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Potentially Functional Polymorphism in the Promoter Region of Prodynorphin Gene May Be Associated With Protection Against Cocaine Dependence or Abuse

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Abstract

It has been demonstrated that the opioid peptide dynorphin plays a role in modulating responses to several psychoactive substances including cocaine. Our laboratory and others have found that mRNA levels of dynorphin in the caudate and putamen are elevated after acute or chronic cocaine exposure in rats. Recently, a 68-base pair (bp) repeat polymorphism within the core promoter region of the human prodynorphin gene has been reported to occur in alleles containing one, two, three, or four copies. This repeat contains a putative AP-1 transcription factor binding site; reporter gene constructs with three or four, but not one or two, copies of the tandem repeats were shown to be associated with increases in transcriptional activation in in vitro cellular assays. We hypothesize that this polymorphism may be associated with individual differences in vulnerability to cocaine dependence or abuse. From an ongoing study of the genetics of addiction, 174 subjects were studied, including individuals with a primary diagnosis (DSM-IV criteria) of cocaine dependence ($N = 61$) or abuse ($N = 22$), and controls with no history of any substance dependence or abuse ($N = 91$). We designed primers for polymerase chain reaction (PCR) to amplify sequences of the promoter region of the prodynorphin gene containing the repeat element. The association of alleles containing three or four repeats with cocaine dependence/abuse was examined. With data stratified by ethnic group, pooled re lative risk (RR) with Mantel-Haenszel Chi square was calculated: RR = 0.59 (95% confidence interval 0.37–0.95), $\chi^2_{(1)} = 4.14$, $P = 0.042$. Our results suggest that this allelic variation at the promoter region of the prodynorphin gene (alleles with three or four repeats), which may result in enhanced transcription of the gene, may contribute to relative protection and decrease individual vulnerability to develop cocaine dependence or abuse.

Keywords

opioid; dynorphin; association study; genetic polymorphism; AP-1 transcription factor binding site human preproenkephalin B “GDB” “X02536”

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INTRODUCTION

Cocaine abuse is a serious problem in the United States and worldwide. According to the 1998 National Household Survey on Drug Abuse, 1.8 million Americans were chronic cocaine users [Substance Abuse and Mental Health Services Administration, 1999]. Clinical observation suggests that individuals exposed to cocaine vary in their vulnerability to become addicted. While the bases for these variations are not well understood, there is strong evidence for a genetic component in the vulnerability to become addicted once self-exposed to drugs of abuse [e.g., Grove et al., 1990; Tsuang et al., 1996, 1998]. However, the specific genetic variations that contribute to these complex disorders have yet to be identified.

Prodynorphin is a member of the opioid neuropeptide family, which has three main precursor gene products: pro-enkephalin A, (PENK) [Noda et al., 1982], proopiomelanocortin (POMC) [Nakanishi et al., 1979], and pro-enkephalin B (prodynorphin) [Horikawa et al., 1983]. The 5′-upstream region of the human prodynorphin gene was cloned two decades ago [Horikawa et al., 1983]. Several transcription factor binding sites were identified in the promoter region of the prodynorphin gene and found to be responsible for enhanced prodynorphin mRNA expression in response to cyclic AMP or protein kinase A in the rat [Douglass et al., 1994; Simpson and McGinty, 1995]. Altered expression of the prodynorphin gene in the central nervous system has been observed in several experimental models of psychiatric diseases, including drug abuse. Our laboratory and others have consistently found that mRNA levels of dynorphin in the brains of rats are elevated following a single injection of cocaine [Hurd et al., 1992], cocaine self-administration [Hurd et al., 1992; Daunais et al., 1993], and acute or chronic “binge” cocaine administration [Spangler et al., 1993, 1996]. In addition, opioid peptides are important neuromodulators that are directly and indirectly associated with the reward pathways in the brain. Using microdialysis in awake freely moving rats, we have shown that direct injection of dynorphin $A_{1–17}$ to the nucleus accumbens, one of the major rewarding sites in the brain affected by cocaine, causes a significant reduction in basal levels of dopamine in the extracellular fluid in this brain region [Claye et al., 1997]. Our laboratory also reported that dynorphin $A_{1–13}$, which is the shortened natural sequence analog to the natural peptide dynorphin $A_{1–17}$ derived from prodynorphin, causes elevation of serum levels of prolactin in healthy human volunteers, indicating that dynorphin peptides act to lower dopaminergic tone in the tuberoinfundibular system [Kreek et al., 1999]. Taken together, these studies suggest that allelic variation of the prodynorphin gene may contribute to individual variation in vulnerability to drug addiction.

Recently a 68-base pair (bp) sequence within the core promoter region of the human prodynorphin gene was found to occur as a polymorphic element, present in one copy or in tandem repeated elements two, three, or four times [Zimprich et al., 2000]. This 68-bp repeat element contains a putative AP-1 transcription factor binding site (TGACTTA), which differs in only one position from the consensus AP-1 site (TGACTCA). Using the electrophoretic mobility shift assay, Zimprich et al. [2000] reported that AP-1 transcription factor binding occurs to this site. More interestingly, in vitro assays in cells transfected with the reporter gene chloramphenicol acetyltransferase (CAT) suggest that there is an allele-

dependent difference in the promoter activity of the prodynorphin gene. Under basal conditions, no difference was observed among the promoter allele constructs that contain one, two, three, or four copies of the 68-bp repetitive element. However, after the AP-1 transcription factor complex was activated, using 12-*O*-tetradecanoylphenol 13-acetate (TPA), a significant increase (~50%) in the promoter activity was observed for the alleles containing three (47% increase) or four repeats (53% increase) to a similar extent, but not one or two repeats [Zimprich et al., 2000].

We hypothesized that dynorphin may act in a counter-regulatory fashion to attenuate the increases in synaptic dopamine levels caused by cocaine [Kreek, 1996; Kreek et al., 1999; LaForge et al., 2000]. Because alleles containing three or four 68-bp repeats might show increased synthesis of dynorphin in response to stimuli that activate AP-1 transcription factors, we hypothesize that the three or four repeat alleles may exert a protective effect against cocaine dependence or abuse by a mechanism of an increased dynorphin “response.”

In this study, we examined the allelic frequencies and genotype distribution of this novel polymorphism in 174 well-characterized unrelated individuals with a primary diagnosis (DSM-IV criteria) of cocaine dependence ($N = 61$) or abuse ($N = 22$), and controls with no history of any psychoactive substance dependence or abuse ($N = 91$). Based on the different promoter activity of the four alleles, we tested whether alleles differing in the number of repeats might be related to cocaine dependence or abuse, which would support the hypothesis that the prodynorphin gene may contribute to individual variation in vulnerability to drug addiction.

MATERIAL AND METHODS

Study Subjects: Recruitment and Diagnostic Procedures

Study subjects were individuals, all unrelated, from a large pool of subjects recruited to an ongoing study of the genetic contribution to addictions conducted in the Laboratory of the Biology of Addictive Diseases at the Rockefeller University. Diagnoses were made using DSM-IV criteria set by the American Psychiatric Association. Subjects with a diagnosis of schizophrenia or any other psychosis were excluded. All subjects in the cocaine dependence or abuse groups met the DSM-IV criteria for cocaine dependence or abuse with criteria as follows to provide a greater stringency: 1) subject demonstrates a pattern of regular cocaine use including either two or more weekly binges or two or more uses per day for a period of at least six months; 2) subject identified cocaine as the current or previous drug of choice; 3) subject suffered major life alterations due to cocaine use, such as problems with relationships, employment, or legal matters; and 4) subject may or may not abuse or be addicted to other licit or illicit drugs (except opiates), either currently or previously, but none of these drugs were identified as a drug of primary focus. A history of opiate dependence or abuse was used as an exclusion criterion for this group.

All subjects in the control group reported no history of any current or previous illicit drug or alcohol dependence or abuse. Individuals who met DSM-IV criteria for any other substance dependence or abuse, or who had any prior period or ongoing drug or alcohol abuse were excluded from the control group. Additional exclusion criteria were as follows: 1) subjects

in clinical management of chronic pain; 2) subjects who do not meet DSM-IV criteria for any substance dependence or abuse, including cannabis, but who have had more than two extended periods of use of alcohol or any illicit drug (excluding cannabis, see below) during their lifetime. Individuals who had abused cannabis for no more than 12 days during the 30-day period before assessment, or whose use during the 30-day period before assessment did not exceed the equivalent of two cannabis cigarettes per day, were not excluded from study. Users of caffeine or nicotine were not excluded from the control group.

A total of 174 subjects were included in this study (Table I). Each subject entering the study demonstrated competence to understand the study procedures and to understand and sign the Rockefeller University Institutional Review Board-approved informed consent. Toxicological analyses for several drugs of abuse and alcohol were performed on urine samples from all subjects, including control subjects. All subjects were interviewed by a research nurse, clinical psychologist, or psychiatrist with regard to medical and psychiatric history, and the severity of symptoms were evaluated using the Addiction Severity Index (ASI) [McLellan et al., 1992], and some subjects were also assessed with the Structured Clinical Interview for DSM-IV Axis I Disorder (SCID). Subjects were ethnically classified as European American, African American, or Hispanic American (approximately half of the subjects in this group were originally from Puerto Rico and the rest were from the Dominican Republic) based on detailed information provided by the subjects. Nine subjects who reported having parents from different ethnic groups (Table I) were genotyped to calculate the overall allelic frequency but were not included in any other analyses for which data was stratified by ethnicity.

Genotyping

Genomic DNA was extracted from blood samples obtained from consenting subjects. Approximately 100 ng of total genomic DNA was amplified by polymerase chain reaction (PCR) with oligonucleotide primers for the promoter region of the prodynorphin gene containing the 68-bp repeat element (accession number: X02536). Sequences of the primers are as follows: 5′-CTG TGT ATG GAG AGG CTG AGT-3′ (upper primer); 5′-AGG CGG TTA GGT AGA GTT GTC-3′ (lower primer). A PCR protocol, commonly referred to as a “step-down” protocol, was performed with an AmpliTaqGold kit (Applied Biosystems, Foster City, CA) in a 50 μl reaction mixture that contained standard PCR buffer, 2.5 mM magnesium chloride, 0.2 mM each dNTPs, 0.5 μM each primer, and 5 U AmpliTaqGold DNA polymerase. An initial denaturation step at 95°C for six min was followed by five sets of three cycles. Every set of three cycles consisted of a denaturation step (94°C for 30 sec) followed by a progressively lower annealing temperature of 66°C, 63°C, 60°C, 57°C, and 54°C for 30 sec each (“step-down”), then an extension step at 72°C for one min. Thirty-five cycles were then performed with an annealing temperature of 52°C. A final extension step was performed at 72°C for six min. All PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide to visualize DNAs. A subset of samples was further verified by direct sequencing by the Rockefeller University DNA Sequencing Center. Genotypes were determined by two independent assessors (A.C.H.C. and K.S.L.), who were blind to diagnostic data (phenotypes). Samples from seven subjects that either could not

produce a consensus genotype or could not be amplified at all after repeated experiments were not included in the 174 subjects in Table I.

Statistical Analyses

The overall frequencies for each allele and genotype were determined. With data stratified by ethnicity, pooled relative risk (RR) and Mantel-Haenszel Chi-square tests were used to evaluate differences in RR between a combined cocaine group (dependence or abuse) and control subjects [Mantel and Haenszel, 1959]. Within each of the European, African, and Hispanic ethnic groups, a Chi-square test was performed to determine whether the allelic distribution was in Hardy-Weinberg equilibrium.

RESULTS

To find the variants of the promoter region of the human prodynorphin gene, DNA samples from individuals with cocaine abuse or dependence, or controls, were amplified with the same pair of primers we designed. Figure 1 shows the positions of primers used for PCR, 68-bp tandem repeat, CAAT, and TATA boxes of the human prodynorphin gene promoter. Four different lengths of PCR products were observed (273, 341, 409, and 477 bp). Direct sequencing of a subset (20) of these samples confirmed that different lengths were due to a 68-bp repeating element occurring either as a single element (273 bp) or as a tandem element that repeated two (341 bp), three (409 bp), or four (477 bp) times. These results are consistent with the previous report [Zimprich et al., 2000], although the amplified region in the previous study was larger (386 to 590 bp). We followed the previous report and designated them as alleles 1–4 according to the number of tandem repeats. Genotype and allelic distribution among control subjects or those with cocaine dependence or abuse, stratified by ethnicity, is shown in Table II. This distribution was in good accord with the Hardy-Weinberg equilibrium and was similar to that in the previous report in Caucasian subjects from the Rhine-Rhur industrial area in western Germany [Zimprich et al., 2000].

Because of small subject numbers, and with the increased stringency of categorization beyond DSM-IV criteria for dependence or abuse in this study, the pooled cocaine dependence and abuse group was compared to controls. Allele frequencies were grouped with alleles 1 and 2 combined vs. alleles 3 and 4 combined, based on the fact that a significant increase (~50%) in the promoter activity had been observed for 3 or 4, but not 1 or 2, alleles in the in vitro study [Zimprich et al., 2000]. Mantel-Haenszel Chi-square analysis was performed for grouped alleles 1 or 2 vs. 3 or 4: RR = 0.59 (95% confidence interval 0.37–0.95), $\chi^2_{(1)} = 4.14$, $P = 0.042$ (Table III). There was no evidence of heterogeneity between ethnic group strata ($P = 0.51$). Each ethnic group stratum had a RR < 1. Only one stratum (African-American) on its own had a borderline significance level sufficient to reject the null hypothesis of no effect (P = 0.049). With the evidence from this study, alleles 3 or 4, which contain three or four copies of the 68-bp tandem repeat in the promoter region of the prodynorphin gene, may contribute to relative protection and decrease individual vulnerability to develop cocaine dependence or abuse.

DISCUSSION

In the present study, we report that specific polymorphisms in the promoter region of the prodynorphin gene, which had been previously identified as functional in cellular assays (alleles containing three or four copies of a 68-bp repeat increase stimulated promoter activity using a molecular cellular construct [Zimprich et al., 2000]), may confer a protective effect against cocaine dependence or abuse.

It is well accepted that social, psychological, and environmental factors contribute to addiction, but it is clear that there is a strong genetic component in the vulnerability to become addicted once self-exposed to substances of abuse [e.g., Grove et al., 1990; Altman et al., 1996; Tsuang et al., 1996, 1998]. The “candidate gene approach,” which is based on the knowledge of the pathophysiology and possible biological or signaling pathways of a disease, is one of the more economic and efficient ways to search for specific genetic variations that contribute to these complex disorders [Lander and Schork, 1994]. Dopamine is one of the most important neurotransmitters involved in the reward pathways in the brain and is thought to be crucial to the action of several substances of abuse [e.g., Koob, 1992; Di Chiara, 1995; Altman et al., 1996]. Cocaine increases the synaptic dopamine levels through blocking the dopamine transporter [e.g., Di Chiara and Imperato, 1988; Di Chiara and North, 1992]. It has been hypothesized that individual variations in genes encod ing proteins involved in dopaminergic transmission may explain some of the genetic bases for individual differences in vulnerability to drug abuse. However, research has so far failed to unequivocally identify any such molecular variation in the genes encoding the protein in the dopamine system [e.g., Blum et al., 1990; Goldman et al., 1992; Uhl et al., 1993; Altman et al., 1996; Comings, 1998]. One possible explanation is that the variations contributing to the differences in vulnerability to drug abuse may occur in the genes encoding proteins that indirectly modulate the dopa-mine systems. We and others have shown that dynorphin lowers dopaminergic tone in both rodents and humans, and hypothesized that the opioid neuropep-tide dynorphin may act in a counter-regulatory fashion to attenuate the increases in synaptic dopamine levels caused by cocaine [Van Vugt et al., 1981; Sivam, 1989; Kreek, 1996; Claye et al., 1997; Kreek et al., 1999]. Several studies in different species have demonstrated that dynorphin may prevent or reverse opiate withdrawal signs and symptoms in morphine-dependent rodents, and this may also pertain in humans [Wen and Ho, 1982; Takemori et al., 1993]. Because alleles containing three or four 68-bp repeats might show increased synthesis of dynorphin in response to stimuli that activate AP-1 transcription factors, we hypothesize that the three or four repeat alleles may exert a protective effect against cocaine abuse.

Our results suggest that allelic variation at the promoter region of the prodynorphin gene may contribute to the individual variation in vulnerability to cocaine dependence or abuse. Alleles containing three or four repeats of the 68-bp tandem, which have a significantly higher promoter activity (~50% increase) of the prodynorphin gene, showed a possible protective effect against cocaine dependence or abuse. The Mantel-Haenszel Chi-square analysis reached a marginally significant level ($\chi^2_{(1)}$ 4.14, P = 0.042), although only one candidate gene was tested due to its functionality. It could be argued that many more candidate genes will be tested with this data set and the α value should be adjusted for

multiple testing. Therefore, a more stringent criterion than $\alpha = 0.05$ should be used to reject the null hypothesis of no effect. For genetic case control studies, as of yet, there is no consensus on how to correct for testing multiple markers at candidate genes and in genome scans.

It is possible that the association that was observed is not due to a polymorphism in the human prodynorphin gene providing a protective effect or because of linkage disequilibrum between these alleles and a functional polymorphism, but instead is a spurious finding due to population stratification. Of the three ethnic groups that were studied, association due to population stratification is most likely to occur in the African American and Hispanic ethnic groups. We cannot rule out the possible effect of admixture in the populations we studied. Additional studies should be carried out using larger sample sizes. For this study, the sample sizes were small, especially for the Hispanic-American stratum. It would also be beneficial in future studies to genotype additional markers with ethnic-specific allele frequencies [Shriver et al., 1997; Parra et al., 1998] and apply methods that control for population stratification [Pritchard and Rosenberg, 1999; Pritchard et al., 2000]. We would recommend that this polymorphism be studied further in other data sets, and when possible, with methods designed to detect linkage, linkage disequilibrium, or both.

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Fig. 1.

(Partial) sequence of the human prodynorphin gene promoter. The 68-bp repeat element (in green, blue, and orange colors; three repeats shown), the caat and tata boxes (in bold font), and the primers used for PCR (bold and underlined) are indicated. Note a putative recognition site for the AP-1 transcription factor (tgactta) within this 68-bp repeat element. This sequence differs only in one position from the consensus AP-1 site (tgactca).

TABLE I.

Demography of Total Subjects Included in Study (N = 174)

	Cocaine abuse	Cocaine dependence	Controls	Totals
European American (E)	7	11	43	61
African American (Af)	13	36	33	82
Hispanic American (H)	2	7	13	22
E/Af	—	2	—	2
E/H	—	—	1	1
Af/H	—	4	1	5
Af/Native American	—	1	—	1
Totals	22	61	91	174

TABLE II.

Genotype and Allelic Distribution Stratified by Ethnicity*

		Genotypes											Alleles				
Ethnicity	**Drug**	**1/1**	**1/2**	**1/3**	**1/4**	**2/2**	**2/3**	**2/4**	**3/3**	**3/4**	**4/4**	**Total**	**1**	**2**	**3**	**4**	**Total**
European American	Cocaine abuse	0	0	0	0	1	4	0	2	0	0	7	0	6	8	0	14
	Coc. dep.	0	0	1	0	0	4	0	6	0	0	11	1	4	17	0	22
	Control	0	1	0	0	3	16	0	23	0	0	43	1	23	62	0	86
Total		0	1	1	0	4	24	0	31	0	0	61	2	33	87	0	122
%		0	1.6	1.6	0	6.6	39.4	0	50.8	0	0	100	1.6	27.1	71.3	0	100
African American	Cocaine abuse	0	0	0	0	7	2	0	4	0	0	13	0	16	10	0	26
	Coc. dep.	0	0	0	0	13	8	1	12	2	0	36	0	35	34	3	72
	Control	1	0	0	0	6	8	2	14	1	1	33	2	22	37	5	66
Total		1	0	0	0	26	18	3	30	3	1	82	2	73	81	8	164
%		1.2	0	0	0	31.7	21.9	3.7	36.6	3.7	1.2	100	1.2	44.5	49.4	4.9	100
Hispanic American	Cocaine abuse	0	0	0	0	0	0	1	1	0	0	2	0	1	2	1	4
	Coc. dep.	1	0	0	0	0	4	0	2	0	0	7	2	4	8	0	14
	Control	0	0	0	0	1	3	0	9	0	0	13	0	5	21	0	26
Total		1	0	0	0	1	7	1	12	0	0	22	2	10	31	1	44
%		4.5	0	0	0	4.5	31.9	4.5	54.6	0	0	100	4.6	22.7	70.4	2.3	
Totals		2	1	1	0	31	49	4	73	3	1	165	6	116	199	9	330
%		1.2	0.6	0.6	0	18.8	29.8	2.4	44.2	1.8	0.6	100	1.8	35.2	60.3	2.7	100

*Individuals of mixed ethnicity are not included.

Coc. dep., cocaine dependence.

TABLE III.

Relative Risk and Mantel-Haenszel Chi-square Test on Allelic Distribution in Combined Cocaine Abuse*

	Cocaine abuse or dependence		Controls		
Alleles	**3 + 4**	**1 + 2**	**3 + 4**	**1 + 2**	**Relative risk, RR (and 95% confidence interval on RR)**
European American	25	11	62	24	0.88
	69.4%	30.6%	72.1%	27.9%	(0.38–1.99)
African American	47	51	42	24	0.53
	48.0%	52.0%	63.6%	36.4%	(0.28–1.00)
Hispanic American	11	7	21	5	0.37
	61.1%	38.9%	80.8%	19.2%	(0.11–1.40)

Overall (pooled) RR = 0.59 (95% confidence interval 0.37–0.95), $\chi^2_{(1)} = 4.14$, $P = 0.042$. Individuals of mixed ethnicity are not included.

*Dependence group and control subjects stratified by ethnicity.