Research Article

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Pharmacogenomics

African-specific variability in the acetylcholine muscarinic receptor M4: association with cocaine and heroin addiction

Aim: This study was designed to determine whether polymorphisms in acetylcholine receptors contribute to opioid dependence and/or cocaine dependence. **Patients & methods:** The sample (n = 1860) was divided by drug and ancestry, and 55 polymorphisms (nine genes) were analyzed. **Results:** Of the 20 SNPs that showed nominally significant associations, the association of the African-specific *CHRM4* SNP rs2229163 (Asn417=) with cocaine dependence survived correction for multiple testing ($P_{corrected}$ = 0.047). *CHRM4* is located in a region of strong linkage disequilibrium on chromosome 11 that includes genes associated with schizophrenia. *CHRM4* SNP rs2229163 is in strong linkage disequilibrium with several African-specific SNPs in *DGKZ* and *AMBRA1*. **Conclusion:** Cholinergic receptors' variants may contribute to drug addiction and have a potential role as pharmacogenetic markers.

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Keywords: African ancestry • cholinergic receptors • *CHRM4* • cocaine addiction • opioid addiction

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Drug addiction is a serious international health and social problem. The pathogenesis of addiction involves interactions between biological, environmental, psychological and drug-related factors [1]. Genetic variation is one of the important determinants of interindividual variability in drug addiction vulnerability [2]. Although dopamine (DA) increases after administration of numerous drugs of abuse, including cocaine and heroin, other neurotransmitter systems contribute to their addictive effects [3]. In this study, we focused on the role of genetic variation in the cholinergic system in heroin and/or cocaine addiction.

Acetylcholine (Ach) participates in various CNS functions including reward, stress response, nociception, memory, synaptic plasticity and response to drugs of abuse [4]. Ach effects are mediated by cholinergic receptors (AChRs) that consist of ionotropic nicotinic (nAChRs) and metabotropic muscarinic (mAChRs) receptors [5]. Neuronal nAChRs modulate the release of dopamine, serotonin, gamma-amino butyric acid (GABA) and glutamate [6]. A dysfunctional central cholinergic system may have serious consequences and has been associated with the pathology and treatment of drug addiction, smoking cessation, stress and psychiatric disorders [7–9].

The nAChRs are heteropentamers consisting of five subunits in various combinations of genetically distinct α and β subunits that produce a spectrum of ion channels that are expressed by various cell types. The nAChRs are the primary targets for the exogenous agonist nicotine; however, alcohol, cocaine and morphine were shown to interact with nAChR as well [10–12]. The predominant nAChR subtypes implicated in drug addiction are those containing α4 and β2 subunits that are highly expressed in DA neurons and regulate DA release.

The mAChRs (encoded by *CHRM1–CHRM5*) are slow acting seven transmembrane domain G-protein-coupled receptors with a

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central role in human physiology, signaling pathways and synaptic plasticity [13]. Both morphine and cocaine conditioned place preference (CPP) were significantly reduced in $M5^{-/-}$ mice, relative to WT mice [14,15]. A recent study in mice showed that M2 and M4 receptors depressed, while M5 receptors potentiated, DA transmission in the nucleus accumbens [16]. Selective M1 and M4 agonists and M5 receptor antagonists were suggested as treatment for psychostimulant addiction [8].

Previous studies of polymorphisms in genes of the cholinergic system reported several associations with drug abuse, addiction and related phenotypes. SNPs in the *CHRNA5*/*CHRNA3*/*CHRNB4* gene cluster located in the 15q25 chromosome region were associated with nicotine dependence (ND), alcohol dependence (AD), opioid dependence (OD) and cocaine dependence (CD) [17–21]. Many studies demonstrated that the nonsynonymous functional *CHRNA5* SNP rs16969968 (Asp398Asn) is associated with ND and related phenotypes [20]. Associations of SNPs in genes encoding other nAChRs with ND and OD were reported in several populations (e.g., see [22–25]). From the genes encoding mAChRs, *CHRM2* SNPs were associated with OD in our previous study of African–Americans (AA) [26], as well as with AD, and nonspecific drug dependence (DD) in AA and European–Americans (EA) by other groups [27–29]. *CHRM3* SNP was associated with specific CD subgroup in AA [30] and *CHRM5* SNP was associated with alcohol consumption [31].

This study was designed to determine whether selected polymorphisms in nine genes which encode nAChRs or mAChRs subunits contribute to the susceptibility to OD and/or CD and to assess whether there is overlap of genetic factors underlying addiction to heroin and cocaine in different populations. The study is an extension of our previous studies of OD in these populations [26,32] with larger sample size and modified SNP content.

Patients & methods

Study population

The sample consisted of 1860 subjects (38% females) divided into five groups according to drugs of abuse (heroin or cocaine) and ancestry: (1) EA OD ± CD, (2) AA OD ± CD, (3) AA CD without OD, (4) EA control and (5) AA control (Table 1). The subjects in the 'OD ± CD' groups (1 and 2) are former heroin addicts in stable methadone maintenance treatment with a history of at least 1 year of multiple daily uses of heroin. About half of them also had a history of cocaine addiction. The 'CD without OD' group (3) included subjects with a history of cocaine addiction but no history of heroin addiction.

Ascertainment of cases and controls was made by extensive personal interview using several instruments: the Addiction Severity Index [33], KMSK [34] and Diagnostic and Statistical Manual of Mental Disorders, 4th Edition (DSM-IV). Diagnosis was based on life-time DSM-IV criteria. Subjects were recruited at the Rockefeller University Hospital, the Manhattan campus of the VA NY Harbor Health Care System and the Dr Miriam and Sheldon G Adelson Clinics for Drug Abuse Treatment and Research in Las Vegas and Israel.

The exclusion criteria from the control sample were: drinking to intoxication and/or using illicit drugs in the last month or more than twice a week for more than 6 consecutive months, and cannabis use for more than 12 days in the last month or more than twice a week for >4 years.

The EA samples included subjects with >50% European, Middle-Eastern (ME) or combined EA/ME ancestry contributions based on Structure analysis (see below) from the US (n = 744) and Israel (n = 315). Combining EA and ME ancestry contributions was based on relative low population differentiation [35,36]. The AA sample included subjects with >50% African ancestry contribution. Self-identified Hispanics and AA subjects with >25% contribution of any major ancestry other than European/Middle Eastern were excluded.

A total of 465 EA subjects and 481 AA subjects were added after the completion of our previous studies [26,32].

The study was approved by the Institutional Review Boards of the Rockefeller University Hospital, the VA New York Harbor Healthcare System and the Tel Aviv Sourasky Medical Center (Helsinki Committee). All subjects signed informed consent for genetic studies.

Table 1. Groups description.

Ancestry	Heroin addiction, n OD ± CD	Cocaine addiction, n CD	Controls, n	Total, n
EA/ME	827 (1)	–	232 (4)	1059
AA	315 (2)	279 (3)	207 (5)	801
Total	1142	279	439	1860

Numbers in brackets are the group assigned numbers.
AA: African–American; CD: Cocaine dependence; EA/ME: European/Middle Eastern; OD: Opioid dependence.

Table 2. Gene list.

Symbol	Description	Chr.	No. of SNP	Excluded
Muscarinic				
CHRM1	Cholinergic receptor, muscarinic 1	11	3	1
CHRM2	Cholinergic receptor, muscarinic 2	7	18	1
CHRM3	Cholinergic receptor, muscarinic 3	1	16	2
CHRM4	Cholinergic receptor, muscarinic 4	11	1	–
CHRM5	Cholinergic receptor, muscarinic 5	15	15	2
Nicotinic				
CHRNA3	Cholinergic receptor, nicotinic, alpha 3 (neuronal)	15	1	–
CHRNA4	Cholinergic receptor, nicotinic, alpha 4 (neuronal)	20	4	–
CHRNA5	Cholinergic receptor, nicotinic, alpha 5 (neuronal)	15	1	–
CHRNB2	Cholinergic receptor, nicotinic, beta 3 (neuronal)	1	3	1
Total		–	62	7

Chr.: Chromosome.

SNPs

A total of 62 SNPs in nine genes (Table 2) encoding nAChRs or mAChRs subunits were selected and genotyped on a modified Illumina® GoldenGate custom panel (GS0013101-OPA, CA, USA). The array content was a modification of the 'Addiction array' in which SNPs were selected based on allele frequencies and linkage disequilibrium (LD) based on HapMap [37]. The nine selected genes were included on the original 'Addiction array'. Thirteen SNPs, in these genes, that were included in the 'Addiction array', were not included in the modified array due to failure or low frequency in EA and AA in our previous studies. Two SNPs (*CHRNA5* SNP rs16969968 and *CHRNA3* rs1051730) were added to the modified array based on functionality and association reports [20,25]. Genotyping was performed at the Rockefeller University Genomics Resource Center and analyzed with Illumina® BeadStudio software v2.3.43 (CA, USA) as described [32].

Structure analysis

Of the original 186 AIMs from the 'Addiction array', 171 SNPs with adequate quality were included in the modified panel, and 155 AIMs with high quality scores were used for analysis. Assessment of ancestry contribution was performed by Structure 2.2 with seven clusters (K). Each subject was anchored against genotypes of 1051 samples from the Human Genome Diversity Cell Line Panel, as described [38].

Statistical analysis

Exact tests for deviation from Hardy–Weinberg equilibrium (HWE) were performed with the PLINK program. Pairwise LD (D′ and r^2) was estimated using Haploview 4.2. LD blocks were identified using the D′ confidence interval bound of 0.7–0.98. Single SNP association analyses were conducted by logistic regression, under dominant or recessive model assumptions using PLINK. Three independent analyses were performed for EA OD ± CD, AA OD ± CD and AA CD without OD. Correction for multiple testing was performed by permutation test (n = 100,000) for each model of inheritance, using PLINK.

Results

The ancestry contributions of all subjects in the cohort were verified using Structure analysis of 155 AIMs and were used to establish two groups (EA and AA) based on the predominant ancestry (see 'Methods'). These groups were then subdivided based on drug addiction phenotype (OD, CD or control). The three AA groups (OD, CD and control) had an average range of 80% (standard deviation [SD]: 0.1) African ancestry and of 10% (SD: 0.08) European ancestry. There was no evidence for substructure between the case (OD) and the control group for the EA group.

A total of 62 SNPs from genes encoding nine subunits of cholinergic receptors (Table 2, Supplementary Table 1) were genotyped in 1860 subjects, and 55 SNPs with high quality were analyzed for association with OD and/or CD. A total of 12 SNPs were excluded from the EA analysis based on low minor allele frequency (MAF < 0.05) in the control group, and one SNP was excluded from the AA analyses based on the same criteria. Two additional SNPs showed significant deviation from HWE ($p < 0.01$) in the EA control group and were excluded from analysis of this group. Strong LD ($r^2 > 0.75$) was found for four SNP pairs in the

EA control group and at least eight haplotype blocks of at least 21 SNPs were identified in both the control groups (Supplementary Figure 1).

Three independent analyses were conducted under two models of inheritance (dominant or recessive) as follows: EA OD ± CD (1 vs 4), AA OD ± CD (2 vs 5) and AA CD without OD (3 vs 5) (Table 1). A total of 20 SNPs in six genes showed nominally significant associations ($p < 0.05$) in at least one analysis (Table 3). One SNP in *CHRM5* 5′-UTR was associated with OD in EA. Eight intronic SNPs in *CHRNA4, CHRM2* and *CHRM5* and one nonsynonymous *CHRM4* SNP were associated with OD in AA, including a *CHRM2* SNP pair in strong LD ($r^2 = 0.75$). A total of 15 SNPs in *CHRNA4, CHRNB2, CHRM2, CHRM3, CHRM4* and *CHRM5* were associated with CD in AA, including *CHRM4* African-specific synonymous SNP, two *CHRNA4* intronic SNPs pairs in strong LD ($r^2 = 0.67$), *CHRM2* 3′-UTR SNP, *CHRM5* 5′-UTR SNP and two SNPs upstream of *CHRNB2* and *CHRM2*, respectively. The one signal that survived correction for multiple testing was the synonymous *CHRM4* SNP rs2229163 (Asn417=) with CD in AA ($P_{corrected} = 0.047$).

Discussion

The study proposes specific genetic contributions to heroin and cocaine addictions in several cholinergic receptor subunits. The study suggests mostly distinct genetic vulnerability in these genes for OD in populations of European and African ancestry as only *CHRM5* SNP was indicated in both groups. Different effects of the same variant in the two populations may be due to differing history and genetic background as well as interactions with other alleles or environmental factors that differ between these populations. There was also limited overlap on the SNP level (four SNPs) between OD and CD in AA, suggesting drug-specific susceptibility. However, this comparison is limited by substance-related comorbidity and the limited power of the study to detect small effects.

Cholinergic receptor, muscarinic 4

The most significant finding of this study is the association of the African-specific synonymous *CHRM4* SNP rs2229163 (C>T, Asn417=) with CD without OD and OD ± CD in AA. To the best of our knowledge this is the first report of association of *CHRM4* SNP with any drug addiction. *CHRM4* (chromosome 11: 46,385,098–46,386,608, hg38) is an intron-less gene that is located in a approximately 300 Kb region of strong LD on chromosome band 11P11.2 that also contains the *AMBRA1*, *MDK* and *DGKZ* genes. Based on HapMap data, strong LD exist in this region across populations. Specifically to our findings, HapMap data in samples of African ancestry indicate that *CHRM4* SNP rs2229163 is in strong LD ($r^2 > 0.9$) with several African-specific SNPs in *DGKZ* (rs11827909 and rs11038880) and *AMBRA1* (rs12290327, rs11038901 and rs7942614). *AMBRA1* SNP rs7942614 is also located in an overlapping ribosomal protein pseudogene (*RPS10P19*).

The correlation of these SNPs is noteworthy since *AMBRA1* SNPs were previously associated with schizophrenia in a large European sample [39]. *AMBRA1* has an important role in the development of the nervous system. Functional studies showed an *AMBRA1* genetic effect on medial prefrontal activation and on impulsivity [39,40]. Nevertheless, as was pointed out by Rietschel *et al.,* it was difficult to determine the susceptibility gene for schizophrenia based on the genetics data alone due to the strong LD in the region and the potential relevance of several genes in this region. Another study reported an association of a synonymous *CHRM4* SNP, which was not analyzed in the current study, with schizophrenia, in a small Australian sample [41]. The implication for the results of the current study is that the *CHRM4* SNP could be a marker in LD with a causative SNP in another gene in this region.

The mAChR4 receptor is the most highly expressed muscarinic receptor in the striatum, where it is present on medium spiny GABAergic output neurons [8]. Preclinical studies showed that *CHRM4* plays a central role in the regulation of corticostriatal glutamatergic transmission [42] and indirectly inhibits DA release [16]. *CHRM4* knockout mice displayed a 'dopamine hypersensitive behavior', an increase in cocaine self-administration and a reduced capacity to stop alcohol-seeking behavior compared with wild type mice [43,44]. Selective targeting of the *CHRM4* is considered for the treatment of neuropsychiatric disorders [8] and *CHRM4* positive allosteric modulator was shown to inhibit cocaine self-administration in mice [45].

Identifying association of ancestry-specific SNPs with drug addiction is relevant for population-specific or personalized diagnosis and treatment. *CHRM4* SNP rs2229163 may potentially be a pharmacogenetic marker in treatment of drug addiction in subjects of African ancestry that predicts response to treatment.

Nominally significant associations

The study identified nominally significant associations of several variants in *CHRM2, CHRM3* and *CHRM5* with OD and/or CD in AA or EA. Some of the signals are probably related based on the LD structure. Although the majority of the findings was only nominally significant and should be considered tentative, several of the SNPs identified in the current study were previously associated with drug addictions

Table 3. Nominally significant associations.

Gene	SNP	Chr.	position	Location	Alleles[¶]	MAF		Model	OR[#]	L95	U95	p-value		
						AA	EA					EA OD	AA OD	AA CD
Nicotinic receptors														
CHRNA4	rs3787138[‡]	20	63347872	Intron	(A/G)	0.43	0.14	R	1.65[††]	1.07	2.55	–	0.0298	0.0488
CHRNA4	rs6011776[‡]	20	63352404	Intron	(A/G)	0.46	0.13	R	1.54[††]	1.03	2.31	–	–	0.0416
CHRNB2	rs4845652	1	154565729	Upstream	(C/T)	0.16	0.10	D	0.57	0.38	0.87	–	–	0.0083
Muscarinic receptors														
CHRM2	rs6957496	7	136867090	Upstream	(T/C)	0.28	0.09	D	0.59	0.41	0.85	–	–	0.0049
CHRM2	rs1424386[†,‡]	7	136895738	5′-UTR	(A/G)	0.21	0.39	D	1.53	1.07	2.20	–	0.0210	–
CHRM2	rs2350780[†,‡]	7	136908222	5′-UTR	(T/C)	0.24	0.36	D	1.46	1.02	2.08	–	0.0365	–
CHRM2	rs7810473	7	136911710	5′-UTR	(A/G)	0.50 (A)	0.44 (G)	R	0.67	0.46	0.97	–	0.0491	–
CHRM2	rs7800170[†]	7	136939573	5′-UTR	(T/G)	0.32	0.50	D	1.53	1.07	2.18	–	0.0197	–
CHRM2	rs324578	7	136963715	5′-UTR	(A/T)	0.34	0.03	R	0.60	0.36	0.99	–	–	0.0467
CHRM2	rs420817	7	137002656	5′-UTR	(A/G)	0.47	0.50	D	0.56	0.37	0.84	–	0.0241	0.0054
CHRM2	rs7357341	7	137006706	5′-UTR	(C/T)	0.16	0.32	D	0.59	0.39	0.89	–	–	0.0121
CHRM2	rs8191993	7	137016816	3′-UTR	(C/G)	0.18	0.32	D	1.58	1.08	2.30	–	–	0.0183
CHRM3	rs1072320[†]	1	239819076	Intron	(A/G)	0.40	0.27	R	0.46	0.27	0.80	–	–	0.0063
CHRM3	rs6701181	1	239906887	Intron	(T/C)	0.28 (C)	0.43 (T)	R	1.80	1.03	3.16	–	–	0.0440
CHRM4	rs2229163[†,§]	11	46385307	Asn417=	(C/T)	0.09	0.002	D	2.09	1.35	3.25	–	0.0399	0.001
CHRM5	rs4041435[†]	15	33969719	5′-UTR	(G/A)	0.31	0.02	D	1.56	1.10	2.23	–	0.0135	–
CHRM5	rs520032	15	33987030	5′-UTR	(C/T)	0.22	0	R	3.12	1.15	8.45	–	–	0.0255
CHRM5	rs4779656[‡]	15	34032402	5′-UTR	(T/C)	0.10	0.20	D	0.56	0.36	0.86	–	0.0449	0.0086
CHRM5	rs2684941	15	34039581	5′-UTR	(C/T)	0.15 (T)	0.38 (C)	R	1.63	1.08	2.47	0.02	–	0.0352
CHRM5	rs8030094[‡]	15	34044681	5′-UTR	(G/A)	0.14	0.20	D	0.57	0.37	0.89	–	–	0.0133

[†]SNPs that were previously identified in a subset of the current OD AA sample (Levran *et al.*, 2009).
[‡]SNPs in strong LD ($r^2 > 0.65$).
[§]The only SNP that survived permutation analysis ($P_{corrected} = 0.047$).
[¶]Major/minor, unless noted in parenthesis next to the MAF for AA and EA.
[#]When more than one group is indicated and the model is identical, OR is listed for the lowest p-value.
AA: African–American; CD: Cocaine dependence; D: Dominant; EA: European/Middle Eastern; L95: 95% CI lower value; MAF: Minor allele frequency; OD: Opioid dependence; OR: Odds ratio; R: Recessive; U95: 95% CI upper value.

and related phenotypes. The study corroborates the associations of six SNPs that were identified in our previous study of OD in a subsample of the current AA group ($p < 0.05$ [Levran *et al.* Unpublished Data]) although only *CHRM2* SNP rs2350780 survived the significance cutoff ($p < 0.01$) of the previous study [26]. *CHRM2* SNP rs8191993 was associated with AD and major depressive syndrome [27]. The intronic *CHRM5* SNP rs8030094 was associated with alcohol consumption [31]. The current study did not identify association of *CHRM2* SNP rs1455858 that was previously indicated in association with drug addiction [46].

In addition, several other *CHRM2* SNPs were previously associated with AD and with nonspecific drug dependence, in AA and EA [27–29], as well as with major depression, cognitive ability, externalizing behavior and brain oscillations [47–50]. The *CHRM2* gene encodes a protein translated from one exon with only rare variants and a large 5′-untranslated region of five exons. Both tissue-specific alternative splicing and complex transcriptional regulation were described [51]. The findings of the current study together with previous data are intriguing since CHRM2 is specifically implicated in learning, memory and cognition, functions hypothesized to be impaired in drug addiction [52]. The associations of *CHRM5* are of potential interest since the rewarding properties of morphine and cocaine were shown to be attenuated in $M5^{-/-}$ mice (e.g., see [14,15]), and selective M5 receptor antagonists are considered for treatment of drug addiction [8]. In a study from our laboratory, the dorsal striatum *Chrm5* mRNA levels were increased after chronic extended access self-administration of the prescription opioid oxycodone, in mice [53].

Two intronic *CHRNA4* SNPs in strong LD and one SNP upstream of *CHRNB2* were nominally associated with OD and/or CD in AA. To the best of our knowledge, there were no reports of association of SNPs in these genes with heroin and cocaine addiction in AA. No SNP showed association with OD in EA. Of the SNPs indicated, the intronic *CHRNA4* SNP rs3787138 was previously associated with a subgroup of tobacco smokers with massive withdrawal symptoms and depression [54]. It is noteworthy that no association was detected with *CHRNA5* SNP rs16969968 which was associated with ND and related phenotypes by numerous studies, and with protection from cocaine dependence in EA [20,55]. This is particularly interesting because of the high prevalence of tobacco smokers among heroin addicts [56] and suggests a nicotine-specific effect.

Conclusion

This study provides evidence for associations of specific cholinergic gene variants, with yet unknown functional consequences, with heroin and cocaine addictions that are mostly specific to the African–American population. The findings suggest the presence of both shared and distinct genetic liability for heroin and cocaine addictions. The identified variants have a potential role as pharmacogenetic markers as nicotinic drugs are being explored for the treatment of addiction [57]. Specific variants may improve treatment outcomes by identifying subjects most likely to benefit from specific treatment or subjects with increased risk for relapse. Ancestry-specific variants like the ones indicated in this study are of particular importance as they may help in the understanding of the interaction between environment and genetics toward reducing health disparities. Further studies are required to corroborate the results and to assess the clinical relevance of the findings.

Supplementary data

To view the supplementary data that accompany this paper, please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/pgs-2016-0028

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Genetic variation is one of the important determinants of interindividual variability in drug addiction vulnerability and response to treatment.
- A dysfunctional central cholinergic system has been associated with the pathology of drug addiction.
- SNPs in the *CHRNA5*/*CHRNA3*/*CHRNB4* gene cluster were previously associated with drug dependence.
- This study was designed to determine whether polymorphisms in genes which encode nAChRs or mAChRs subunits contribute to the susceptibility to OD and/or CD in different populations.
- The ancestry contributions were verified using Structure analysis of 155 AIMs.
- A total of 55 SNPs were analyzed in 1860 subjects in three independent analyses: EA OD ± CD, AA OD ± CD and AA CD without OD.
- The one signal that survived correction for multiple testing was the synonymous *CHRM4* SNP rs2229163 (Asn417=) with CD in AA ($P_{corrected}$ = 0.047).
- *CHRM4* is located in a region of strong LD on chromosome 11 that includes genes associated with schizophrenia (*DGKZ* and *AMBRA1*).
- The study suggests mostly distinct genetic vulnerability in these genes for OD in populations of European and African ancestry.

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