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Association of polymorphisms in the melanocortin receptor type 2 (*MC2R*, ACTH receptor) gene with heroin addiction

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Abstract

The melanocortin receptor type 2 (*MC2R* or adrenocorticotrophic hormone, ACTH receptor) gene (*MC2R*) encodes a protein involved in regulation of adrenal cortisol secretion, important in the physiological response to stressors. A variant of *MC2R*, –179A>G, results in reduction of promoter activity and less adrenal action. We hypothesize that altered stress responsivity plays a key role in the initiation of substance abuse. By direct resequencing of the promoter region and exons 1 and 2 of the *MC2R* gene in 272 subjects including Caucasians, Hispanics and African Americans with approximately equal numbers of former heroin addicts and normal volunteers, we identified five novel variants each with allele frequency < 2%. Previously reported polymorphisms –184G>A (rs2186944), –179A>G, 833A>C (rs28926182), 952T>C (rs4797825), 1005C>T (rs4797824) and 1579T>C (rs4308014) were each in allelic frequency ≥2% in one or more ethnic groups. These polymorphisms were genotyped in 632 subjects (260 Caucasians, 168 Hispanics, 183 African Americans and 21 Asians) using TaqMan assays. Significant differences in genotype frequency among ethnic groups studied were found for each of the six variants analyzed. We found a significant association ($p=0.0004$, experiment-wise $p=0.0072$) of the allele –184A with a protective effect from heroin addiction in Hispanics. Also, in Hispanics only we found the haplotype GACT consisting of four variants (–184G>A, –179A>G, 833A>C and 1005C>T) to be significantly associated with heroin addiction ($p=0.0014$, experiment-wise $p=0.0168$), whereas another haplotype, AACT, consisting of the same variants, was associated with a protective effect from heroin addiction ($p=0.0039$, experiment-wise $p=0.0468$).

Keywords

ACTH receptor; *MC2R* gene; polymorphisms; haplotypes; genotypes; heroin addiction

Previous studies have demonstrated that individuals with depression, stress and anxiety are more vulnerable for the initiation and development of drug addiction [e. g., 10]. Our working hypothesis is that dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis may contribute to acquisition, persistence of and relapse to drug addiction specifically. HPA hyper

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reactivity has been observed in medication-free illicit drug-free former heroin addicts [8] and in cocaine addicts [20]. The melanocortin 2 receptor (MC2R or adrenocorticotrophic hormone, ACTH receptor [14]), which is located in the adrenal cortex, activates the production of cortisol, which acts in a negative-feedback manner on both the hypothalamus and pituitary to inhibit the production and release of CRF, β -endorphin and ACTH [9].

ACTH, the 39-amino acid peptide derived from anterior pituitary peptide proopiomelanocortin (POMC), is the major hormone that regulates adrenal glucocorticoid and androgen synthesis in the zonae fasciculata and reticularis in the adrenal cortex. ACTH binds to its specific MC2R, a seven-transmembrane domain receptor that belongs to the melanocortin subfamily of the G protein-coupled receptor family. This leads to activation of the adenylate cyclase pathway followed by activation of protein kinase A [2]. Among five known melanocortin receptors, only MC2R specifically binds ACTH and is located in the adrenal cortex. However, ACTH as well as α -MSH binds to MC1R. ACTH along with α -MSH and β -MSH binds to MC4R in the brain and regulates appetite. To a lesser extent, ACTH as well as α -MSH and β -MSH and γ -MSH also binds to MC5R and MC3R (for review see [4]).

The *MC2R* gene is located in chromosome 18p11.2 and has two exons. The coding region for the 297-amino acid protein is located in the second exon. Several polymorphisms in the *MC2R* gene [3,24,26] were reported in patients with familial glucocorticoid deficiency (FGD). In healthy volunteers the polymorphism -179A>G (-2T>C according to the authors' nomenclature [18,22]) reduces the promoter activity resulting in less adrenal activity, that is, cortisol production after ACTH stimulation [18]. This polymorphism is located 2 nucleotides upstream from the start of transcription of exon 1. A number of putative transcription factor binding sites including AP1, CRE and Sp1 have been identified in the promoter region of the *MC2R* gene [15].

For this investigation, unrelated subjects from an ethnically diverse population, including African Americans, Caucasians, Hispanics and Asians were recruited in New York City between February 7, 1995 and November 9, 2004. All subjects signed informed consent for genetic studies using DNA, approved by the Institutional Review Board of The Rockefeller University Hospital. Subjects provided self-reported ethnicity. Individuals from mixed ethnicities were not included in this study. Subjects with a primary diagnosis of heroin addiction were patients in methadone maintenance treatment programs in New York City, or were individuals who met the stringent Federally-regulated criteria for admission into such programs: subjects must have self-administered illicit opioids, primarily heroin, daily for one year or more and continued to use in spite of potential negative consequences to themselves and others [19]. Subjects were not excluded from this group for prior or ongoing co-dependence on other substances of abuse as long as heroin addiction was their primary diagnosis. A total of 632 case and control subjects who met the criteria were identified in this study cohort.

To provide more detailed characterization of the subjects involved in our research, all subjects who met methadone maintenance criteria as well as control subjects recruited after June 26, 2000 (130 subjects total), were also administered the Kreek-McHugh-Schluger-Kellogg (KMSK) scale which quantifies frequency, mode, amount, and duration of self-exposure to opiates, cocaine, alcohol, and tobacco [7].

Control subjects (264 individuals) had no ongoing or prior abuse of illicit drugs or alcohol and were assessed using the Addiction Severity Index [12]. Subjects were excluded from the control group if they used any illicit or licit drug, or alcohol to intoxication, at least once during the past 30 days. Subjects who used cannabis for up to three days during the previous thirty days were not excluded (eleven subjects total). For prior abuse, subjects were excluded for any illicit

drug use, or alcohol use to intoxication, for three or more times per week during six months or longer period with the exception of cannabis.

DNA was isolated from peripheral blood lymphocytes [13]. Exon-intron structure of the gene was found by alignment of NM_000529.2 and chr18. The numbering of the polymorphism positions is based upon the system [11] that assigns value +1 to the first nucleotide of the ATG initiation codon; bases belonging to the coding region have positive values.

For the search of novel polymorphisms by direct resequencing, we analyzed DNA of a subset of subjects of Hispanic, African American and Caucasian ethnicities (272 DNA samples total). We resequenced exon 1, exon 2, 50–80 nt long flanking regions of exon 1 and exon 2, and the 500 nt part of 5'-untranslated region (UTR) upstream from the transcription initiation site with a set of oligonucleotide primers designed using Primer Express software (Applied Biosystems, ABI, Foster City, CA, Supplement 1) and custom synthesized by ABI. This was done using both forward and reverse primers for each amplicon in a step-down protocol as previously described [27]. PCR products were then purified on Qiagen BioRobot 9600® (Venlo, Netherlands) and sequenced in both forward and reverse directions using BigDye Ready Reaction Kit (v3, ABI) on ABI 3700 DNA analyzer (ABI). Electropherograms were assembled using Vector NTI Advance 9 software (Invitrogen) and analyzed by two independent investigators.

Polymorphisms of high frequency were analyzed by 5' Fluorogenic Exonuclease Assay (TaqMan) in this and an additional group of 360 DNA samples, so that 632 samples total were used (Table 1). Genotyping by (TaqMan) was performed in 5 µl volumes in 384-well plates using Platinum® quantitative PCR SuperMix-UDG (Invitrogen) and 1 to 10 ng of DNA per reaction with oligonucleotide primers and TaqMan probes designed using Primer Express software (ABI, Supplement 1) and custom synthesized by ABI. Amplification reactions for genotyping of polymorphisms -184G>A, 952T>C, 1005C>T and 1579T>C were done on a GeneAmp® PCR system 9700 and the dual 384-well sample block module (ABI) according to the manufacturer's protocol (ABI). Genotyping of polymorphisms 833A>C and -179A>G was done in the same manner with extension at 54°C for 2 min. Upon completion of PCR cycling, genotype analysis was performed on the ABI Prism® 7900 sequence detection system using SDS 2.2 software (ABI).

The results of fluorescent PCR assay were verified by comparison with the results of direct sequencing using over 200 DNA samples. We found >99.2% concordance between the results provided by these two methods.

Tests for Hardy-Weinberg equilibrium (HWE) were done in the groups of control individuals as well as in cases. If any subject group significantly deviated from HWE, genotyping errors were looked for; TaqMan results were reexamined. The statistically inferred haplotype pairs were determined using the method implemented in the PHASE program [23]. Tests for association of history of severe heroin addiction with genotypes, alleles, and haplotypes in different ethnicities were performed using Fisher exact tests.

To study pairwise linkage disequilibrium (LD) in control subjects we evaluated D' and r^2 (Δ^2) values. These are displayed using Graphical Overview of Linkage Disequilibrium (GOLD) software [1]. We also used the four-gamete test [6], where we determined the number of two-locus haplotypes with a relative frequency greater than 1%. For haplotype analysis in each ethnic group we excluded those polymorphisms for which genotypes were not in HWE in control subjects. We did not exclude genotypes that were in linkage disequilibrium with each other since this would not significantly change the results of analysis. Experiment-wise p-values were calculated using the Bonferroni correction. We adjusted for the number of ethnic groups studied and one of the following: the number of haplotype (3 ethnicities $\times$ 4 haplotypes

= correction factor 12), or genotype (3 ethnicities $\times$ 6 genotypes = correction factor 18) or allelic tests (3 ethnicities $\times$ 6 genotypes = correction factor 18); we did not adjust for all three types of tests (genotype, haplotype, allelic) concurrently because these tests are correlated.

As the result of the direct resequencing, we identified five novel variants in cases and controls (see Supplement 3) 318G>A, 715C>T, 754C>A, 795G>A, 880T>C and also, the previously reported high frequency variants ($\geq 2\%$, polymorphisms) –184G>A, –179A>G, 833A>C and 952T>C (Supplements 2, 3). Polymorphisms 1005C>T and 1579T>C from the 3'-UTR were selected from the dbSNP database. Variants 715C>T, 754C>A, 833A>C and 880T>C result in non-homologous substitution of amino acids V239I, A252S, F278C and S294G, respectively.

There was significant deviation from HWE ($\alpha=0.05$) in the controls in both Hispanics and Asians for the polymorphisms 952T>C and 1579T>C that are in complete LD ($p=0.006$ for Hispanics and $p=0.002$ for Asians). The possible reason for this observation might be a small representation of these ethnic groups in the study, or admixture. This pattern was not observed either in Caucasians or in African Americans for these SNPs. For the polymorphism –184G>A we found significant deviation from HWE in controls in the African American group ($p<0.001$). To evaluate the presence of genotype errors in these study groups, the results of TaqMan analysis were reexamined. For those subjects for whom both TaqMan and direct resequencing data were available, we found complete concordance in genotype calls provided by these two independent methods.

Deviation from HWE in cases may be indicative of an association [16]. We found evidence of significant deviation from HWE in the African American group of former heroin-addicted subjects ($p=0.005$) but not controls for polymorphism –179A>G. However, we did not find an association of this polymorphism with heroin addiction in any ethnicity tested.

There was a significant difference overall in allele frequency of control subjects among all four ethnic groups analyzed using 4×2 contingency tables in all six polymorphisms tested (Table 2).

In Hispanics, we found increased frequency of the minor allele –184A in the control group (point-wise $p=0.0004$, Table 3 and Supplement 4; corrected for three ethnicities and six polymorphisms tested $p=0.0072$), which might be an indication of a protective effect from heroin addiction. A similar effect was also found in Hispanics in a genotypes ($p=0.0012$, corrected for multiple tests $p=0.0216$, Table 3 and Supplement 4) and specific combinations (GG vs. GA+ AA, $p=0.001$, corrected for multiple tests $p=0.018$, Supplement 5). In Hispanics the allele 1005T was associated with heroin addiction ($p=0.031$, not significant experiment-wise).

We found that polymorphisms 952T>C and 1579T>C are in almost complete linkage disequilibrium in African Americans, Caucasians, Hispanics and Asians (r^2 values are 0.9704, 0.9666, 0.9999 and 0.6440, respectively, see Supplements 6, 7). In Caucasians polymorphism 833A>C was monomorphic in the controls, and therefore we were unable to obtain linkage disequilibrium between this and any other polymorphism.

To analyze the collective effect of different alleles, we performed association studies of inferred haplotypes with heroin addiction. Haplotypes having frequency less than 5% in both heroin-addicted and control groups were combined in the separate category “others” (Table 4, Supplement 8). In each ethnicity, each haplotype was tested versus the sum of all other haplotypes. Polymorphisms 952T>C and 1579T>C were excluded from haplotype analysis in the Hispanic group since the distribution of genotypes of these polymorphisms in the control group was not in HWE. Since we found significant deviation from HWE in the African

American control group for polymorphism $-184G>A$, this polymorphism was eliminated from the haplotype analysis in this ethnic group.

The results of the association analyses of statistically inferred haplotypes with heroin addiction for African Americans, Caucasians and Hispanics are shown in Table 4 and Supplement 8. In Hispanics, associations of the haplotype GACT (at positions -184 , -179 , 833 and 1005) with heroin addiction ($p=0.0014$, corrected for multiple tests $p=0.0168$) and haplotype AACT (at the same positions) with the protective effect from heroin addiction ($p=0.0039$, corrected for multiple tests $p=0.0468$), were found. These two haplotypes differ from each other only by $-184G>A$; allele $-184A$ was found to be associated with the protective effect from drug addiction in Hispanics (see above). The observed associations of haplotype GACT with heroin addiction and AACT with a protective effect from heroin addiction are likely to be a result of single $-184G>A$ SNP effect.

In previous studies variants in the *MC2R* gene [e. g., 3, 24, 26] have been linked to familial glucocorticoid deficiency (FGD), a condition characterized by cortisol deficiency despite a high plasma level of ACTH with normal aldosterone response [e.g., 21]. Some variants of *MC2R* associated with FGD, including S74I, I44M and R146H, result in impaired maximal cAMP response; others, including D107N, R128C and T159K, result in loss of sensitivity for cAMP generation compared to the prototype receptor [5].

The polymorphisms $-184G>A$ and $-179A>G$ are located in the promoter region of the *MC2R* gene and therefore may potentially influence the initiation of transcription. Several potential transcription initiation binding sites including SF-1 (positions -212 and -275), AP-1 (-279) and CRE (-285 , -355) are located in proximity to these polymorphisms [22]. However, influence of the polymorphisms on the function of these sites may not be inferred from location alone. In Hispanics, we found an experiment-wise significant association of the minor allele A of the polymorphism $-184G>A$ and the haplotype AACT bearing $-184A$ allele with a protective effect from heroin addiction. A strong effect was also found in association of individual genotype frequencies of this polymorphism or combination GA+AA vs. GG with a protective effect from heroin addiction. These findings may provide genetic evidence supporting our working hypothesis that dysregulation of the HPA axis contributes to the initiation and development of drug addiction. Reduced cortisol production would reduce negative feedback by cortisol in CRF and in POMC. However, the precise impact of these two SNPs on any perturbation of cortisol production has not been fully elucidated.

Substitution A to G in polymorphism $-179A>G$ ($-2T>C$) discovered by another group [22] results in lower promoter activity *in vitro* and is found in association with impaired cortisol response to ACTH stimulation *in vivo*. Heterozygous genotype was found in 19% frequency and homozygous variant of the minor allele of this polymorphism was found in less than 1% frequency in German subjects [22]. A clinical study with the six-hour ACTH stimulation test showed that homozygous prototype AA carriers have a significantly higher dehydroepiandrosterone (DHEA) response than homozygous GG carriers, while baseline DHEA concentrations did not differ between groups [18]. We found distributions of the $-179A>G$ polymorphism in Caucasian (MAF 9.3%, Table 2) and Hispanic controls (11.5%) similar to each other, although in the African American control group the frequency of this polymorphism (both genotype and allele) was considerably lower (2.9%). No association of $-179A>G$ alone with heroin addiction was found in any ethnic group in our current study.

We found the polymorphism $-184G>A$ to be in high frequency in African American (MAF 12.6%), Hispanic (16.7%) and Asian (10.0%) control groups with minor allele frequency (Table 2). However, only in the Hispanic group both polymorphisms $-184G>A$ and $-179A>G$ were in high frequency (16.7% and 11.5%, respectively).

According to the protein structure suggested by the homology to the beta-2 adrenergic receptor (ADRB2), the non-synonymous variants 833A>C (F278C) and 880T>C (S294G) are located in the COOH- intracellular tail of the receptor (Supplement 2). Variant 833A>C was found in relatively high frequency only in the African American control group (6.3%; Table 2). Other rare variants that were identified in the current study may affect the secondary structure of the receptor, although due to their low representation we were unable to use them in the association studies.

In conclusion, in an examination of several polymorphisms in the promoter, 3'-untranscribed and coding regions of the *MC2R* gene, we found an association of the allele -184A with a protective effect from heroin addiction in Hispanics. Further studies are required to evaluate the functionality of this polymorphism, located in proximity to the functional -179A>G.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Demography of study subjects

Ethnicity	Number of subjects						Subtotal
	Control			Heroin Addicted			
	Female	Male	Total	Female	Male	Total	
African American	41	46	87	43	53	96	183
Caucasian	55	63	118	49	93	142	260
Hispanic	26	13	39	42	87	129	168
Asian	9	11	20	1	0	1	21
Total	131	133	264	135	233	368	632

Table 2
Allelic distribution of selected polymorphisms of the *MC2R* gene in control subjects of different ethnicities

Ethnicity	-184G>A (rs2186944)			-179A>G			833A>C (rs28926182)		
	Allele G counts (frequency)	Allele A counts (frequency)		Allele A counts (frequency)	Allele G counts (frequency)		Allele A counts (frequency)	Allele C counts (frequency)	
African Americans	152 (0.873)	22 (0.126)		169 (0.971)	5 (0.029)		163 (0.937)	11 (0.063)	
Caucasian	235 (0.996)	1 (0.004)		214 (0.907)	22 (0.093)		236 (1.000)	0 (0.000)	
Hispanic	65 (0.833)	13 (0.167)		69 (0.885)	9 (0.115)		77 (0.987)	1 (0.013)	
Asians	36 (0.900)	4 (0.100)		31(0.775)	9 (0.225)		40 (1.000)	0 (0.000)	
χ^2 (P)		33.1657 (<0.0001)			18.2490 (0.0004)			19.6074 (0.0002)	
Ethnicity	952T>C (rs4797825)			1005C>T (rs4797824)			1579T>C (rs4308014)		
	Allele C counts (frequency)	Allele T counts (frequency)		Allele C counts (frequency)	Allele T counts (frequency)		Allele T counts (frequency)	Allele C counts (frequency)	
African Americans	46 (0.264)	128 (0.736)		138 (0.793)	36 (0.207)		129 (0.741)	45 (0.259)	
Caucasian	122 (0.517)	114 (0.483)		153 (0.648)	83 (0.352)		112 (0.475)	124 (0.525)	
Hispanic	40 (0.513)	38 (0.487)		63 (0.808)	15 (0.192)		38 (0.487)	40 (0.513)	
Asians	21 (0.525)	19 (0.475)		29 (0.725)	11 (0.275)		19 (0.475)	21 (0.525)	
χ^2 (P)		30.3187 (<0.0001)			13.6970 (0.0033)			33.1047 (<0.0001)	

Table 3

Allelic and genotype association of the polymorphisms –184G>A and 1005C>T of the *MC2R* gene with heroin addiction^a

A. Polymorphism –184G>A							
Ethnicity	Group of subjects	Number of alleles G (frequency)	Number of alleles A (frequency)	P (Allele test)	Genotype GG (frequency)	Genotype GA (frequency)	P (Genotype Test)
African American	Controls	152 (0.874)	22 (0.126)	NA	72 (0.828)	8 (0.092)	NA
	Heroin-addicted	175 (0.911)	17 (0.089)		84 (0.875)	7 (0.073)	5 (0.052)
	Controls	235 (0.996)	1 (0.004)		117 (0.992)	1 (0.008)	0
	Heroin-addicted	280 (0.986)	4 (0.014)		138 (0.972)	4 (0.028)	0
Caucasian	Controls	65 (0.833)	13 (0.167)	0.0004	28 (0.718)	9 (0.231)	2 (0.051)
	Heroin-addicted	248 (0.961)	10 (0.039)		120 (0.930)	8 (0.062)	1 (0.008)
B. Polymorphism 1005C>T							
Ethnicity	Group of subjects	Number of alleles C (frequency)	Number of alleles T (frequency)	P (Allele test)	Genotype CC (frequency)	Genotype CT (frequency)	P (Genotype Test)
African American	Controls	138 (0.793)	36 (0.207)	0.7048	57 (0.655)	24 (0.276)	6 (0.069)
	Heroin-addicted	149 (0.776)	43 (0.224)		57 (0.594)	35 (0.364)	4 (0.042)
	Controls	153 (0.648)	83 (0.352)		50 (0.424)	53 (0.449)	15 (0.127)
	Heroin-addicted	183 (0.644)	101 (0.356)		60 (0.422)	63 (0.444)	19 (0.134)
Caucasian	Controls	63 (0.808)	15 (0.192)	0.0331	24 (0.615)	15 (0.385)	0
	Heroin-addicted	176 (0.682)	82 (0.318)		61 (0.473)	54 (0.419)	14 (0.108)

^a this table shows results of association studies for those polymorphisms only, which were found to be in significant association with heroin addiction (point-wise p.0.05); the complete results of analysis for all polymorphisms tested are shown in Supplement 4; NA, statistical analysis of genotype or allele frequencies was not performed since genotype frequencies in control group were not in HWE; significant association (P<0.05) is shown in bold italic

Table 4
Association analysis of statistically inferred haplotypes of the *MC2R* gene (four polymorphisms^a tested: -184G>A, -179A>G, 833A>C and 1005C>T) with heroin addiction in Hispanics^b

Haplotype	Haplotype count (frequency)		95% Confidence interval	OR	p
	Control	Severe heroin addicted			
GGCT	9 (0.115)	27 (0.105)	0.402 to 1.996	0.896	0.8347
GACT	19 (0.244)	116 (0.450)	1.431 to 4.496	2.537	0.0014
GAAC	37 (0.474)	104 (0.403)	0.450 to 1.245	0.748	0.2575
AACT	10 (0.128)	9 (0.035)	0.096 to 0.629	0.246	0.0039
Other ^c	3 (0.038)	2 (0.008)	0.032 to 1.191	0.195	

^aPolymorphisms 952C>T and 1579T>C in control Hispanics were not in HWE, therefore these two polymorphisms were excluded from haplotype analysis;

^bsignificant association of haplotypes of the *MC2R* gene with heroin addiction was found in Hispanics only and shown in this table; complete results of association of various haplotypes with heroin addiction in African Americans and Caucasians are shown in Supplement 8;

^chaplotypes having allele frequencies less than 5% in both, control and heroin-addicted groups combined, were used as a separate group "others"; point-wise significant p-values are indicated in bold italic; OR, odds ratio.