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Evidence for association between low frequency variants in CHRNA6/CHRNB3 and antisocial drug dependence

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Abstract

Common SNPs in nicotinic acetylcholine receptor genes (*CHRN* genes) have been associated with drug behaviors and personality traits, but the influence of rare genetic variants is not well characterized. The goal of this project was to identify novel rare variants in *CHRN* genes in the Center for Antisocial Drug Dependence (CADD) and Genetics of Antisocial Drug Dependence (GADD) samples and to determine if low frequency variants are associated with antisocial drug dependence. Two samples of 114 and 200 individuals were selected using a case/control design including the tails of the phenotypic distribution of antisocial drug dependence. The capture, sequencing, and analysis of all variants in 16 *CHRN* genes (*CHRNA*1-7, 9, 10, *CHRNB*1-4, *CHRND, CHRNG, CHRNE*) were performed independently for each subject in each sample. Sequencing reads were aligned to the human reference sequence using BWA prior to variant calling with the Genome Analysis ToolKit (GATK). Low frequency variants (minor allele frequency < 0.05) were analyzed using SKAT-O and C-alpha to examine the distribution of rare variants among cases and controls. In our larger sample, the region containing the *CHRNA6/CHRNB3* gene cluster was significantly associated with disease status using both SKAT-O and C-

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CONFLICT OF INTEREST DISCLOSURES

The authors declare that they have no conflict of interest.

INFORMED CONSENT

The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 and 2008. All participants provided informed consent.

alpha (unadjusted p values < 0.05). More low frequency variants in the *CHRNA6/CHRNB3* gene region were observed in cases compared to controls. These data support a role for genetic variants in *CHRN* genes and antisocial drug behaviors.

Keywords

Low frequency variants; antisocial drug dependence; association; sequence; nicotinic acetylcholine receptor

INTRODUCTION

Prior studies have demonstrated a comorbidity among externalizing disorders, such as conduct disorder and adult antisocial behaviors, and drug use (Elkins et al., 2007; Goldstein et al., 2007; Schubiner et al., 2000). Biometrical studies have provided support for the idea that comorbidity between these traits may be attributed to common genetic factors (Button et al., 2006; Kendler et al., 2003; McGue et al., 2006). Genetic variation in *CHRN* genes (genes that encode the nicotinic acetylcholine receptor subunits) is widely known to be associated with nicotine behaviors (Bierut et al., 2008; Hoft et al., 2009a; Kamens et al., 2013; Saccone et al., 2007; Schlaepfer et al., 2008; Thorgeirsson et al., 2010; Zeiger et al., 2008). Moreover, data have emerged to suggest that these genes are associated with other drug phenotypes including alcohol use (Coon et al., 2014; Ehringer et al., 2007; Hällfors et al., 2013; Lubke et al., 2012; Schlaepfer et al., 2008), cocaine dependence (Haller et al., 2014) and behavioral traits such as novelty seeking (Landgren et al., 2011), neuroticism (Criado et al., 2014; Grazioplene et al., 2013), and externalizing behaviors (Stephens et al., 2012). Thus, in addition to their well-established association with nicotine behaviors, *CHRN* genes may contribute to a general liability for both externalizing behaviors and drug use, as has been shown by Corley and colleagues (2008).

Nicotinic acetylcholine receptors are ligand-gated ion channels that allow the flux of cations through the cell membrane. These receptors are activated by the endogenous neurotransmitter acetylcholine and by nicotine. Several reviews detail the structure and function of these receptors (Gotti et al., 2009; Papke, 2014). Briefly, there are seventeen known α, β, δ, γ, and ε nicotinic receptor subunits. Of these, sixteen form functional pentameric channels in mammals.

Targeted sequencing studies allow for the evaluation of rare genetic variants and their association with phenotypes of interest. In the past few years, five sequencing studies of the *CHRN* genes have been reported. Most of these studies have found associations with nicotine dependence (Haller et al., 2012; Wessel et al., 2010; Xie et al., 2011; Yang et al., 2015), but a recent study observed a significant association with rare variants in *CHRN* genes and alcohol and cocaine dependence (Haller et al., 2014). These initial sequencing studies of the *CHRN* genes have provided important insights regarding the complexity of variation in these genes, but two limitations need to be addressed. First, these studies examined only a subset of the acetylcholine receptor subunits, so they did not include *CHRND, CHRNG*, and *CHRNB1*, for which modest evidence for association of common genetic variants with nicotine behaviors has been reported (Keskitalo-Vuokko et al., 2011;

Lou et al., 2006; Philibert et al., 2009; Saccone et al., 2009, 2010). Second, published studies have focused exclusively on the exons and adjacent flanking regions, so previous studies were limited primarily to protein-coding regions. Although there is an *a priori* rationale to support the hypothesis that protein-coding variants are likely to confer functional relevance, a recent paper by Schork highlights the importance of untranslated regions (UTRs) and other non-coding variation for involvement in complex traits (Schork et al., 2013). Non-coding regions in the *Chrn* genes in rodents have been shown to play an important role in gene regulation (Bruschweiler-Li et al., 2010; McDonough and Deneris, 1997; McDonough et al., 2000; Scofield et al., 2010; Xu et al., 2006). Moreover, *in vitro* gene expression studies using cell culture models to examine human genetic variants in the non-coding regions of *CHRN* genes have demonstrated differential functional effects for SNP alleles (Ehringer et al., 2010; Flora et al., 2013; Gallego et al., 2013; Kamens et al., 2015). Because expression of individual nicotinic receptor subunits is tightly regulated, both temporally and spatially (Albuquerque et al., 2009; Gotti et al., 2006, 2009), the importance of genetic variants in non-coding regions must also be explored.

To address these limitations and expand the scope of work of the existing studies, we investigated the role of low frequency genetic variation in the *CHRN* genes and association with antisocial drug dependence. In this study we sequenced all sixteen mammalian *CHRN* genes, including all exons, introns and 5kb up- and down-stream of the coding region in two samples (the CADD and GADD samples are described below).

MATERIALS AND METHODS

Sample selection

The subjects used in the current analysis were collected as part of the Center on Antisocial Drug Dependence (CADD) or Genetics on Antisocial Drug Dependence (GADD) studies. Details of the ascertainment of the CADD (Rhea et al., 2006; Stallings et al., 2003, 2005) and GADD (Kamens et al., 2013) have been described in detail elsewhere. Briefly, all subjects in both studies participated in a structured interview that assessed drug use behavior with the Composite International Diagnostic Interview-Substance Abuse Module (CIDI-SAM) (Robins, Cottler et al. 2000). From the CIDI-SAM a measure of dependence vulnerability was constructed. Dependence vulnerability was defined as the number of dependence symptoms (DSM-IV criteria endorsed) across ten classes of drugs divided by the number of classes of drugs used (Stallings et al., 2003). Additionally, conduct disorder symptoms was assessed using the Diagnostic Interview Schedule for DSM-IV (DIS) (Robins et al., 2000) or the Diagnostics Interview Schedule for Children (DISC) (Shaffer et al., 2000) based on age of the individual (DIS when older than 18 years). The dependence vulnerability and conduct disorder symptoms were standardized for sex, age, and age^2 based on beta-weights derived from the CADD community-based (unselected) sample using a linear regression analysis in SAS (SAS Institute Inc., Cary, NC, USA). Subjects were selected for sequencing based on a composite score that included the sum of dependence vulnerability and conduct disorder symptoms, termed antisocial drug dependence (Stallings et al., 2005). This composite score was used because of the known common genetic factors for these two behaviors (Button et al., 2006; Krueger et al., 2002; McGue and Iacono, 2008;

Young et al., 2000) and known associations with genetic variants in *CHRN* genes and multiple drugs (Bierut et al., 2008; Ehringer et al., 2007; Lubke et al., 2012; Sherva et al., 2010), childhood externalizing disorder (Stephens et al., 2012), and novelty seeking (Landgren et al., 2011). Since one goal of the current study was to identify novel genetic variants in these genes, the sample was enriched for ethnic diversity. In order to achieve this, the composite score was given more weight among individuals who were not of white non-Hispanic descent. To individuals in the positive end of the phenotypic distribution, one point (equivalent to 1 standard deviation unit) was added to the score of non-Caucasian individuals. In addition, those who self-identified as Hispanic were given 0.5 extra points. Thus an individual who identified as Hispanic African American would have 1.5 points added to their score. An equivalent number of points were subtracted from subjects on the negative end of the phenotypic distribution. Secondary analyses were conducted in the subset of individuals identified as non-Hispanic white. Importantly, this weighting scheme was only use to identify extreme subjects to sequence, while the analysis was conducted on the binary case/control variable.

Two groups of individuals were selected for sequencing. Individuals in the first study (Sample 1) were only chosen from the CADD sample and ranged in age from 14 to 30 years (descriptive statistics of the sample can be found in Table I). One hundred and fourteen individuals (58 cases/56 controls) were identified from the extreme ends (actual z-score cut off used to define: controls $z \leq -1.02$, cases $z \geq 3.42$) of the phenotypic distribution.

For the second study (Sample 2) individuals were selected from among both CADD and GADD subjects who had been previously genotyped in a GWAS study and ranged in age from 14 to 20 years (sample descriptive statistics can be found in I; (Derringer et al., 2015). The restricted age range in comparison to Sample 1 reflects our desire to include only subjects who had GWAS data available and the age range for inclusion in that study was less than 20 years of age. As for Sample 1, individuals from both phenotypic extremes (actual z-score cut off used to define: controls $z \leq -0.66$, cases $z \geq 3.88$) were selected (100 cases/ 100 controls). Individuals in Samples 1 and 2 were completely independent with the exception of five subjects sequenced in Sample 1 that we purposefully included in Sample 2 to confirm variant calls.

Sequencing

Sample 1—Genomic DNA was amplified using the Repli-G kit according to the manufacturer's recommendations (Qiagen, Hilden, Germany). The genomic regions containing the *CHRN* genes (Supplementary Table I) were enriched with either RainDance© target enrichment (http://raindancetech.com/) or Agilent's SureSelect© target enrichment selection (Agilent, Santa Clara, CA; http://www.genomics.agilent.com/article.jsp?pageId=2094&_requestid=492072) technologies at SeqWright (http://www.seqwright.com/aboutus/aboutseqwright.html). DNA samples were barcoded so that genotypes could be linked with behavioral data for each individual. Only 7 (of 114; 4 controls and 3 cases) subjects were enriched for the desired sequences using the RainDance© technology in a pilot study, while the remaining subjects was enriched with the SureSelect© protocol. Each gene was targeted to include 5000 base pairs upstream of the coding region, all introns and

exons, as well as 5000 base pairs downstream of the coding region. Following enrichment of the *CHRN* gene regions, samples were run on a SOLiD V4 platform (Applied Biosystems, Foster City, CA).

Sample 2—Genomic DNA samples were sent to Centrillion Bioscience (https://www.centrillionbio.com/) for enrichment of the *CHRN* genes using a proprietary custom enrichment strategy, targeting the same regions as described in Sample 1, and were then sequenced on an Illumina HiSeq 2000 (Illumina, San Diego, CA). As with Sample 1, DNA was barcoded to allow for identification of individual subjects.

Mapping, Variant Detection, and Annotation

Raw sequencing reads were mapped to the human genome using the Burrows-Wheeler Aligner (BWA, v0.7.3a-r367) (Li and Durbin 2009), duplicate reads were identified with Picard (v1.85, http://picard.sourceforge.net), and single nucleotide variants were identified using the Genome Analysis ToolKit (GATK, v2.3) (DePristo et al., 2011; McKenna et al., 2010) following the Broad Institute's best practices recommendations (http://www.broadinstitute.org/gatk/guide/best-practices). Briefly, reads were realigned locally to known indels, followed by a recalibration of base quality scores based on known variant locations. Variants were called using GATK's UnifiedGenotyper, and then recalibrated to facilitate identification of true variants. The final variant set was annotated with ANNOVAR (www.openbioinformatics.org/annovar) against Human Genome Build hg19/dbSNP Build 137. The consequences of nonsynonymous variants were examined in Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml).

Variant Confirmation

To confirm the presence of detected genetic variants in our sample, a subset of variants were confirmed by Sanger sequencing. Briefly, PCR primers were designed to amplify the region containing the nucleotide of interest. Sanger sequencing of the potential variants of interest was performed on both strands for verification. Sequencing chromatograms were examined using Sequencher 4.8 (GeneCodes Corporation, Ann Arbor, MI, US) to confirm the presence of a polymorphism.

Association Analysis

To determine the impact of low frequency variants on the binary case/control outcome (defined here as having a minor allele frequency of less than 5% in our sample), SKAT-O (Lee et al., 2012a, 2012b) and C-alpha (Neale et al., 2011; run in Variant Analysis Tools (Wang et al., 2014)) were utilized. These two burden method tests were selected because there is no requirement that rare variants will increase risk in cases. This is important in the context of *CHRN* genes, because prior studies have shown a protective effect of rare variants for nicotine dependence (Xie et al., 2011). Both approaches were used because SKAT-O can correct for the ethnic diversity included in this sample with the inclusion of covariates in the analysis, but this function is not available with c-alpha. Ethnic diversity was included as a covariate in Sample 2 by including the first five principal components derived from the previous GWAS study (Derringer et al., 2015) that included subjects in Sample 2. Nominal

p-values reported were based on the value obtained from 10,000 (SKAT-O) or 1,000 (C-alpha) permuted tests.

RESULTS

Quality Control

The sequencing coverage in Sample 2 was much better than Sample 1 due to increasing sequence capacity. Sample 1 was processed in late 2011 and yielded an average read depth of 128 across all *CHRN* genes (Table II). With the exception of *CHRNA4*, all *CHRN* genes had an average read depth greater than 50. *CHRNA4* had an average read depth of 23, consistent with difficulties observed in prior experiments in sequencing this gene (Wessel et al., 2010). Examination of the coverage across genes for each individual (data not shown) revealed that one subject (a case) had most reads map to a single genetic locus. We speculate that this was due to a bad capture of the other *CHRN* genes, thus we excluded this individual from further analysis. Sample 2 was completed in 2013 and due to greater sequencing capacity yielded better coverage across all genes, with an average read depth of 412 across all *CHRN* genes (Table II). The Ti/Tv ratio was similar between the two experiments: 2.33 ± 0.13 versus 2.27 ± 0.10 (mean ± SD, Sample 1 and Sample 2, respectively).

To ensure that our variant analysis pipeline was performing correctly, we used Sanger sequencing to verify SNPs identified in our analyses. We confirmed 20 of 21 variants examined, for an overall confirmation rate of 95%. All variants confirmed were selected from Sample 1, since this experiment had a lower average coverage.

Five samples (2 cases/3 controls) were sequenced in both studies. Comparison of the variant calls between the two studies revealed high congruency (91%) when the read depth was above 10X. Since there was greater overall coverage in Sample 2 these individuals were included in only this dataset for analysis.

Genetic Associations

We report uncorrected, nominal p-values throughout presentation of the results. One highly conservative approach to define a multiple testing threshold would be to base it on testing 12 gene clusters, two samples, two subgroups within each sample (full and white non-Hispanics), and two analytical approaches (SKAT-O and C-alpha) yielding 12 x 2 x 2 x 2 = 96 tests for a threshold of 0.05/96 = 0.00052. However, the two subgroups are not independent, nor are the two analytical approaches, so this cut-off is very conservative.

Sample 1—Due to subjects excluded (see above) from the analysis, 108 subjects were included in the analysis (55 cases/53 controls). SKAT-O and C-alpha were both tested in this sample. There were no statistically significant genetic associations observed in the full dataset (Table III). However, in the subset of white non-Hispanics only, a nominally significant association for *CHRNB1* was observed with both SKAT-O (p=0.02) and C-alpha (p=0.04). Additionally, a nominally significant association with the *CHRNA5/CHRNA3/CHRNB4* gene cluster was observed (p=0.02), but only with SKAT-O (Table VI).

Sample 2—In contrast to Sample 1, in Sample 2 (100 cases/100 controls) there were nominally significant associations with low frequency genetic variants in *CHRN* genes and antisocial drug dependence in both the full sample (Table V) and in the subset of white non-Hispanic subjects (Table VI). In the full sample, the region including the *CHRNA6* and *CHRNB3* genes had a nominal association p-value when analyzed using either SKAT-O ($p = 0.02$) or C-alpha ($p = 0.02$). Moreover, this region was associated with antisocial drug dependence when the subset of white non-Hispanic subjects was examined with SKAT-O ($p < 0.001$), reaching the threshold for multiple testing significance, but no evidence for association was seen using C-alpha (Table VI). An additional nominally significant p-value was observed with in the full sample using only one of the two statistical tests. An association was observed between variants in the *CHRNA5/CHRNA3/CHRNB4* gene cluster and antisocial drug dependence, but only with C-alpha ($p = 0.03$). Since this association was not observed with SKAT-O, which takes into account the ethnic diversity in our sample, the association observed with this region may solely be due to population stratification. Finally, a significant association with *CHRNA7* and antisocial drug dependence was observed with SKAT-O ($p < 0.01$), but only in the white non-Hispanic subjects.

We used ANNOVAR to determine genomic features where the detected low frequency variants were located. Figure 1 provides the locations of the 405 low frequency variants observed in Sample 2 in the *CHRNA6/CHRNB3* gene region and Table VII presents the number of variants observed in cases versus controls. Overall, cases carried more low frequency variants than controls (1910 and 800, respectively), and cases carried more variants in all genomic regions. The large number of variants in cases and controls compared to the relatively smaller number of distinct variants observed is due to subjects carrying more than one variant. Specifically, cases had twice as many variants than controls in *CHRNB3* introns and exons and in *CHRNA6* introns and UTR regions. We observed seven variants that lead to a nonsynonymous amino acid change, and six of these were observed in cases while only two were observed in controls. Of these seven nonsynonymous coding variants, four were classified as “probably damaging” with Polyphen-2 (Table VIII).

We identified two novel low frequency variants in the coding region of *CHRNB3*, one nonsynonymous SNP and the other resulting in a synonymous change. To determine if these variants were *de novo* we sequenced available family members. In both cases these novel variations were inherited from the mother and did not represent *de novo* variants.

DISCUSSION

In the current study we tested for association between low frequency genetic variation in *CHRN* genes and antisocial drug dependence in two separate samples. All sixteen *CHRN* genes, including exons, introns and 5kb up and downstream of the coding region were sequenced. We observed modest evidence for association with low frequency variants in the *CHRNB1* gene and antisocial drug dependence in Sample 1 and in the *CHRNA6/CHRNB3* region in Sample 2. However, these associations did not replicate in both datasets, and thus future studies to expand samples sizes are warranted.

The role of genetic variants in the *CHRNA6/CHRNB3* gene region in nicotine behaviors is well established. Common genetic variants in this gene region have been associated with many nicotine behaviors including: nicotine dependence (Cui et al., 2013; Culverhouse et al., 2014; Greenbaum et al., 2006; Hoft et al., 2009a; Rice et al., 2012; Saccone et al., 2010, 2007; Wei et al., 2012), subjective response to nicotine (Ehringer et al., 2010; Pedneault et al., 2014; Zeiger et al., 2008), and number of cigarettes smoked per day (Thorgeirsson et al., 2010). Furthermore, there is emerging evidence that this region also influences sensitivity to other drugs of abuse and related behavioral phenotypes. Common variants in this gene region have been associated with cocaine use (Sadler et al., 2014), alcohol consumption (Hoft et al., 2009b), alcohol use (Landgren et al., 2009), and novelty seeking (Landgren et al., 2011). In addition, a recent study provided evidence that rare variants in *CHRNB3* are associated with alcohol and cocaine behaviors (Haller et al., 2014). Together with the current data, these studies suggest that genetic variation in this region may influence a general susceptibility to use drugs of abuse.

Nicotinic receptors containing α6 or β3 subunits (coded by the *CHRNA6* and *CHRNB3* genes, respectively) are found in brain regions known to be important for drug behaviors. Receptors containing these subunits are localized in both the ventral tegmental area and substantia nigra, and have been shown to be involved in dopamine release (Grady et al., 2007). Genetic variation in these genes may alter sensitivity to drugs of abuse in a number of ways. It is possible that a genetic variant may change the amino acid structure of the protein or cause alterations in transcription or translation regulation. Changes in the coding sequence which lead to changes in the pharmacological properties of the receptor have been observed with genetic variants in other nicotinic receptor subunits (Bierut et al., 2008; McClure-Begley et al., 2014). In addition, common genetic variation upstream of *CHRNB3* has been shown to alter gene expression using an *in vitro* reporter assay (Ehringer et al., 2010; Kamens et al., 2015). Given that these are low frequency variants, it is possible that different variants in each individual may affect the biology of this system in unique ways.

Unfortunately, neither SKAT-O nor C-alpha methods can indicate which variants are contributing the most to the observed association, or whether any of them are functional. Examination of the number of low frequency variants observed in cases and controls suggest there are overall more low frequency variants in cases. These data add to the existing literature that low frequency variants in *CHRN* genes are important in drug phenotypes (Haller et al., 2012, 2014; Wessel et al., 2010; Xie et al., 2011). It is difficult to predict which variant(s) may cause this effect, but prior work has highlighted the enrichment of variants in the UTRs and exons as associated with complex traits (Schork et al., 2013). We observed seven rare nonsynonymous variants that lead to amino acid changes (five in *CHRNB3* and two in *CHRNA6*). Six of these variants are observed in cases where as only two were observed in controls; these variants may warrant further exploration. The four nonsynonymous variants classified as probably damaging by Polyphen-2 may be of interest for further analysis. We found twice as many variants in *CHRNA6* introns and UTRs in cases compared to control, which may lead to subtle differences in transcription (Ehringer et al., 2010; Flora et al., 2013; Gallego et al., 2013; Kamens et al., 2015).

Interestingly, a recent study by Vrieze and colleagues (Vrieze et al., 2014) examined the association between rare exonic variation and addiction traits and behavioral disinhibition. Vrieze et al were unable to observe an association between rare genetics variants in the *CHRNA6/CHRNB3* genes and behavioral disinhibition or the use of nicotine, alcohol or drugs. In addition to differences in phenotypes examined and the sample population, there are at least two other reasons why we may have observed an association in our sample compared to this work. First, these authors used the Illumina HumanExome BeadChip to genotype known rare exonic variants. We may have detected more rare exonic variants in our sequencing based approach. Alternatively, these data could point to the importance of rare genetic variation in non-coding regions since Vrieze and colleagues focused solely on exonic variants. Future work will be needed to disentangle these possibilities.

Much less has been reported with regard to the role of *CHRNB1* variants and drug behaviors. Significant associations with common genetic variants in *CHRNB1* and nicotine dependence have been reported in samples of primarily European ancestry (Lou et al., 2006; Philibert et al., 2009; Saccone et al., 2009, 2010). In contrast, studies of African American samples have yielded mixed results (Lou et al., 2006; Saccone et al., 2010). We found a nominal association with *CHRNB1* and antisocial drug dependence in the white non-Hispanic subgroup of Sample 1 (see Table IV). This finding was not replicated in the full analysis of Sample 1 or in Sample 2. The sample size (25 controls/24 cases) of this subgroup is very small, so replication of these findings will be critical. Although there have been a number of published reports examining rare variants in nicotinic acetylcholine receptors genes, here we present the first report of variants in the *CHRNB1* gene and potential associations with behavior.

The association between low frequency genetic variants in *CHRNA6/CHRNB3* and antisocial drug dependence was only observed in Sample 2. There are several reasons why we may not have observed an association in Sample 1. First, the sample size was approximately half that of Sample 2, thus there was lower power to detect an association. *Post hoc* power calculations revealed that under an optimal scenario with 10% of the cases having at least one rare variant and 5% of controls carrying a variant compared to cases we only had 40% power to detect an association in Sample 1. Under the same conditions we had 80% power to detect an association in our larger sample (Sample 2). Even though we had 80% power under this ideal condition, power decreases dramatically when a greater proportion of controls has the variant or the proportion of cases with the variant decreases. Second, Sample 1 included subjects 14–30 years of age while subjects selected in Sample 2 were limited to 14–19 years of age. With a more narrow age range, the individuals who were defined as cases may have a slightly different, perhaps more extreme, behavioral phenotype compared to those with the larger age range. Third, Sample 1 was analyzed using earlier enrichment and sequencing technologies. Visualization of the read coverage of Sample 1 revealed that there were regions of the genes that were not well covered. It is possible that enrichment was not consistent across all regions, leading to regions with lower coverage and missed rare variant calls. Similar to Sample 2, we did observe more low frequency variants in cases compared to controls in the *CHRNA6/CHRNB3* gene region in Sample 1 (495 versus 415), but this was not statistically significant. Finally, as noted above these are relatively small samples and significant results could be attributed to false positives.

Although this is a possibility, it is important to recognize that these data are consistent with the contribution of common and rare genetic variation in this region to drug and personality phenotypes (Culverhouse et al., 2014; Haller et al., 2014; Hoft et al., 2009b; Landgren et al., 2009; Sadler et al., 2014; Thorgeirsson et al., 2010). Moreover, the association with variants in this region in the subset of white non-Hispanic subjects in Sample 2 would withstand the Bonferroni multiple comparisons correction for all 96 tests performed.

We chose to examine antisocial drug dependence because it represents a composite score taking into account both the use of multiple drugs and conduct disorder that is very well-characterized in our unique sample. Common genetic factors have been demonstrated to contribute to these two behaviors (Button et al., 2006) and this phenotype was found to be the most heritable in our CADD sample (Stallings et al., 2005). An additional limitation of this approach is the inherent assumption that any functional alleles confer the same direction of effect for all drugs. However, this may not be the case. For example, the minor allele of the well-characterized common variant rs16969968 in *CHRNA5*, is the risk allele for nicotine dependence, but is protective for cocaine dependence (Grucza et al., 2008; Sherva et al., 2010).

Limitations

We report here modest associations with *CHRN* genes and antisocial drug dependence, but this study is not without important limitations. First, we acknowledge that the sample sizes are small. To our knowledge there are no publicly available datasets that have measures of antisocial drug dependence with sequencing coverage of the nicotinic receptors. Second, the sequencing coverage of sample 1 was not as good as sample 2, but in most cases (Table II) exceed the 50X coverage required for variant calling (Ajay et al., 2011). Finally, our sample was enriched for ethnic diversity, and this may have decreased our ability to detect significant associations.

Overall conclusion

Here we provide suggestive evidence that low frequency genetic variants in the *CHRNA6/CHRNB3* gene region are associated with antisocial drug dependence. This is consistent with prior literature showing that common genetic variants in this region are associated with multiple drugs of abuse and novelty seeking (Hoft et al., 2009b; Landgren et al., 2009, 2011; Rice et al., 2012; Sadler et al., 2014; Thorgeirsson et al., 2010). Additional sequencing studies of these genes should be conducted to expand the sample size and collection of low frequency variants. Such future studies will increase the power for detecting associations with other receptor genes. Likewise, the novel variants identified here should be further characterized using laboratory approaches and mouse models to determine their functional role. Variants with verified biological significance may potentially lead to novel therapies for these costly and disruptive behavioral disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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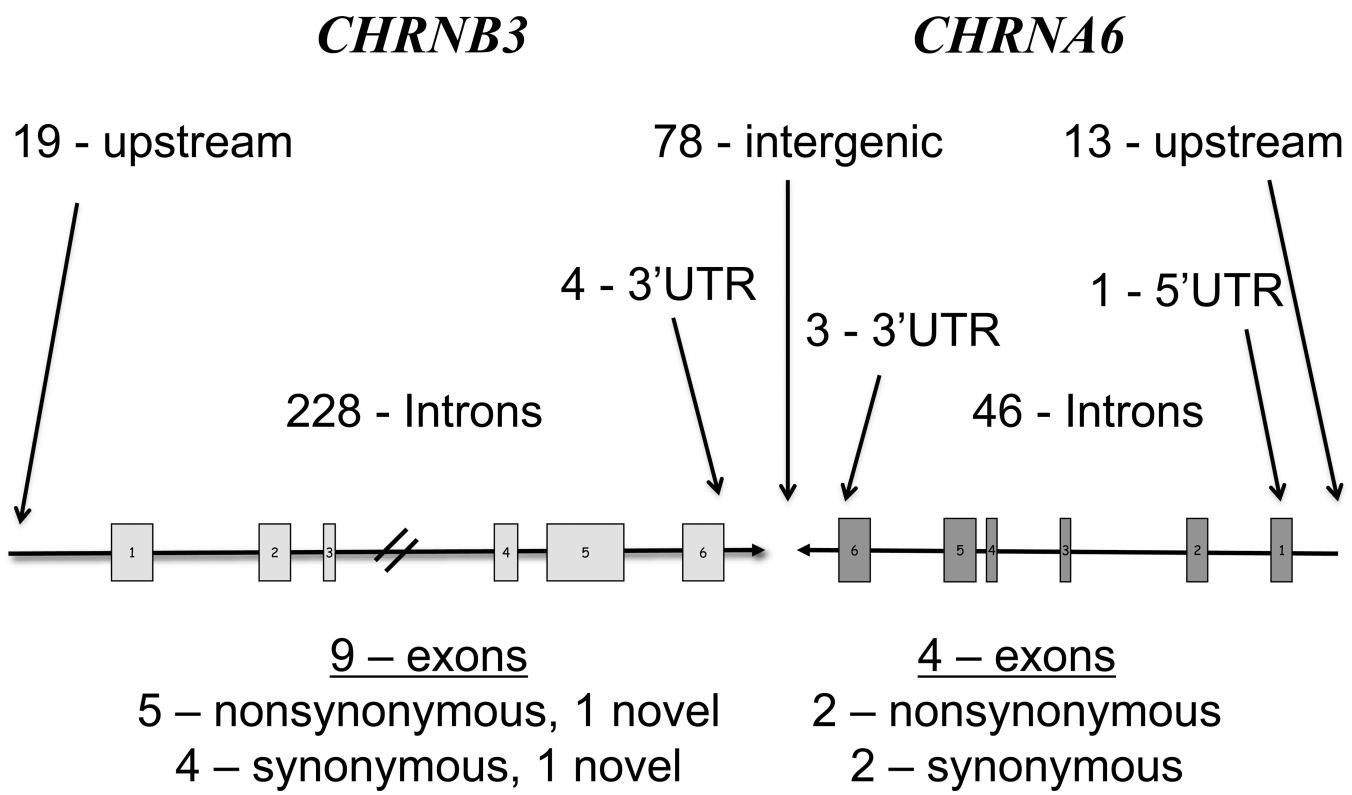

Figure 1.
Location of low frequency genetic variants observed in the *CHRNB3/CHRNA6* gene region in Sample 2.

Table I

Descriptive statistics of the test populations.

	Sample 1		Sample 2	
	Control	**Case**	**Control**	**Case**
Male (%)	35 (62.6)	44 (75.9)	74 (74)	68 (68)
Age (years ± SD)	19.91 (3.42)	16.62 (2.38)	17.48 (1.30)	15.40 (1.13)
Caucasian (%)	25 (44.6)	24 (41.4)	66 (66)	45 (45)
Hispanic (%)	26 (46.4)	22 (37.9)	23 (23)	36 (36)
Non-white, non-Hispanic	5 (8.9)	12 (20.7)	11 (11)	19 (19)

Table II

Average read depth.

Gene	Sample 1	Sample 2
CHRNA1	99	413
CHRNA2	161	468
CHRNA5/CHRNA3/CHRNB4	151	348
CHRNA4	23	438
CHRNA6/CHRNB3	136	331
CHRNA7	223	362
CHRNA9	159	349
CHRNA10	156	464
CHRNB1	127	418
CHRNB2	161	511
CHRNE	76	461
CHRND/CHRNG	65	383

Table III

Results of association analyses with low frequency genetic variants in *CHRN* genes and antisocial drug dependence in all subjects in Sample 1.

Gene	Num of Low Frequency Variants	SKAT-O p-value	C-alpha p-value
CHRNA1	96	1	0.93
CHRNA2	81	0.59	0.47
CHRNA5/CHRNA3/CHRNB4	217	0.64	0.81
CHRNA4	63	0.89	0.67
CHRNA6/CHRNB3	243	0.93	0.83
CHRNA7	384	0.90	0.88
CHRNA9	83	0.44	0.54
CHRNA10	47	0.10	0.25
CHRNB1	30	0.21	0.11
CHRNB2	52	0.41	0.62
CHRNE	51	1	0.64
CHRND/CHRNG	62	0.47	0.46

Table IV

Results of secondary association analyses with low frequency genetic variants in *CHRN* genes and antisocial drug dependence in white non-Hispanic subjects only from Sample 1.

Gene	Num of Low Frequency Variants	SKAT-O p-value	C-alpha p-value
CHRNA1	31	0.85	0.76
CHRNA2	45	0.53	0.34
CHRNA5/CHRNA3/CHRNB4	68	0.02*	0.13
CHRNA4	32	1	0.89
CHRNA6/CHRNB3	137	0.68	0.72
CHRNA7	157	0.68	0.36
CHRNA9	38	0.62	0.26
CHRNA10	20	0.33	0.37
CHRNB1	14	0.02*	0.04*
CHRNB2	19	0.83	0.46
CHRNE	18	0.82	0.54
CHRND/CHRNG	29	0.42	0.33

* indicates nominal significant association of uncorrected p-value.

Table V

Results of association analyses with low frequency genetic variants in *CHRN* genes and antisocial drug dependence in all subjects in Sample 2.

Gene	Num of Low Frequency Variants	SKAT-O p-value	C-alpha p-value
CHRNA1	159	1	0.78
CHRNA2	201	0.85	0.36
CHRNA5/CHRNA3/CHRNB4	397	0.69	0.03*
CHRNA4	198	0.26	0.21
CHRNA6/CHRNB3	405	0.02*	0.02*
CHRNA7	622	0.23	0.06
CHRNA9	145	0.89	0.06
CHRNA10	104	0.50	0.64
CHRNB1	84	0.47	0.26
CHRNB2	107	0.70	0.31
CHRNE	110	0.07	0.14
CHRND/CHRNG	150	0.54	0.39

* indicates nominal significant association of uncorrected p-value.

Table VI

Results of secondary association analyses with low frequency genetic variants in *CHRN* genes and antisocial drug dependence in white non-Hispanic subjects only from Sample 2.

Gene	Num of Low Frequency Variants	SKAT-O p-value	C-alpha p-value
CHRNA1	107	0.71	0.71
CHRNA2	149	0.78	0.42
CHRNA5/CHRNA3/CHRNB4	299	0.54	0.78
CHRNA4	143	0.65	0.34
CHRNA6/CHRNB3	348	<0.001**	0.27
CHRNA7	392	<0.01*	0.31
CHRNA9	107	0.89	0.71
CHRNA10	86	0.06	0.50
CHRNB1	52	0.08	0.62
CHRNB2	84	0.40	0.26
CHRNE	75	0.82	0.45
CHRND/CHRNG	107	0.16	0.18

* indicates nominal significant association of uncorrected p-value.

** indicates significant association when p-value is corrected for multiple comparisons using a Bonferroni correction.

Table VII

Number of low frequency genetic variants in the *CHRNB3/CHRNA6* gene region in 100 cases and 100 controls in Sample 2.

	Case	Control
Upstream *CHRNB3*	46	33
CHRNB3 intron	1514	545
CHRNB3 exon	28	10
CHRNB3 3'UTR	6	7
Intergenic	188	141
CHRNA6 3'UTR	5	1
CHRNA6 intron	97	48
CHRNA6 exon	2	2
CHRNA6 5'UTR	3	1
Upstream CHRNA6	21	12
Total	1910	800

Table VIII

Results from Polyphen-2 analysis of nonsynonymous variants from Sample 2 in the *CHRNB3* and *CHRNA6*.

rs#	Location	Gene	Prediction	Score	Sensitivity	Specificity
NA	42586877	*CHRNB3*	Probably damaging	1	0	1
rs75384358	42587301	*CHRNB3*	Probably damaging	1	0	1
rs149775276	42587435	*CHRNB3*	Probably damaging	0.999	0.14	0.99
rs56198260	42587552	*CHRNB3*	Benign	0.005	0.97	0.74
rs35327613	42591735	*CHRNB3*	Benign	0.245	0.91	0.88
rs55662044	42608381	*CHRNA6*	Benign	0.012	0.96	0.78
rs144350308	42611524	*CHRNA6*	Probably damaging	1	0	1