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Deep resequencing of 17 glutamate system genes identifies rare variants in *DISC1* and *GRIN2B* affecting risk of opioid dependence

Pingxing Xie¹, Henry R. Kranzler², John H. Krystal³, Lindsay A. Farrer⁴, Hongyu Zhao⁵, and Joel Gelernter⁶

¹Department of Genetics, Yale University School of Medicine, New Haven, CT; VA CT Healthcare Center, West Haven, CT, USA

²Department of Psychiatry, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104; VISN 4 MIRECC, Philadelphia VAMC, Philadelphia, PA, USA

³Department of Psychiatry, Yale University School of Medicine, New Haven, CT; VA CT Healthcare Center, West Haven, CT, USA

⁴Departments of Medicine (Biomedical Genetics), Neurology, Ophthalmology, Genetics and Genomics, Epidemiology, and Biostatistics, Boston University School of Medicine and Public Health, Boston, MA, USA

⁵Departments of Genetics and Biostatistics, Yale University School of Medicine, New Haven, CT, USA

⁶Departments of Genetics and Psychiatry, Yale University School of Medicine, New Haven, CT 06511; VA CT Healthcare Center, West Haven, CT, USA

Abstract

The *N*-methyl-D-aspartate (NMDA) glutamate receptors play important roles in the pathophysiology of substance dependence (SD), but no strong genetic evidence has associated common variants in NMDAR-related genes to SD. We hypothesized that rare variants (RVs) with minor allele frequency < 1% in the NMDAR-related genes might exert large effects on SD risk. We sequenced 34,544 bp of coding and flanking intronic regions of 17 genes involved in the NMDA system in 760 subjects, all with co-occurring alcohol dependence (AD), cocaine dependence (CD) and opioid dependence (OD), and 760 healthy control subjects. One hundred percent of the target regions were sequenced at > 1000× coverage. We identified 454 variants, including 380 RVs. Based on case-control allele count differences, we genotyped 11 exonic RVs in 6751 additional subjects, and the 1520 subjects from the sequencing stage for validation. All

Correspondence to: Joel Gelernter, MD, Yale University School of Medicine, Department of Psychiatry, Division of Human Genetics in Psychiatry; VA CT Healthcare Center 116A2; 950 Campbell Avenue; West Haven, CT 06516; Telephone: 203-932-5711; Fax: 203-937-4741; joel.gelernter@yale.edu.

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alleles of the 11 rare variants called in the sequencing stage were confirmed. We found a statistically significant association of the 11 RVs with OD in AAs ($P=0.00080$). Results from gene-based association tests showed that the association signal derived mostly from *DISC1* ($P=0.0010$) and *GRIN2B* ($P=0.00085$). *DISC1* is a well-validated schizophrenia risk gene. This is the first demonstration that RVs affect the risk of OD and the first demonstration of biological convergence of schizophrenia and OD risk -- via *DISC1*.

Keywords

addiction; *DISC1*; NMDA; opioid dependence; rare variants

Introduction

Genomewide association studies (GWAS) have had limited success at identifying risk genes for complex traits (Chambers et al., 2001). With the noteworthy exception of nicotine dependence (ND), published GWAS for drug dependence traits (excluding alcohol dependence) have for the most part not produced compelling results (Liu et al., 2010). The reservoir of the unexplained heritable risk for most complex traits is an area of much discussion. Clearly, some of this reservoir is composed of rare variants (RVs), either too infrequent, or of too recent origin, or both, to be detected by GWAS. It is unlikely that the full set of RVs will be sufficient to account for all or even most of the “missing” heritability, but RVs have been shown to account for at least some of the risk in a growing set of complex traits, including inflammatory bowel disease (Rivas et al., 2011), autism (Sanders et al., 2012), schizophrenia (Girard et al., 2011), impulsivity (Bevilacqua et al., 2010), and nicotine dependence (Xie et al., 2011). Next-generation high throughput sequencing technologies have recently rendered deep sequencing projects more feasible. However, whole-exome or whole-genome sequencing a large number of patients are still costly. We therefore focused on genes involved in the glutamatergic pathway and investigated the RV contribution to substance dependence (SD) risk, using deep high throughput sequencing.

Genes coding for glutamate receptors and genes coding for proteins that regulate glutamate receptor function are very important in addiction biology and are therefore promising candidates as addiction risk genes. The *N*-methyl-D-aspartate (NMDA) glutamate receptor, for example, is one of the most potent brain targets for ethanol (Grant and Lovinger, 1995; Manolio et al., 2009). Ethanol's blockade of glutamate receptors contributes to features of human ethanol intoxication (Girard et al., 2011). Similarly, neuroadaptations in NMDA, AMPA, and several metabotropic glutamate receptors contribute to features of tolerance, sensitization, and withdrawal from ethanol, opiates, nicotine, and cocaine (Kalivas, 2009; Krystal et al., 2003b; Liechti and Markou, 2008; Manolio et al., 2009; Siggins et al., 2003). Some of these neuroadaptations may be downstream consequences of the capacity of drugs of abuse to stimulate the release of dopamine and, as a downstream consequence of dopamine-related signaling mechanisms, to regulate NMDA receptors (Rasmussen et al., 1991).

NMDA receptors are regulated via phosphorylation sites on receptor subunits (Mao et al., 2011). Protein kinase A (PKA) and protein kinase C (PKC) are the most studied serine and threonine kinases that upregulate NMDAR function (Cerne et al., 1993; Chen and Huang, 1992), while protein phosphatases 1 (PP1), 2A (PP2A), and 2B (PP2B) are the major protein serine/threonine phosphatases to suppress NMDAR activity (Wang et al., 1994). For protein tyrosine kinases upregulating NMDAR function, the Src family, especially Src and Fyn, are the major members of this group, while the most investigated protein tyrosine phosphatase reducing NMDAR activity is striatal-enriched protein (STEP). Recently, palmitoylation was also found to regulate NMDAR trafficking (Hayashi et al., 2009). NMDAR functions are linked to many proteins directly or indirectly including dysbindin (Tang et al., 2009), neuregulin 1 (Hahn et al., 2006), and Disc1 (Hayashi-Takagi et al., 2010; Ron, 2004).

Despite some encouraging initial findings (Carter, 2006), there is not yet compelling evidence of the involvement of genes encoding proteins involved in glutamate signaling in addiction vulnerability. In this study, we hypothesized that NMDAR-related RVs (minor allele frequency (MAF) < 1%) play important roles in SD. To test the hypothesis, we sequenced the coding exons and their flanking intronic regions of 17 genes related to NMDARs and NMDAR function for 760 individuals with co-occurring diagnoses of alcohol dependence (AD), cocaine dependence (CD) and opioid dependence (OD), and 760 healthy controls. We chose subjects who were dependent on all three substances for the purpose of increasing statistical power, because sampling individuals with extreme phenotypes can enrich the presence of risk rare variants (Barnett et al., 2013). We followed up 11 RVs in an independent sample of 6751 subjects with a high percentage of SD (where contributions to the individual SD traits could also be disambiguated). We observed that in African Americans (AAs), RVs in *DISC1* and *GRIN2B* affect the risk of OD, with stronger evidence in *DISC1* than *GRIN2B*.

Materials and methods

Study subjects

Subjects were recruited at five US sites for studies of the genetics of AD, CD and OD: Yale University School of Medicine (n=3461), the University of Connecticut Health Center (n=3336), the University of Pennsylvania School of Medicine (n=669), the Medical University of South Carolina (n=594), and McLean Hospital, an affiliate of Harvard Medical School (n=211). The institutional review boards at each of the participating sites approved the study. Written informed consent was obtained from all participants. All subjects were interviewed by trained interviewers using the Semi-Structured Assessment for Drug Dependence and Alcoholism (SSADDA) to derive diagnoses for lifetime psychiatric and substance use disorders based on *DSM-IV* criteria (Pierucci-Lagha et al., 2007; Pierucci-Lagha et al., 2005). The inter-rater and test-retest reliabilities of the SSADDA for the lifetime diagnoses of AD, CD and OD based on *DSM-IV* were excellent (Pierucci-Lagha et al., 2005).

In the sequencing stage, the coding regions and the flanking intronic regions of 17 genes associated with NMDARs were sequenced using DNA samples collected from 760 SD cases and 760 controls for a total of 1520 research subjects. The 760 SD cases all had lifetime

diagnoses of dependence on three substances: alcohol, cocaine and opioids. Some SD subjects were also dependent on other drugs, such as nicotine and cannabis. The 760 controls were healthy subjects with no lifetime SD or substance abuse diagnoses. In addition to these 1520 subjects, another 6751 subjects were studied in the genotyping stage. Among them, 5482 had phenotype information for AD (1269 subjects who met criteria for alcohol abuse, but not for AD, were excluded from the analyses), 6538 had phenotype information for CD (213 subjects with cocaine abuse, but not CD, were excluded from analyses), and 6608 had phenotype information for OD (143 subjects with opioid abuse, but not OD, were excluded from analyses). None of these 8271 subjects had lifetime diagnoses of schizophrenia, schizoaffective disorder, or mental retardation.

DNA sequencing

DNA was extracted from blood, saliva, or cell lines. A detailed description regarding DNA quality and quantity is provided in the supplementary material. A total of 1520 DNA samples were selected for sequencing based on the subjects' phenotypes (Table 1) and the DNA quality and quantity.

We adopted a DNA pooling strategy to reduce the cost and maximize the utility of the high-throughput DNA sequencing technology. This strategy has been proven to be very successful in a number of previous studies (Bevilacqua et al., 2010; Rivas et al., 2011). First, four DNA samples with the same concentrations and volumes were pooled. The four pooled DNA samples were from subjects with the same sex, population, and phenotypes of AD, CD and OD. This resulted in 380 pooled DNA samples for PCR enrichment of the targeted genomic regions.

The target regions are the coding exons and their flanking intronic regions (at least 50bp from the exon) of the 17 genes. A total of 173 PCRs with a cumulative length of 75,152 bp were performed to cover the targeted regions of 34,544 bp. PCR conditions of all the primers were optimized individually. Then, the 173 PCR products from each of the 380 pooled DNA samples were combined. Five pools with the same sex, population group, and phenotypes of AD, CD and OD were pooled. In the end, 76 DNA pools with 20 individuals per pool were prepared for sequencing, including 16 AA case pools, 19 AA control pools, 22 EA case pools, and 19 EA control pools.

The 76 DNA pools were barcoded, and paired-end sequenced with read-length 2×100 bp in seven lanes of an Illumina HiSeq analyzer at the Yale Center for Genome Analysis. Each of the first six lanes contained 11 barcoded DNA pools, and the seventh lane contained 10 bar coded DNA pools. Every lane included both case and control pools from AAs and EAs.

Validation and follow-up genotyping

Seventeen coding RVs observed in the sequencing stage were selected to be genotyped in the 1520 subjects for validation and in another 6751 subjects for follow up. The criteria for selecting these RVs were: first, MAFs less than or equal to 1% in both EAs and AAs; second, location in coding regions or introns within 5 bp of a splice junction; third, the variants were in at least three more case pools than control pools, or vice versa, in the

sequencing stage. Eleven of the 17 RVs were successfully genotyped using the TaqMan method.

Data analysis

The population group of each participant was ascertained by one of two methods: First, for 6310 subjects, 41 ancestry informative markers (AIMs), including 36 short tandem repeats markers and 5 single nucleotide polymorphisms (SNPs) were genotyped (Yang et al., 2005). We used STRUCTURE software (Pritchard and Rosenberg, 1999) to analyze the AIMs, and to generate an ancestry proportion score for each individual. Subjects with African ancestry proportion scores < 0.5 were classified as EAs; otherwise, they were classified as AAs. Second, 96 AIMs, all SNPs, were genotyped for the remaining 1961 subjects. These AIMs were selected from SNPs included in the Illumina OminQuad genotyping microarray to maximize allele frequency differences between European, African, Asian, and other ancestry. STRUCTURE software was used to analyze the AIMs and group the subjects. In total, 4240 of the 8271 subjects were AAs, and 4031 subjects were EAs.

In the sequencing stage, reads were aligned to human reference genome 19 using BWA software (Li and Durbin, 2009). The per-base quality scores were recalibrated, and reads were locally realigned using the Genome Analysis Toolkit (DePristo et al., 2011). Syzygy software (Rivas et al., 2011) was used to call variants from reads generated from pooled DNA samples.

Sequence Kernel Association Test (SKAT) (Wu et al., 2011) was adopted to test for association between the 11 RVs and three SD phenotypes, namely, AD, CD and OD. When one SD phenotype was used as the outcome, the other two phenotypes were adjusted in the model along with sex and age. SKAT is a powerful method to test for association between SNP sets and phenotypes, and is designed to perform well when the SNP set is a mixture of protective and deleterious variants (Ladouceur et al., 2012). At first, SKAT analyses were performed for RVs in the same genes. Since these 11 RVs are located in 7 genes, and there were three phenotypes and two populations, the threshold of significance was $0.05/(7 \times 3 \times 2) = 0.0012$. Then, all 11 RVs were analyzed together to test whether genes in the NMDAR system associated with AD, CD or OD. Bonferroni correction was used here also to adjust for multiple comparisons, and the threshold of significance was $0.05/(3 \times 2) = 0.0083$.

We performed power analysis of our association tests based on the sample size of each SD phenotype, and the type I errors determined by Bonferroni correction. The risk allele frequency was assumed be to 1% with relative risk of 2.

Following these analyses, the associations between *DISC1* RVs and whether subjects had ever used opioids and whether users had experienced OD were investigated in AAs. In the SSADDA interview, all subjects were asked “have you ever used any of the following opiate/drugs? Heroin, codeine, Demerol, morphine, Percodan, Percocet, methadone, Darvon, opium, fentanyl or P-dope, Dilaudid or other opiate.” Subjects who answered yes were considered to have been exposed to opioids. SKAT was used to test the association between *DISC1* RVs and opioid exposure. To explore the role of *DISC1* RVs in the development of

OD after exposure, we restricted the SKAT analysis to subjects who had used opioids before. All tests adjusted for sex, age, AD and CD.

Results

Many genes are linked to NMDAR functions. We selected seventeen genes closely related to NMDAR functions for study: three NMDAR subunit genes (*GRIN1*, *GRIN2A*, and *GRIN2B*), ten genes encoding protein kinases, protein phosphatases and protein palmitoyltransferase, which perform post-translational modifications of NMDAR (*FYN*, *SRC*, *PRKCG*, *PTPN5*, *PPP1CA*, *PPP1CB*, *PPP1CC*, *PPP1R1B*, *PPP1R9B*, and *ZDHHC3*), and four genes encoding proteins that regulate NMDAR function (*DISC1*, *NRG1*, *DAOA*, and *DTNBP1*).

In the sequencing stage, the coding exons and their flanking intronic regions of the 17 genes were sequenced in 320 AA cases with co-occurring diagnoses of AD, CD and OD and 380 AA controls, and 440 EA cases with co-occurring diagnoses of AD, CD and OD and 380 EA controls (Table 1). Each DNA pool contained DNAs from 20 subjects of the same sex, population, and phenotypes (AD, CD and OD or none of these). The total length of the target regions was 34,544 bp. One hundred percent of the target regions were sequenced at $> 1000\times$ coverage, and the mean coverage of all of the bases in the target regions per pool was $41,869\times$ (about $1,000\times$ per individual chromosome; Figure S1) indicating very high depth.

A total of 454 variants were found in the coding exons of the 17 genes, including 380 RVs (MAF $\leq 1\%$ in both EAs and AAs), and 74 common variants (MAF $> 1\%$ in at least of one of the two populations). As expected, AAs had more rare variants than EAs (Table 2). Genes with longer cDNA tend to have more exonic variants ($r=0.87$, $P=4.9\times 10^{-6}$). However, some genes are less conserved than others, although they have similar length of cDNA. Among these 454 variants, 61.5% were already in dbSNP version 137, and the transition/transversion ratio (Ti/Tv) was 3.50. The Ti/Tv is consistent with the results from the 1000 Genomes Exon Pilot Project (Marth et al., 2011), indicating low false-positive variant calling from our sequencing results. In addition to these 380 exonic RVs, eight RVs were found in introns within 5 bp of a splice junction.

Because of the moderate sample size in the sequencing stage and our focus on rare variants, the power to detect a significant association with a single RV and SD is very low. The discovery stage was employed only for variant discovery, not for association discovery. Based on the sequencing results, we selected a subset of the RVs and genotyped them individually in an independent sample of 6751 similarly assessed subjects for follow up, and in the 1520 subjects included in the sequencing stage, to validate the genotype calls in that sample. The criteria for selecting these RVs were: first, MAFs $\leq 1\%$ in both EAs and AAs; second, location in coding regions or introns within 5 bp of a splice junction; third, variants were found in at least three more case pools than control pools, or vice versa, so they could be either deleterious or protective. A total of 17 RVs met these criteria. Because three TaqMan assays failed manufacturing quality control, and another three assays performed poorly, in the end, 11 RVs were successfully genotyped.

The call rate of the 11 RVs ranged from 96.1% to 98.0%, with an average call rate of 97.1%. In the sequencing stage, the 11 RVs were observed in 88 DNA pools. TaqMan genotyping successfully validated all of the RVs called in the sequencing stage. Moreover, no additional alleles were found in the 1520 sequenced subjects. This indicates very high sensitivity and specificity of the call set. Our pooled DNA sequencing results were of high quality based on available metrics, mainly due to the extreme high coverage in our sequencing data. After genotyping an additional 6751 subjects, we observed a total of 766 alleles of these 11 RVs in AAs and 543 alleles in EAs (Table 3). Since these RVs exist in at least three individuals in the sequencing stage, none of them were singletons, and 11 of them were already in dbSNP database. The largest MAFs were 0.85% in AAs, and 1.0% in EAs. The average African ancestry proportion was 0.922 in AAs who did not carry any alleles of these 11 RVs, and 0.938 in AAs who were carriers. In EAs, the average African ancestry proportion was 0.063 in non-carriers of these 11 RVs, and 0.069 in carriers.

Associations between the RVs and AD, CD and OD were evaluated using SKAT (Wu et al., 2011), for the 8271 subjects. The analysis was adjusted for age, sex and SD phenotypes (when one SD phenotype was used as the outcome, the other two phenotypes were adjusted in the model).

At first, RVs in different genes were analyzed separately. Based on the power analysis of the association tests, the power to detect RVs with relative risk of 2 for AD, CD and OD was 55%, 47% and 60% in AAs; 52%, 58% and 65% in EAs (Figure S2A and S2B). We observed that in AAs, *DISC1* and *GRIN2B* were significantly associated with OD ($P=0.0010$ and 0.00085 , respectively) (Table 3). Both were highly significance based on Bonferroni correction ($P=0.0012$). No statistically significant associations were observed in EAs. Three RVs in *DISC1* were investigated in this study. In AAs, differences in MAF between opioid dependence cases and controls at two stages are very similar (Table 3). In the combined sample, the minor allele of the first RV, rs139667828, was observed in 64 of the 6386 chromosomes (1.00%) in OD controls, and 5 of the 1832 chromosomes (0.27%) in OD cases; the minor allele of the second RV rs116628628 occurred in 26 of the 6334 chromosomes (0.41%) in OD controls, and 13 of the 1838 chromosomes (0.71%) in OD cases; the minor allele of the third RV rs61737326 occurred in 58 of the 6352 chromosomes (0.91%) in OD controls, and 8 of the 1834 chromosomes (0.44%) in OD cases. Therefore, two of the three rare variants in *DISC1* were protective, and another was deleterious. These three RVs in *DISC1* were independent of each other, with only one AA subject carrying two different minor alleles (rs139667828 and rs61737326) and no subjects carrying three different minor alleles. In EAs, because each of the three *DISC1* RVs was observed only once, we could not test for association (Of course, other RVs in *DISC1* may affect OD risk in EAs). For *GRIN2B*, only one RV (rs146792012) was studied. In AAs, its minor allele was not observed in any of the 6320 chromosomes in OD controls, but was found in 3 of the 1814 chromosomes (0.17%) in OD cases. This RV was also observed at a low frequency in EAs, but its association with OD was not statistically significant.

Next, these 11 RVs were analyzed together by the SKAT method. The power to detect RVs with relative risk of 2 for AD, CD and OD increased to 76%, 70% and 80% in AAs; 75%, 80% and 85% in EAs (Figure S3A and S3B). This indicates that the test had adequate

power. A statistically significant association was observed in AAs for OD, with $P=0.00080$ (Table 3). This was highly significance based on Bonferroni correction for multiple testing ($P=0.0083$).

We further investigated the role of three *DISC1* RVs in the initiation of opioid use and the development of OD in AAs. First, we tested the association between the RVs and opioid initiation, and no significant association was observed ($P=0.1686$). Then, we restricted the analysis to AAs who had initiated an opioid ($n=1701$), and found a significant association between the three RVs and OD ($P=0.0041$). rs139667828 had the strongest protective effect ($P=0.0013$). Its minor allele existed in 18 of the 1514 chromosomes (1.19%) in OD controls, and 5 of the 1832 chromosomes (0.27%) in OD cases.

On the contrary, the RV rs146792012 in *GRIN2B* was observed to be significantly associated with opioid initiation ($P=0.0155$) in AAs. However, possibly due to the small sample size and low MAF, no significant association was found for OD among opioid users ($P=0.158$).

Discussion

In this study, we sequenced the coding regions and the flanking intronic regions of 17 NMDAR-related genes in 1520 subjects to study the relationship of RVs at these loci to SD phenotypes (AD, CD and OD). A total of 388 of RVs (MAF $\geq 1\%$ in both EAs and AAs) were observed with high confidence in coding exons and introns within 5 bp of a splice junction (Table 2). Seventeen exonic RVs, the frequency of which were observed in at least three more case pools than control pools, or vice versa, were selected for follow up in an independent sample of 6751 subjects. Eleven RVs were successfully genotyped using the TaqMan method. All variants called in the sequencing stage were validated by TaqMan genotyping and no additional alleles were observed in the subjects of the sequencing stage, indicating high quality of the sequencing data where it could be confirmed directly. SKAT analyses of the 8271 subjects showed a statistically significant association between these 11 RVs and OD in AAs ($P=0.00080$). Results from gene-based association tests showed that the association signal derived mostly from *DISC1* ($P=0.0010$) and *GRIN2B* ($P=0.00085$) (Table 3). It is possible that these RVs influence protein expression and/or function, and thus contribute to OD risk in AAs.

There is strong evidence from functional studies that the NMDA system plays important roles in AD, CD and OD (Kalivas, 2009; Krystal et al., 2003b; Liechti and Markou, 2008; Manolio et al., 2009; Siggins et al., 2003), and we identified RVs associated with OD. We did not identify RVs in genes of the NMDA system that affect risk for AD and CD; this could have been due to larger statistics power in identifying RVs associated with OD than that in AD and CD. In addition, since only a subset of RVs were explored in this study, whether they are associated with SD remains unknown.

Three of the 11 RVs genotyped were located in *DISC1*. Rs139667828 and rs116628628 are 242kb apart, and rs116628628 and rs61737326 are 73kb apart. The three RVs are independent of each other. In this study, we did not have a prior hypothesis of whether the

RVs are protective or deleterious, because each mutation can have different functions in terms of gene expression level and protein function.

For rare variants rs139667828 and rs61737326 located in *DISC1*, differences in MAF between OD cases and controls were very similar when observed at the sequencing stage and again at the genotyping stage. Because the direction of the association was the same for the two rare variants, we used a regular collapsing method to test their association with OD. Based on the Fisher's exact test, the association p-value was 0.035 in the sequencing stage and 0.0028 in the genotyping stage. When subjects from the two stages were combined, the association p-value was 0.0001. Although combining the subjects yielded a smaller p-value, we posit that it was not, most likely, because of bias from including subjects from the first stage, but due to increased sample size. This is supported by the allele frequency similarities noted above.

For rs116628628, the minor allele of the rare variant was observed more frequently in OD cases than in controls at the sequencing stage. However, the difference was not observed at the genotyping stage. Thus, it is likely that rs116628628 has little actual effect on OD risk and its inclusion did not contribute to the association signal. As stated above, without considering rs116628628, the association p-value for the combined sample was 0.0001. However, after including this rare variant, the p-value increased to 0.0010. Because we followed-up three *DISC1* rare variants, we included all of them in the association test.

The above details RV support for *DISC1* association with OD. We have also reported common-variant support, for variants at the same risk locus: in our GWAS for OD, *DISC1* was identified as a possible risk locus, based on supporting evidence in both EAs and AAs (Gelernter et al, submitted manuscript). We believe that the presence of both rare and common variants at the same locus associated with risk for the same trait, adds greatly to the overall support for association, although naturally replication by other research groups is necessary.

Rs61737326 is non-synonymous, while rs139667828 and rs116628628 are synonymous variants. Although synonymous variants do not change protein coding directly they can have a significant impact on protein expression and function (Chamary et al., 2006), via, for example, altering mRNA splicing, mRNA stability, mRNA secondary structure, translation efficiency, and protein folding.

Among the 11 RVs genotyped, only one was located in *GRIN2B* (rs146792012). Compared to the three RVs in *DISC1*, which were observed in a total of 172 AAs, the minor allele of rs146792012 was found in only three AAs, all OD cases. It was also observed in seven EAs (five OD cases, and two controls). When AAs and EAs were analyzed separately, the association between rs146792012 and OD was statistically significant in AAs only ($P=0.00085$). When AAs and EAs were analyzed together, the association remained significant, but was weaker ($P=0.0031$). Despite the significant P value, due to the extremely small MAF, the association between this RV in *GRIN2B* and OD is not as well supported, compared to the association between *DISC1* and OD in AAs.

DISC1 has been extensively studied in schizophrenia and mood disorder patients (Millar et al., 2000). It is very interesting that some loci associated with SD in this population are also implicated in schizophrenia. Schizophrenia is associated with increased rates of substance dependence, perhaps due to many traits associated with schizophrenia including impulsive decision making, disturbances in reward-related processes, and the propensity to self-medicate dysphoria (Krystal et al., 2006). NMDA receptor function has been implicated in both disorders (Harrison and Weinberger, 2005; Liechti and Markou, 2008). However, schizophrenia appears to be associated with decreased NMDA receptor function, while alcohol dependence, for example, is associated with enhanced NMDA receptor function (Krystal et al., 1995; Krystal et al., 2003a; Krystal et al., 2003c). Characterizing the impact of each variant on NMDA receptor function in vivo will be an important step in putting the current findings in a neurobiological context.

Our study has limitations that must be noted. Although the sample size was large in comparison with other published population genetics studies of OD, when one sets out to study RVs, unconventionally-large samples may be required to identify associations with particular variants – because they are, definitional, rare. Thus, statistical techniques such as binning must be relied upon. While we feel the support for association presented in this article is compelling, there is wide latitude for disagreement on this point, and little that can be done to address this statistically practically, until larger samples of OD subjects become widely available. Also, this study was by nature a candidate gene study focused on particular pathways. As is always the case with pathways-based studies, selection of genes for inclusion is somewhat subjective, and more so for brain-based systems which are particularly complex. It is entirely possible, perhaps even likely, that other glutamate-system genes will in future be shown to be associated with OD and other substance dependence traits. Learning if this is true will have to await other even more extensive gene based studies, of full exome or genome sequencing studies.

We conclude that in this, the most extensive direct sequencing study for substance dependence traits to date (to our knowledge), we have identified association to sets of RVs in two glutamate-system genes, to OD. Despite the amount of data included in this study, our conclusions rest on a relatively small number of allele observations, and thus will require replication prior to wide acceptance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Dr. Krystal has served as a consultant or advisory board member for Alkermes, Lilly, Lundbeck, Pfizer and Roche. He is also a member of the American Society of Clinical Psychopharmacology's Alcohol Clinical Trials Initiative, which is supported by Lilly, Lundbeck, Abbott, and Pfizer. Dr. Krystal reports serving as a scientific consultant and/or on the scientific advisory board to the following companies: AbbVie, AMGEN, AstraZeneca, Bristol-Myers Squibb, Eisai, Eli Lilly and Co, Lundbeck, Otsuka, Quintiles Consulting, Sage Therapeutics, Shire, Sunovion, Takeda, Teva, Lohocla, Mnemosyne, Naurex, and Pfizer. Dr. Krystal is a cosponsor for 3 patents under review for glutamatergic agents targeting the treatment of depression, including one that involves the antidepressant effects of ketamine.

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Table 1

Substance dependence phenotypes of the subjects

	Alcohol dependence		Cocaine dependence		Opioid dependence	
	Cases (n)	Controls (n)	Cases (n)	Controls (n)	Cases (n)	Controls (n)
Sequencing stage						
AA	320	380	320	380	320	380
EA	440	380	440	380	440	380
Genotyping stage						
AA	1784	1177	2633	824	615	2862
EA	1379	1142	1809	1272	1452	1679

Table 2

Sequencing stage results

Gene	Chr	Length of cDNA (bp)	African American			European American				
			Exonic common variants	Rare variants		Exonic common variants	Rare variants			
				Exonic nonsyn	Exonic syn		Intronic within 5bp of an exon	Exonic nonsyn	Exonic syn	Intronic within 5bp of an exon
<i>DISC1</i>	1	2562	9	13	8	0	8	25	5	0
<i>PPP1CB</i>	2	981	1	0	1	0	1	1	2	1
<i>ZDHHC3</i>	3	981	2	2	2	1	2	2	0	0
<i>DTNBP1</i>	6	1053	3	1	4	0	3	6	5	1
<i>FYN</i>	6	1611	2	0	5	0	2	6	2	0
<i>NRG1</i>	8	1920	12	18	4	0	12	12	3	0
<i>GRIN1</i>	9	2814	6	2	12	0	6	3	5	1
<i>PTPN5</i>	11	1695	2	13	7	1	2	16	6	0
<i>PPP1CA</i>	11	990	1	0	5	0	1	0	4	0
<i>GRIN2B</i>	12	4452	13	14	17	1	12	5	14	1
<i>PPP1CC</i>	12	969	1	0	4	0	1	1	4	0
<i>DAOA</i>	13	459	3	1	0	0	3	5	0	0
<i>GRIN2A</i>	16	4392	2	10	22	0	2	19	10	0
<i>PPP1R1B</i>	17	612	2	2	3	0	2	2	0	0
<i>PPP1R9B</i>	17	2445	6	7	13	1	6	4	14	1
<i>PRKCG</i>	19	2091	8	6	9	0	8	6	5	0
<i>SRC</i>	20	1608	1	5	12	0	1	4	5	0
Total			74	94	128	4	72	117	84	5

Table 3

Sequence Kernel Association Test (SKAT) results of 11 rare variants followed up in the genotyping stage

Gene	Position (hg19)	dbSNP	Function	Alcohol dependence						Cocaine dependence						Opioid dependence							
				Sequencing Stage			Combine			Genotyping Stage			Combine			Genotyping Stage			Combine				
				cases*	controls		cases	controls	P	cases	controls		cases	controls	P	cases	controls		cases	controls		cases	controls
AA:																							
GRIN2A	chr16:9858173	rs61758995	nonsyn	1/601	1/725	3/3449	1/2293	4/4050	2/3018	0.44	5/5083	2/1616	6/5684	3/2341	0.64	3/1193	4/5546	4/1794	5/6271	0.05			
GRIN2B	chr12:13716059	rs146792012	syn	1/599	0/740	0/3488	2/2308	1/4087	2/3048	0.44	1/5127	1/1623	2/5726	1/2363	0.45	2/1212	0/5580	3/1811	0/6320	0.00085			
PPP1CA	chr11:67166102	rs114854848	syn	1/601	5/735	5/3495	9/2315	6/4096	14/3050	0.05	9/5135	4/1640	10/5736	9/2375	0.46	3/1205	12/5610	4/1806	17/6345	0.47			
	chr11:67166211	rs17881020	syn	0/616	0/744	1/3503	1/2313	1/4119	1/3057		3/5151	0/1638	3/5767	0/2382		2/1206	1/5621	2/1822	1/6365				
PPP1CC	chr12:111160015	rs61748070	syn	2/618	0/740	8/3500	3/2309	10/4118	3/3049	0.23	8/5156	5/1623	10/5774	5/2363	0.4	1/1231	11/5589	3/1849	11/6329	0.83			
PPP1R9B	chr17:48226570	rs188506814	syn	3/609	8/734	14/3462	8/2278	17/4071	16/3012	0.95	17/5074	6/1614	20/5683	14/2348	0.16	2/1188	22/5540	5/1797	30/6274	0.21			
	chr17:48226604	rs181028466	syn	1/605	0/740	3/3483	3/2295	4/4088	3/3035		5/5117	2/1624	6/5722	2/2364		3/1205	4/5572	4/1810	4/6312				
NRG1	chr8:31497511	new	nonsyn	0/604	0/726	0/3434	1/2279	0/4038	1/3005	0.23	1/5089	0/1588	1/5693	0/2314	0.64	1/1195	0/5516	1/1799	0/6242	0.12			
DISC1	chr12:31830059	rs139667828	syn	2/618	11/731	27/3497	14/2298	29/4115	25/3029	0.17	46/5128	11/1627	48/5746	22/2358	0.13	3/1209	53/5591	5/1827	64/6322	0.0010			
	chr12:231902964	rs116628628	syn	6/616	1/739	12/3466	15/2303	18/4082	16/3042		24/5112	6/1624	30/5728	7/2363		7/1209	25/5569	13/1825	26/6308				
	chr12:232144751	rs61737326	nonsyn	2/612	4/736	26/3468	27/2291	28/4080	31/3027		49/5121	11/1609	51/5733	15/2345		6/1212	54/5558	8/1824	58/6294				
Combine										0.15					0.13					0.0008			
EA: GRIN2A	chr16:9858173	rs61758995	nonsyn	8/802	4/708	17/2651	17/2213	25/3453	21/2921	0.8	26/3448	18/2472	34/4250	22/3180	0.13	15/2771	28/3242	23/3573	32/3952	0.24			
GRIN2B	chr12:13716059	rs146792012	syn	2/820	0/724	3/2697	1/2227	5/3517	1/2951	0.43	3/3511	2/2484	5/4331	2/3208	0.71	3/2815	2/3274	5/3635	2/4000	0.33			
PPP1CA	chr11:67166102	rs114854848	syn	0/830	0/732	0/2668	0/2250	0/3498	0/2982	0.71	0/3526	0/2484	0/4356	0/3216	0.91	0/2822	0/3276	0/3652	0/4008	0.76			
	chr11:67166211	rs17881020	syn	5/825	1/735	2/2668	6/2240	7/3493	7/2975		3/3493	5/2487	8/4318	6/3222		2/2812	6/3264	7/3637	7/4001				
PPP1CC	chr12:111160015	rs61748070	syn	6/832	4/734	31/2673	22/2226	37/3505	26/2960	0.5	38/3484	27/2477	44/4316	31/3211	1	31/2811	37/3247	37/3643	41/3983	0.89			
PPP1R9B	chr17:48226570	rs188506814	syn	3/831	0/734	1/2649	2/2226	4/3480	2/2960	1	2/3464	1/2483	5/4295	1/3217	0.82	2/2784	1/3249	5/3615	1/3985	0.23			
	chr17:48226604	rs181028466	syn	6/824	4/736	10/2618	10/2186	16/3442	14/2922		15/3441	13/2431	21/4265	17/3167		9/2751	20/3206	15/3575	24/3944				
NRG1	chr8:31497511	new	nonsyn	0/824	3/725	0/2656	1/2205	0/3480	4/2930	0.34	0/3492	1/2443	0/4316	4/3168	0.12	1/2801	0/3220	1/3625	3/3947	0.72			
DISC1	chr12:31830059	rs139667828	syn	0/836	0/740	0/2708	0/2258	0/3544	0/2998	0.12	0/3554	1/2495	0/4390	1/3235	0.06	1/2851	0/3294	1/3687	0/4036	0.21			
	chr12:231902964	rs116628628	syn	0/834	0/732	0/2714	1/2249	0/3548	1/2981		1/3539	0/2498	1/4373	0/3230		0/2848	1/3285	0/3682	1/4019				
	chr12:232144751	rs61737326	nonsyn	0/827	1/732	0/2682	0/2240	0/3509	1/2972		0/3506	0/2488	0/4333	1/3220		0/2834	0/3258	0/3661	1/3992				
Combine										0.92					0.56					0.54			

AA: African American; EA: European American; syn: synonymous; nonsyn: nonsynonymous.

* At the sequencing stage, all cases were dependent on three substances: alcohol, cocaine and opioid.