luttrell and Lefkowitz, 2002).

Seven known GRK subtypes exists which are classified into three subfamilies (GRK1/7, GRK2/3 and GRK4/5/6) on the basis of sequence and functional similarity (Gainetdinov *et al.*, 2004). The GRKs are widely distributed in brain and periphery (Arriza *et al.*, 1992). Studies have demonstrated that the neuronal GPCRs, including D1-type, D2-type and D3-type dopamine receptors are substrates for the regulation of the *GRK3* subfamily (Tiberi *et al.*, 1996; Kim *et al.*, 2001). Animal studies have demonstrated a 14-fold increase in *GRK3* mRNA in the prefrontal cortex of rats, after methamphetamine administration (Niculescu *et al.*, 2000). Moreover, mice lacking the *GRK3* gene do not demonstrate enhanced locomotor or climbing responses to cocaine. Indeed, cocaine induced reduced response in these animals (Gainetdinov *et al.*, 2004). Taken together, these data suggest that *GRK3* may play a major role in the control of dopaminergic response, and may modulate the effects of stimulants on the brain.

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Thus, we hypothesized that genetic variation at the *GRK3* gene may be associated with an increased risk of cocaine addiction. We tested this by genotyping three variants located in 5′ untranslated (5′ UTR) and promoter regions of the gene (rs576895, rs558934 and rs5761116) in our sample of cocaine addicts and healthy control individuals.

Methods

Participants

Patients

Seven hundred and eleven cocaine abusers, 681 men, 30 women (mean age: 26.70 years; SD = 7.23) were ascertained (Turchi *et al.*, 2002). The study group consisted of drug users who were in treatment from August 1997 to October 1998 in one outpatient and six inpatient units located in the city of Sao Paulo, Brazil. Inclusion criteria were age ≥ 18, previous history of cocaine abuse. Individuals with another psychiatric diagnosis or a chronic physical illness were excluded. All current cocaine users undertook a structured interview dealing with socio-demographic characteristics, sexual behaviours, and drug use profile. All participants satisfied an ICD10 diagnosis of cocaine dependence. For further details see Guindalini *et al.* (2006).

Controls

Eight hundred and sixty-two healthy controls, 589 men and 273 women (mean age: 31.65 years; SD = 9.89) were recruited from the Blood Transfusion Unit of the Hospital das Clinicas, Faculty of Medicine, University of São Paulo. Each blood donor was screened using a short questionnaire investigating contagious diseases and the use of licit, illicit or prescribed drugs. Participants who reported daily drinking or any other type of alcohol abuse behaviour, past history of drug abuse or recent use of an illegal drug were excluded. At the time of donation, a short structured interview was conducted and participants with a lifetime history of a psychiatric disorder requiring admission to hospital or suffering from a psychiatric condition at time of interview were excluded.

Ethics

All the participants included in this study gave written informed consent and this project was approved by the Ethical Committee of the Federal University of São Paulo and other relevant ethics committees.

Genotyping

GRK3 SNPs

We decided to genotype three *GRK3* promoter single nucleotide polymorphisms (SNPs) in our sample: rs576895, rs558934 and rs5761116. The genotyping of all SNPs selected for this study was performed blind to status using an amplifluor assay, and was performed under contract by K-Biosciences (Cambridge, UK; *http://www.kbioscience.co.uk/*).

Markers for population stratification analysis

To detect the presence of genetic stratification in our sample, we selected a total of 71 (64 SNPs and seven microsatellites) ancestry informative markers, for example, markers that exhibit large allele frequency differences among the three main Brazilian ancestral populations (Europeans, Africans and Native American). The genotyping of all SNPs selected for this study was performed blind to status using allele-specific PCR with molecular beacons (Myakishev *et al.*, 2001) and was performed under contract by Prevention Genetics (Marshfield, USA; *http://www.preventiongenetics.com*). Names, primers and conditions for the microsatellites used can be obtained on request.

Statistical analysis

Hardy–Weinberg equilibrium, genotype and allele frequencies were compared using a χ^2 test. Odds ratios were derived from logistic regression carried out in SPSS (v.12) (Statistical Package for Social Sciences, version 12.0, for Windows. (SPSS Inc., Chicago, Illinois, USA)). Pair-wise linkage disequilibrium (LD) was calculated using LD pairs program from GC utilities (Zhao, 2004). In addition, WHAP software v.2.06 (*http://www.genome.wi.mit.edu/~shaun/whap*) was used to estimate haplotype frequencies. The effect of population stratification was tested using the ADMIXMAP program (McKeigue *et al.*, 2000; Hoggart *et al.*, 2003).

Results

The genotype frequencies were in Hardy–Weinberg equilibrium in both, case and control groups for the three polymorphisms. Genotyping failure rate was around 2% for the rs558934, 4% for the rs576116 and 2% for the rs576895. Genotype counts and frequencies of the three available SNPs, and allele wise odds ratio analyses, can be found in Table 1. No statistical difference was observed between cases and controls, allele or genotype wise.

Pairwise LD analyses demonstrated that the three markers were in significant LD. Markers rs558934 and rs5761116 higher linkage ($D' = 0.7$; $P < 0.0001$) than markers rs558934 and rs576895 ($D' = 0.2$; $P < 0.0001$) and markers rs5761116 and rs57689 ($D' = 0.2$; $P < 0.0001$). Subsequently, we conducted haplotype analyses for the three markers, and for each pairwise combination. No significant associations were observed between cocaine addiction and the haplotypes comprised of alleles of the three markers (LRT = 5.0; $P = 0.65$), the rs576895/rs558934 combination (LRT = 1.7; $P = 0.62$), the rs576895/rs5761116 combination (LRT = 3.2; $P = 0.36$) and rs558934/rs5761116 combination (LRT = 1.2; $P = 0.74$).

The program ADMIXMAP was used to correct the association tests for the presence of population stratification in the sample. The score test calculated by

Table 1 Genotype and allele counts and frequencies (%) of the three promoter polymorphisms in the *GRK3* gene among healthy controls and cocaine addicts

	Genotype wise								Allele wise						
	11		12		22		Total	*P* value	1		2		Total	*P* value	OR (95% CI)
rs576895															
Controls	320	38%	384	46%	138	16%	842		1024	61%	660	39%	1684		
								0.81						0.73	0.99 (0.85–1.14)
Cases	261	37%	333	47%	111	16%	705		855	61%	555	39%	1410		
rs558934															
Controls	420	50%	344	41%	78	9%	842		1184	70%	500	30%	1684		
								0.73						0.48	0.94 (0.81–1.10)
Cases	341	49%	286	41%	73	10%	700		968	69%	432	31%	1400		
rs5761116															
Controls	604	73%	201	24%	18	2%	823		1409	86%	237	14%	1646		
								0.35						0.23	1.13 (0.92–1.4)
Cases	518	76%	159	23%	9	1%	686		1195	87%	177	13%	1372		

Odds ratio (OR) analysis with 95% confidence intervals (CI) and *P* values for the χ^2 distribution are also shown.

ADMIXMAP verifies the association of the outcome variable with the alleles at each locus, adjusting for age, sex and admixture. The latter was performed by averaging over the posterior distribution of individual admixture proportions as estimated using the observed ancestry informative marker genotypes. As the three markers were in close LD, ADMIXMAP considered *GRK3* as a compound locus and conducted the association test not only between the outcome variable and the alleles at each locus, but also between each possible haplotype, correcting for effect of population stratification. The score tests did not provide evidence for association between cocaine addiction and any of single markers or any of the eight possible haplotypes (data not shown), demonstrating that the detected population substructure was not responsible for the negative finding.

Discussion

In this study, we hypothesized that *GRK3* sequence variants could influence susceptibility to cocaine addiction. Genotypic, allelic and haplotypic analyses, however, provided no evidence for an association between alleles at these polymorphisms and cocaine addiction in the Brazilian population.

GRK3 is an interesting candidate gene for cocaine addiction because it has been demonstrated to be involved in the regulation of numerous neuronal GPCRs, including dopamine receptors D1 and D3 (Tiberi *et al.*, 1996; Kim *et al.*, 2001). Functional genetic variants may influence the ability of *GRK3* to desensitize these receptors and consequently augment the responsiveness of dopamine and other neurotransmitters signal transduction in the brain. Barrett and colleagues (2003) demonstrated evidence of association between a variant in the promoter of *GRK3* and bipolar disorder in families of Caucasian ancestry. Their study included the three markers analysed here and three additional SNPs. A second study, Yu *et al.* (2004), examining schizophrenia families of Japanese and Chinese origin, failed to demonstrate an association with schizophrenia using a slightly different marker set.

Our data did not provide evidence that the variants we tested are associated with cocaine addiction. Population stratification was evaluated for and that detected did not affect the association test results, suggesting that a false positive effect owing to this is unlikely. Of course, it is possible that further undetected or cryptic population stratification may serve to mask a positive association and thus we cannot rule out a minor role of the gene or a role for a polymorphism not insignificant LD with those genotyped. Given the sample size tested, however, we can conclude that our results do not support a major role for *GRK3* gene promoter variants in cocaine.

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