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SNPs in CHRNA6 and CHRNB3 are associated with alcohol consumption in a nationally representative sample

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Abstract

The CHRNA6 and CHRNB3 genes have been associated with nicotine dependence and early subjective response to nicotine (Bierut *et al.*, 2007; Hoft *et al.*, 2008; Saccone *et al.*, 2007; Zeiger *et al.*, 2008). Here we present evidence, using a nationally representative sample of adults, that this region is also associated with alcohol behaviors. Six SNPs spanning the CHRNB3/A6 genes were analyzed using the statistical genetics software FBAT-PC (Lange et al., 2004b), which allows one to examine a collection of multiple phenotypes to generate a maximally heritable composite phenotype for each SNP. The six SNPs were tested using FBAT-PC including four alcohol phenotypes: average number of drinks, blackouts, total number of DSM-IV abuse and dependence symptoms endorsed, and quit attempts. Three SNPs in CHRNA6 (rs1072003 p=0.015, rs892413 p=0.0033, and rs2304297 p=0.012) and one SNPs in CHRNB3 (rs13280604 p = 0.0053) were associated with a composite of the alcohol phenotypes. The association was primarily driven by the average number of drinks.

Keywords

Nicotinic receptors; SNP; Genetic association; Alcohol use; Alcohol Dependence

Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) have recently been implicated in several human studies of nicotine-related behaviors (Amos *et al.*, 2008; Berrettini *et al.*, 2008; Bierut *et al.*, 2007; Ehringer *et al.*, 2007; Hung *et al.*, 2008; Saccone *et al.*, 2007; Schlaepfer *et al.*, 2008a; Thorgeirsson *et al.*, 2008; Zeiger *et al.*, 2008) and they have been associated with alcohol dependence in the COGA sample as well (Wang *et al.*, 2008). An emerging body of evidence suggests that nAChRs may be a common site of action for both nicotine and alcohol (Schlaepfer et al., 2008b). Several studies have shown that nAChRs are modulated by ethanol (Aistrup *et al.*, 1999; Cardoso *et al.*, 1999; Jerlhag *et al.*, 2006). Furthermore, receptors containing the nAChR subunits β2 and/or β3 appear to be important

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to the dopamine-enhancing effects of ethanol, particularly those located in the ventral tegmental area (VTA) (Jerlhag *et al.*, 2006). More recently, a partial nicotinic agonist known to affect both α4β2 containing receptors and α6β3 containing receptors, Varenicline, was shown to decrease voluntary ethanol consumption in rats (Steensland *et al.*, 2007). Likewise, mice selectively bred for high and low response to ethanol show differences in expression of α6 and β4, but not α3 and β2 receptor subunits (Kamens & Phillips, 2008).

However, only four studies have looked directly for an association between human polymorphisms in nAChRs and alcohol behaviors (Ehringer *et al.*, 2007; Schlaepfer *et al.*, 2008a; Wang *et al.*, 2008; Zeiger *et al.*, 2008). Evidence for alcohol-related behaviors was found in three of these (Ehringer *et al.*, 2007; Schlaepfer *et al.*, 2008a; Wang *et al.*, 2008), but not in the only study which examined the CHRNB3/A6 genes (Zeiger *et al.*, 2008). Among the few genome wide association studies conducted on alcoholism in adult samples, genes for the nAChRs have not emerged as top candidates (Johnson *et al.*, 2006; Zhu *et al.*, 2005). Among the receptor types expressed on dopaminergic nerve terminals, only the α6 and β3 subunits show localized expression to dopamine neurons, whereas α4 and β2 are expressed throughout the brain and α5 is primarily expressed in the periphery (Putz *et al.*, 2008). Furthermore, α6 and β3 are likely to be co-regulated since they are adjacently located in the genome and co-localized to the substantia nigra, ventral tegmental area, striatum, and locus coeruleus (Gotti *et al.*, 2006). However, *in vitro* studies of the α6 and β3 subunits are challenging since they are not widely expressed in cell culture lines, difficult to transfect, and difficult to separate from α4β2-containing receptors. Therefore, a focus on these two subunits in humans is one line of research that would complement limitations of lab-based studies. In previous work, we found an association between SNPs in the CHRNB3 gene and tobacco behavior in a large nationally representative adult sample (Hoft *et al.*, 2008). It was of interest to consider whether this finding is specific to tobacco, or whether it might generalize to the other most readily available substance, alcohol. Therefore, the current study explores the possibility that variations within these genes are also associated with alcohol behaviors.

Materials and Methods

Subjects

Subjects were from the National Youth Survey Family Study (NYSFS), a nationally representative household sample of 1725 original respondents across 1044 households who were between the ages of 11 and 17 in 1976 and living in the United States in 1977 (Elliott *et al.*, 1989). In 2002 a follow up interview was conducted at which buccal cell DNA was collected on a voluntary basis. At the time participants were between 35 and 44 years of age. In addition to DNA collection, as part of a face-to- face structured interview, alcohol and tobacco use behaviors were assessed using an adaptation of the Composite International Diagnostic Interview - Substance Abuse Module (CIDI-SAM) (Cottler & Keating, 1990). Overall phenotype information and DNA was available for 1071 subjects, 48.1% male and 51.9% female, of whom 227 belonged to families with sibships ranging from 2 to 5 offspring (592 individuals) and 479 were individuals without siblings in the study (siblings may have been too old or too young to be recruited in 1977). The majority of the subjects are Caucasian (80.3%), with 12.3% African American, and 5.4% of Hispanic or other ancestry.

Genotyping

Eight single nucleotide polymorphisms (SNPs) in CHRNA6 and CHRNB3 were chosen and genotyped as described in Zeiger *et al.* (2008). Briefly, SNPs were chosen to include those that have been previously examined by our group or others, and for which reliable

genotyping assays were available. Figure 1 presents a diagram of the genes and the locations of the selected SNPs. It also shows linkage disequilibrium (r^2) between SNPs in the Caucasian sub-sample and the predicted haplotype blocks based on current HapMap data (data release #21a). No LD data are given for rs35489610 due to the very low allele frequency. The SNPs previously genotyped were sufficient to capture the majority of known genetic variation. While some gaps exist, all of the SNPs in the regions of low LD are very rare with the exception of rs7017612 which shares high LD with SNPs in both LD blocks 1 and 2, but is flanked by rare SNPs. Following preamplification using the method of Zhang et al. (Zhang et al., 1992), TaqMan assays (ABI) were performed according to the manufacturer's instructions in a 384 well format using a Biomek 3000 Laboratory Automation Workstation (Beckman Coulter) for sample preparation, an Applied Biosystems 9700 thermocycler for temperature cycling, and an Applied Biosystems 7900 PRISM Genetic Analyzer for genotype calling with the 7900HT Sequence Detection System software version 2.3 (Applied Biosystems).

Analytic Methods

Genotypes were previously checked for quality by concordance between monozygotic twins and percent of genotypes successful for each individual and each SNP, tested for Hardy Weinberg equilibrium, and linkage disequilibrium between SNPs was estimated within ethnic groups using Haploview (Barrett *et al.*, 2005), as previously described (Hoft *et al.*, 2008).Two SNPs were rare, rs35389610 and rs4953 (minor allele frequencies = 0.006 and 0.04), and were removed from subsequent analyses. Phenotype descriptors and summary statistics were generated in SPSS version 16.0 and SAS version 9.1.

Single marker Genotype x Phenotype analysis was done using the statistical genetics software FBAT-PC (Lange *et al.*, 2004a), which allows one to generate a maximally heritable composite phenotype for each SNP from a collection of multiple phenotypes. This data-driven approach provides a way to minimize multiple testing of phenotypes in the context of multiple SNPs and was derived from methods originally developed by Fulker *et al.* (Fulker *et al.*, 1999). A maximally heritable composite phenotype is generated using information obtained from the between-family association for each SNP. This composite phenotype is tested subsequently for genetic association using the within-family association test, which is statistically independent of the between-family information (Lange *et al.*, 2004a). The six SNPs in the CHRNB3-CHRNA6 region were tested using FBAT-PC to form a composite of four quantitative alcohol phenotypes: average number of drinks, number of blackouts, total number of DSM-IV abuse and dependence symptoms, and number of unsuccessful quit attempts. The four phenotypes were chosen to capture quantitative measures of alcohol-specific behaviors. It is worth noting that the last three of these phenotypes are lifetime measures whereas average drinks measures past year use. Data were available for these items on 980 people. Ten individuals reported having tried alcohol but did not answer other alcohol related questions. Average number of drinks was assessed by the question: “On days when you drank alcoholic beverages in the past year, on average how many drinks would you have?”. Number of quit attempts was assessed with the question “How many times were you unable to quit or cut down?”, after the individual had indicated desire to and being unable to quit or cut down in the past. Finally, number of blackouts was assessed with the question “How many blackouts have you had?”, which was asked only if the individual had said yes to the question: “Have you ever had blackouts while drinking, that is where you drank enough so that you couldn't remember the next day what you had said or done?”. Following the initial test of association with the composite phenotype, the most highly correlated (weighted) individual phenotype was analyzed post-hoc. Due to the known correlations between smoking and the nicotinic receptors, as well as the co-morbidity between smoking and alcohol consumption a final post-hoc test was done

to assess whether any associations were specific to smokers or never-smokers. Never-smokers were individuals who reported never having tried any tobacco, smokers were individuals who reported ever having tried cigarettes or other tobacco. Although the family based approach employed by FBAT-PC inherently controls for stratification, sex and ethnicity were included as covariates in the association model.

Results

Allele frequencies were similar to those reported in the literature and on public databases (dbSNP), shown in Table 2. None of the SNPs differed significantly from Hardy-Weinberg proportions within the groups defined by self-reported ethnicity. It is worth noting that in this region there are striking differences in allele frequencies between Caucasians and African Americans, but family-based association methods such as FBAT inherently control for population stratification. Two SNPs, rs4953 and rs35389610 were too rare (MAF < 0.006 and 0.04) to be informative in a sample of this size and so were omitted from further analyses.

No major differences were observed in the distribution of phenotypes in the three most represented ethnic populations, Caucasians, African Americans, and Hispanics, shown in Table 3a. Most subjects reported drinking about two drinks on days that they drank, and endorsed fewer than two abuse or dependence symptoms total. The distribution of lifetime number of blackouts was greatly skewed, which inflated the mean, but otherwise seemed in agreement with other samples (Nelson et al., 2004). The averages for each of the phenotypes were higher in the population of smokers than in individuals who had never smoked, as seen in Table 3b. This increase was significant for both the number of alcohol abuse and dependence symptoms as well as the number of blackouts, but not for average number of drinks nor number of quit attempts. The four phenotypes are somewhat correlated with the exception of average number of drinks with number of blackouts, which is uncorrelated, Table 3c.

Since six SNPs were tested, level of significance for nominal p-values was set at 0.05/6=0.0083. One SNP in intron 2 of the CHRNA6 gene reached this conservative threshold, SNP rs892413 (p=0.0033) while two others in CHRNA6 attained suggestive significance: rs1072003 (also in intron 2) and rs2304297 in exon 6 (p= 0.015 and p= 0.012). One SNP, rs13280604, in intron 1 of CHRNB3 also reached the conservative threshold (p= 0.0053). The correlation of the individual phenotypes with the composite is given in Table 4.

The correlations of the composite phenotype with each individual phenotype clearly show that the association is dominated by the average number of drinks reported. In post-hoc testing to examine the origin of the composite associations, average drinks was tested as a single phenotype for genetic association. Table 5 shows that while the signal was consistent, more information is gained by including all four alcohol-related phenotypes in a composite compared to average drinks alone, as evidenced by the less strong p-values (rs13280604 (p=0.04), rs2304297 (p=0.054), rs892413 (p=0.011) and rs1072003 (p = 0.035)). This highlights the advantage of using the FBAT-PC approach, since testing each trait individually would have failed to yield sufficiently significant p-values to overcome multiple testing.

Given the high co-morbidity of alcohol and tobacco use, we repeated the original analysis within smokers only to ensure that the signals were not merely a reflection of an association with smoking, shown in Table 6. Each composite was created specifically for the smokers, using FBAT-PC which led to small differences in weights for each phenotypic item compared to what was derived for the entire sample. There was modest evidence for

significant associations with a few of the SNPs in the smokers only, but none of these were as significant as in the full sample. A similar trend was found for the average drinks phenotype (Table 7).

Discussion

This study provides the first evidence for association between the CHRNB3/A6 genes and alcohol behaviors in humans. In CHRNA6, SNP rs892413 (p=0.0033) reached statistical significance using a conservative threshold, as did SNP rs13280604 (p=0.0053) in CHRNB3. Two other SNPs in CHRNA6 (rs1072003 and rs2304297) were nominally significant. Our findings, coupled with the recent evidence for polymorphisms in these receptors contributing to susceptibility to nicotine dependence (Bierut *et al.*, 2007; Hoft *et al.*, 2008; Saccone *et al.*, 2008; Saccone *et al.*, 2007) supports the hypothesis that nAChR genes may contribute to the co-morbidity of smoking and alcohol problems. This is consistent with results from *in vitro* systems and animal work showing that alcohol modulates the binding of nicotine to nAChRs (Aistrup *et al.*, 1999; Dohrman & Reiter, 2003; Jerlhag *et al.*, 2006).

Our post hoc analysis examining smokers and never-smokers was limited in its power to determine whether the association with alcohol in this sample is driven primarily by the smokers, because of the reduced sample size of the never-smoking group. Future studies examining a larger sample of non-smokers for association of these polymorphisms and alcohol phenotypes are merited. If association of CHRNA6 and CHRNB3 with alcoholism is found to be limited to only smokers it raises some interesting questions as to whether there is an underlying simultaneous genetic effect on both phenotypes, or whether the association is truly with smoking and other factors lead to a correlation between smoking and alcoholism. It is interesting that in previous associations of nicotine behaviors with CHRNB3/A6, the strongest signals have been found in the putative promoter and 5'UTR of CHRNB3 or in exon 6 of CHRNA6. In this study, the association with alcohol is with SNPs in intron 2 of CHRNA6 and intron 1 of CHRNB3, with only a moderate level of association with exon 6 of CHRNA6 and no association with the upstream areas of CHRNB3. Nicotine acts at the binding site of nAChRs whereas ethanol is believed to act allosterically to modulate receptor sensitivity. Therefore, the finding that SNPs in different regions of the subunit genes are associated with different drug behaviors is consistent with idea that distinct molecular mechanisms may be associated uniquely with the two different substances. This would suggest that these polymorphisms have a direct effect on alcohol behavior beyond a correlation with tobacco use, although additional human genetic research followed by functional studies will be necessary to tease this apart.

Previous studies of nAChR genes have examined alcohol phenotypes including age of initiation, DSM-IV alcoholism diagnosis, maximum number of drinks when drinking the most and subjective effects to early alcohol exposure (Ehringer *et al.*, 2007; Johnson *et al.*, 2006; Schlaepfer *et al.*, 2008a; Wang *et al.*, 2008; Zeiger *et al.*, 2008; Zhu *et al.*, 2005). Three of these studies focused on populations of young adults (Ehringer *et al.*, 2007; Schlaepfer *et al.*, 2008a; Zeiger *et al.*, 2008). Ehringer *et al.* found an association between early subjective effects of alcohol and SNPs in the CHRNB2 gene, and Schlaepfer *et al.* found association between age of initiation of alcohol use and SNPs in CHRNA3 and CHRNB4 in two separate samples. However, no evidence for linkage or association has been observed between alcohol abuse and dependence related symptoms and nAChRs. To our knowledge, only the COGA studies have examined nAChR genes and adult alcoholism, and only the CHRNA5 gene has been associated with alcohol dependence (Johnson *et al.*, 2006; Wang *et al.*, 2008; Zhu *et al.*, 2005). Here we used FBAT-PC to combine four quantitative measures of alcohol-related behaviors in an attempt to capture a complex

phenotype driven by genetics. Interestingly, the item most highly correlated with the composite phenotypes was the quantity variable "average number of drinks when drinking in the past year". This item differs slightly from the other three in that it measures recent rather than lifetime behavior. Some of the studies mentioned above (Ehringer *et al.*, 2007; Schlaepfer *et al.*, 2008a; Zeiger *et al.*, 2008) have examined a "maximum drinks" measure, which is defined as the "number of drinks a day at a time when drinking the most." Although this measure had been collected in the NYS sample, it was not included in our original analysis because we felt the "average number of drinks" might be more reflective of overall drinking patterns for an individual. In this sample, the "max drinks" phenotype is modestly correlated with "average drinks" ($r^2 = 0.25$; $p < 0.001$). In an effort to examine why our results might have differed from those previously reported a post-hoc examination of "max drinks" was undertaken. As expected, there was no evidence for association with any of the SNPs (all $p>0.065$). These results underscore the importance of phenotype definition and illustrate the possible limitations of approaches focused on clinical diagnosis or simple quantity/frequency measures. They also illustrate the utility of using a family-based approach with elegant statistical tools such as those implemented in FBAT-PC to reduce the dimensionality of the data and obtain informative associations with composite phenotypes.

In summary, this work provides the first evidence for an association between SNPs in CHRNA6 and CHRNB3 with quantitatively measured alcohol-related behaviors. This finding is an extension of the existing literature using pharmacological experiments and animal models which has shown that alcohol modulates the binding properties of nicotine at nAChRs. Additional work will be necessary to tease apart whether this finding is dependent on smoking status and whether it can be replicated in other samples. Ultimately, studies aimed at understanding the putative functional mechanisms of these SNPs will be key to drawing strong conclusions about their possible roles in alcohol and tobacco co-morbidity.

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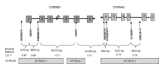

Figure 1.
Illustration of the CHRNA6-CHRNB3 locus. Boxes represent exons separated by intronic regions (not drawn to scale). Eight SNPs were genotyped shown in bold, with their reference sequence numbers and gene locations indicated. The number of nucleotide base pairs (bp) between each SNP, the estimated linkage disequilibrium (r^2) in the Caucasian subsample and LD blocks as determined from HapMap project data (NCBI build 36) are also indicated.

Table 1

Sample characteristics for the NYS-FS sample.

Sample	Female (%)	Male (%)	Ever Alcohol (%)	Ever Tobacco(%)
Pooled (1051)	545 (51)	506 (49)	990 (94)	856 (81)
Caucasian (860)	450 (52)	410 (48)	824 (96)	727 (85)
Hispanic (28)	17 (61)	11 (39)	27 (96)	23 (82)
African-American (132)	65 (49)	67 (51)	109 (83)	82 (62)

Table 2

Allele frequencies and ethnic differences in the NYS-FS

Gene	Reference sequence (rs)	Position Chr. 8	Location	MAF sample	MAF CEU*	MAF AA*	Minor Allele CEU*	Minor Allele AA*	P-value Eth. diff
CHRNB3	rs7004381	42670318	Upstream	0.285	0.227	0.399	A	G	0.0084
CHRNB3	rs4950	42671790	3'UTR	0.291	0.215	0.258	G	A	<0.0001
CHRNB3	rs13280604	42678743	Intron 1	0.298	0.218	0.299	G	A	<0.0001
CHRNB3	rs4953	42706816	Exon 5	0.041	0.03	0.06	C	C	0.3
CHRNA6	rs2304297	42725148	Exon6	0.295	0.237	0.271	C	G	<0.0001
CHRNA6	rs35389610	42726954	Intron 5	0.006	0.007	0	T	C	-
CHRNA6	rs892413	42727356	Intron 2	0.267	0.212	0.308	A	C	<0.0001
CHRNA6	rs1072003	42729008	Intron 2	0.236	0.186	0.345	G	C	<0.0001

*CEU = Caucasian, AA = African American, "MAF sample" is the frequency of the CEU minor allele across the entire sample.

Table 3

Phenotype characteristics by (a) ethnicity and (b) smoking status 3a

	Average drinks		Abuse + Dependence		Quit attempts			Blackouts		
	μ ± σ	n	μ ± σ	n	μ ± σ	n	Number endorsing	μ ± σ	n	Number endorsing
All	2.20 ± 2.87	980	1.76 ± 2.55	980	0.18 ± 0.89	980	53	2.64 ± 12.13	980	202
Caucasian	2.15 ± 2.72	806	1.72 ± 2.51	806	0.15 ± 0.84	806	34	2.85 ± 12.85	806	169
AA	2.32 ± 3.52	105	1.72 ± 2.49	105	0.35 ± 1.08	105	12	1.32 ± 8.77	105	16
Hisp.	2.59 ± 2.52	27	1.96 ± 2.81	27	0.30 ± 0.87	27	3	0.32 ± 0.95	27	4

All: entire sample, AA: African American, Hisp. Hispanic

3b

	Average drinks		Abuse + Dependence *		Quit attempts		Blackouts*	
	μ ± σ	**n**	**μ ± σ**	**n**	**μ ± σ**	**n**	**μ ± σ**	**n**
Smokers	2.34 ± 2.93	807	1.89 ± 2.59	807	0.19 ± 0.91	807	3.04 ± 13.38	807
Never-Smokers	1.59 ± 4.37	135	0.78 ± 2.02	136	0.12 ± 0.76	133	0.65 ± 7.17	135

*: indicates significant difference between groups

3c

	Average drinks	Abuse + Dependence	Quit attempts	Blackouts
Average drinks	1	0.307*	0.083*	0.034
Abuse + Dependence	0.307*	1	0.392*	0.470*
Quit attempts	0.083*	0.392*	1	0.431*
Blackouts	0.034	0.470*	0.431*	1

*Significant at $p<0.01$

Table 4

Results of association between alcohol phenotypes and CHRNA6/B3 SNPs.

SNP	Gene	Fam	p-value	Risk allele	power	Average drinks	Alcohol ABD	Alcohol Quit	# Blackouts
rs7004381	CHRNB3	92	0.1644		0.276				
rs4950	CHRNB3	79	0.056	G	0.283	0.830	-0.255	-0.140	-0.102
rs13280604	CHRNB3	80	**0.005**	G	0.120	0.826	-0.262	-0.147	-0.106
rs2304297	CHRNA6	88	0.012	C	0.132	0.994	0.240	0.102	0.155
rs892413	CHRNA6	78	**0.003**	A	0.114	0.939	0.622	0.275	0.362
rs1072003	CHRNA6	71	0.015	G	0.149	0.931	-0.037	-0.044	0.004

Fam indicates the number of informative families for each SNP, power indicates the statistical power to detect an association with the data. The last four columns give the correlation of each item with the composite phenotype, where the association is at least nominally significant. This composite phenotype is used in the final within-family test for association to obtain p-values shown in column 4. Bold indicates p-values are significant at the 0.0083 level.

Table 5

Results of association between average drinks and CHRNA6/B3 SNPs

marker	allele	fam	pvalue
rs7004381		102	0.65
rs4950	G	89	0.026
rs13280604	G	91	0.041
rs2304297	C	95	0.054
rs892413	A	85	0.012
rs1072003	G	76	0.036

Table 6

p-values for significant SNP-phenotype associations within smokers

SNP	Fam	pvalue	allele	power	Average drinks	Alcohol ABD	Alcohol quit	# blackouts
rs7004381	84	0.051	A	0.130	0.938	0.137	-0.106	0.230
rs4950	71	0.124		0.212				
rs13280604	74	0.285		0.085				
rs2304297	82	0.018	C	0.065	0.948	0.360	-0.031	0.231
rs892413	71	0.060	A	0.061	0.106	0.956	0.303	0.574
rs1072003	64	0.114		0.082				

Fam.: Number of informative families used in analysis.

Table 7

Association of average drink within smokers

SNP	Fam	Pvalue*	allele	power
rs7004381	84	0.123		0.056
rs4950	71	**0.045**	G	0.067
rs13280604	74	**0.028**	G	0.050
rs2304297	82	0.052	C	0.050
rs892413	71	**0.028**	A	0.067
rs1072003	64	0.084	G	0.084

* Bold indicates pvalues significant at the 0.05 level.