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Associations among types of impulsivity, substance use problems and *Neurexin-3* polymorphisms

Scott F. Stoltenberg[a], **Melissa K. Lehmann**[b], **Christa C. Christ**[a], **Samantha L. Hersrud**[c], and **Gareth E. Davies**[d,e,f]

[a]Department of Psychology, University of Nebraska-Lincoln, Lincoln, NE 68588-0308, USA

[b]Department of Biology, Black Hills State University, Spearfish, SD 57799, USA

[c]Sanford School of Medicine University of South Dakota, Vermillion, SD 57069, USA

[d]Department of Psychiatry, Sanford School of Medicine University of South Dakota, Vermillion, SD 57069, USA

[e]College of Pharmacy, South Dakota State University, Brookings, SD 57007, USA

[f]Avera Institute for Human Genetics, Sioux Falls, SD 57108, USA

Abstract

Background—Some of the genetic vulnerability for addiction may be mediated by impulsivity. This study investigated relationships among impulsivity, substance use problems and six neurexin-3 (*NRXN3*) polymorphisms. Neurexins (NRXNs) are presynaptic transmembrane proteins that play a role in the development and function of synapses.

Methods—Impulsivity was assessed with the Barratt Impulsiveness Scale Version 11 (BIS-11), the Boredom Proneness Scale (BPS) and the TIME paradigm; alcohol problems with the Michigan Alcoholism Screening Test (MAST); drug problems with the Drug Abuse Screening Test (DAST-20); and regular tobacco use with a single question. Participants (N = 439 Caucasians, 64.7% female) donated buccal cells for genotyping. Six *NRXN3* polymorphisms were genotyped: rs983795, rs11624704, rs917906, rs1004212, rs10146997 and rs8019381. A dual luciferase assay was conducted to determine whether allelic variation at rs917906 regulated gene expression.

Results—In general, impulsivity was significantly higher in those who regularly used tobacco and/or had alcohol or drug problems. In men, there were modest associations between rs11624704 and attentional impulsivity (p = .005) and between rs1004212 and alcohol problems (p = .009). In women, there were weak associations between rs10146997 and TIME estimation (p = .03); and between rs1004212 and drug problems (p = .03). The dual luciferase assay indicated that C and T alleles of rs917906 did not differentially regulate gene expression *in vitro*.

Corresponding Author: Scott F. Stoltenberg, 402-472-7861 (Phone), 402-472-4637 (Fax), sstoltenberg2@unl.edu .

Contributors

Scott Stoltenberg contributed to all aspects of the study, data analysis and manuscript preparation. Melissa Lehmann contributed substantially to all aspects of the study and the manuscript preparation. Samantha Hersrud contributed to writing the manuscript. Gareth Davis contributed to the dual luciferase assay. Christa Christ contributed to the collection of genotype data and manuscript preparation. All authors contributed to the editing and final review of the manuscript. All authors approved the final paper.

Conflict of Interest

The authors report no financial interests or potential conflicts of interest.

Conclusions—Associations between impulsivity, substance use problems and polymorphisms in *NRXN3* may be gender specific. Impulsivity is associated with substance use problems and may provide a useful intermediate phenotype for addiction.

Keywords

NRXN3; cell adhesion protein; substance use disorder; impulsiveness

1. Introduction

There is abundant evidence that an individual's vulnerability to addiction is influenced by their genetic makeup but identifying the genes that contribute to that risk has been and continues to be a vexing task (Li and Burmeister, 2009). For a given substance use disorder, there is an expectation that there are genes that affect addiction vulnerability to that particular substance, and that there are also genes that influence traits that increase vulnerability to addiction, in general. For example, polymorphisms in genes that code for enzymes involved in alcohol metabolism (i.e., alcohol dehydrogenase [ADH] and aldehyde dehydrogenase [ALDH]) have been convincingly shown to influence risk for alcohol dependence, and a gene that codes for an enzyme involved in catecholamine metabolism (i.e., catechol-o-methyl transferase [COMT]) appears to influence addiction risk through its influence on behavioral traits such as impulsivity and anxiety (Ducci and Goldman, 2008).

Impulsivity is an interesting trait in the context of addiction vulnerability. The general construct of "impulsivity" represents several independent facets such as response inhibition, resistance to delay of reinforcement, timing, behavioral switching, motor impulsivity, cognitive impulsivity, preparation, execution outcome, premature responding and lack of persistence (Evenden, 1999). Individuals with elevated levels of impulsivity are at increased risk for problems with alcohol (Dick et al., 2009; Lejuez et al., 2010), stimulants (Ersche et al., 2010), and nicotine (Doran et al., 2009; Spillane et al., 2010). In animal models, individual differences in different facets of impulsivity predict drug self-administration and exposure to drugs and increase impulsivity (see Winstanley et al., 2010 for a review). Slow developing behavioral control in children is associated with increased risk for adolescent substance use (Wong et al., 2006) and gender appears to modify the association between different types of impulsivity and alcohol problems (Stoltenberg et al., 2008). The relations among different facets of impulsivity and aspects of substance use or problems are not yet fully characterized, but there is growing appreciation of their complexity (Lejuez et al., 2010). There is some emerging evidence that individual differences in certain facets of impulsivity are influenced by genes in neurotransmitter systems (e.g., Stoltenberg et al., 2006; Walderhaug et al., 2010), but little is known about the underlying genetic architecture of impulsivity.

Recent empirical evidence suggests that the gene that codes for Neurexin-3 (*NRXN3*) may be a good candidate for general addiction vulnerability. Certain alleles of three single nucleotide polymorphisms (SNPs) within the fifth splice site of the *NRXN3* gene were more common in alcohol dependent subjects than in matched controls (Hishimoto et al., 2007). A genome wide association study found suggestive evidence that a NRXN3 SNP (rs2221299) was associated with nicotine dependence (Bierut et al., 2007). Another *NRXN3* SNP (rs1004212) was associated with the amount of nicotine consumption in schizophrenia patients (Novak et al., 2009). A genome-wide linkage study indicated an area on chromosome 14q, on which the *NRXN3* gene is located, was linked to opioid dependence (Lachman et al., 2007).

Neurexins (NRXNs) are presynaptic transmembrane proteins that function as cell adhesion molecules, binding with neuroligins to stabilize the synapse (Hata and Südhof, 1995). There is growing evidence that neurexins are key elements properly functioning synapses and that NRXN dysfunction may play a role in diseases with a cognitive component (Südhof, 2008). The genes that code for NRXNs are large, contain numerous polymorphisms and are subject to alternative splicing. Regulatory region and splice site variants are likely to have a substantial impact on NRXN expression. NRXN proteins are encoded by three separate, unlinked genes: *NRXN1* (2p16.3), *NRXN2* (11q13), and *NRXN3* (14q31). Each of the three *NRXN* genes has two promoters from which a longer alpha and shorter beta NRXNs are transcribed (Rowen et al., 2002). The alpha promoters are located at the 5′ end of the genes, while the beta promoters are located between exons 17 and 18 (Rowen et al., 2002). Each of these *NRXN* genes also has multiple alternative splice sites and thousands of possible isoforms (Tabuchi and Südhof, 2002). The *NRXN3* gene is one of the largest genes in the human genome containing 1,826,818 base pairs (Rowen et al., 2002).

Lines of mice with the *α-NRXN* gene knocked out showed that α-NRXN is responsible for the coupling of Ca^{2+}-channels to synaptic vesicles in preparation for exocytosis, and that they are essential for normal neurotransmitter release (Missler et al., 2003). These *α-NRXN* knockout mice had low survival rates, and those that did survive had decreased neurotransmitter release at both inhibitory (gamma-amino butyric acid; GABA) and excitatory (glutamate) synapses. GABA is the brain's major inhibitory neurotransmitter, and alcohol had been shown to mimic its effects on the $GABA_A$ receptor (Lovinger and Homanics, 2007). During development, α-NRXNs on pre-synaptic neurons promote post-synaptic specialization of GABAergic neurons by clustering $GABA_A$ receptors (Kang et al., 2007). The involvement of NRXNs in normal neurotransmitter release and synaptic integrity suggests that variation in their genes may have widespread and substantial effects on key behavioral phenotypes such as impulsivity. To our knowledge, there have been no studies to date to examine potential associations between *NRXN3* polymorphisms and impulsivity.

This study was designed to investigate potential associations among types of impulsivity, substance use problems, and genetic polymorphisms in *NRXN3*. Our hypothesis is in line with the notion that impulsivity is a key construct in the pathways from genes to risky behaviors, which can lead to behavioral disorders such as addiction.

2. Methods

2.1. Identifying Single Nucleotide Polymorphisms for Genotyping

The SNP@Promoter database was used to identify SNPs in the *NRXN3* α-promoter that may affect gene regulation (http://variome.kobic.re.kr/SNPatPromoter/; Kim et al., 2008). We identified 20 SNPs found within the α-promoter, and selected one of these SNPs (rs917906; chromosome 14 position 77,939,227) because of its location in a transcription factor (TF) binding site [the CCAAT/ Enhancer Binding Protein-gamma (C/EBPγ)] 619 base pairs upstream of the start codon. Though termed "enhancer" the C/EBPγ TF can act as both an activator and repressor depending on the cell and promoter context (Parkin et al., 2002).

Recently, a report was published that identified several areas in putative regulatory regions upstream of the NRXN3 structural gene (Pedrosa et al., 2010). Using HapMap, we identified 21 SNPs located in those regions and then used SNP Cutter software (Liu et al, 2009), to identify two (rs983795, position 77,767,309 and rs11624704, position 77,855,830) for which we could design restriction fragment length polymorphism assays.

We also genotyped three other SNPs, using TaqMan SNP Genotyping Assays because they were previously identified as being associated with alcohol dependence (rs8019381, position

79,390,336; Hishimoto et al. 2007), quantity of nicotine intake (rs1004212 position 78,250,979; Novak et al., 2009) and obesity (rs10146997, position 79,014,915; Heard-Costa et al., 2009).

2.2 Participants and Measures

All participants were recruited at a small Midwestern university via posters and in-class presentations about the study, were informed about the particular study and signed informed consent documents. Participants (N = 477) completed multiple questionnaires that assessed impulsivity and involvement in health-risk behaviors, four computer tasks, and donated buccal cells for genotyping. Participation in this study was voluntary, and all participants were compensated $20. The study was approved by the Institutional Review Board (IRB). In this manuscript we will report only analyses focused on testing the associations between impulsivity, *NRXN3* polymorphisms, and substance use problems; other findings from this study will be reported elsewhere.

To reduce the risk of population stratification data was analyzed from only the 92% who self-identified as Caucasians (N = 439; 64.7% female). Age ranged from 18 to 67 (Mean= 22.49, SD= 6.12; 81.6% were age 24 or younger). Four individuals did not report age.

The Barratt Impulsiveness Scale (BIS-11) is a 30-item self-report instrument that uses a 4-point Likert scale from Rarely/Never to Almost Always (Patton et al., 1995). A total score is calculated by summing three subscale scores. The subscales are: Motor ("I act on the spur of the moment"), Attentional ("I have outside thoughts when thinking") and Nonplanning ("I plan trips well ahead of time" reverse scored). The total score is reliable (in terms of internal consistency, Cronbach's $\alpha = .86$ in this study).

The Boredom Proneness Scale (BPS) is a 28-item self-report instrument that uses a 5-point Likert scale from "highly disagree" to "highly agree" (Farmer and Sundberg, 1986). A total score is calculated by summing five subscales. The subscales include: External Stimulation ("When I was young, I was often in monotonous and tiresome situations"), Internal Stimulation ("It is easy for me to concentrate on my activities" reverse scored), Affect ("Frequently when I am working I find myself worrying about other things"), Time Perception ("Time always seems to be passing slowly") and Constraint ("I am good at waiting patiently" reverse scored). The total score has good reliability (Cronbach's $\alpha = .86$ in this study).

The The Time Paradigm (Dougherty et al., 2005) was administered on a desk top personal computer with a cathode ray tube display with a refresh rate of 60 Hz. The Time Paradigm is a behavioral assessment of the passage of time. Participants were asked to estimate the passage of 60 s five times. Participants were instructed to press the space bar to start the timer and to press it again when they thought that 60 s had passed. We analyzed the mean of the five estimates. Participants did not receive any feedback regarding the accuracy of their estimates or any payments based on their performance.

We assessed tobacco use with a single yes/no item: "Do you regularly use tobacco (cigarettes, cigars, chewing tobacco)?" We assessed alcohol problems with 22 items from the Michigan Alcohol Screening Test (MAST; Selzer, 1971). It consists of yes or no questions about alcohol consumption and related behaviors that were scored as either 1 (yes) or 0 (no), and are summed for a total score. We assessed drug problems with the Drug Abuse Screening Test (DAST-20), which is a 20 item self-report questionnaire that measures an individual's involvement with drugs (not alcohol or tobacco) over the preceding 12 months (Skinner, 1982). Responses to the items were scored as either 1 (yes) or 0 (no), and are summed for a total score. For both the MAST and the DAST we used a

cutoff score of five to identify those with probable problems (i.e., 0-5 = no problems, 6+ = problems).

2.3. Genotyping

DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen Inc, Valencia, CA, USA). Twenty μL PCR reactions for rs8019381 (Applied Biosystems, Inc., genotyping assay ID, C_29283249_10), rs917906 (C_2674044_20), rs1004212 (C_16183691_10) and rs10146997 (C_30288512_10), were prepared in 2x ABI TaqMan Universal Master Mix no UNG (Applied Biosystems, Inc., Foster City, CA) adding the corresponding 20x Taqman SNP genotyping assay containing Fam and Vic-labelled allele-specific TaqMan MGB probes and forward and reverse primers. The PCR was performed with the following cycling parameters: 95°C for 10 min, followed by 40 cycles of 92°C for 15 sec and 60°C for 1 min. End point FAM and VIC florescence levels were measured StepOnePlus Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA), and genotype calls were made based on this level of fluorescence signal.

rs983795 was amplified using the primers: forward: 5′-AGG GAT ACC TGT TGG GAG AAC C-3′ and reverse: 5′-TTA GAG CCAA GGC ACT ACA CCC-3′. PCR reactions (25μL) contained 20ng of DNA, 1X GoTaq Green Master Mix (Promega), and 400nM final concentration of each primer. The PCR was performed with the following cycling parameters: 95°C for 6 min, followed by 35 cycles of 95°C for 30 sec, 56.6°C for 30 sec, and 72°C for 1 min, with a final extension at 72°C for 10 min. Ten μL of the PCR product was digested for 1 hour at 37°C with 20 units of NdeI enzyme (New England Biolabs) and run on a 1% agarose gel at 100V for 1.5 hours. Fragments were visualized under UV light with ethidium bromide stain. Fragment sizes for alternative alleles are A= 373bp and G= 259bp and 114bp.

rs11624704 was amplified using the primers: forward: 5′-TTG CCT TAC ACA CTG GTG GTT G-3′ and reverse: 5′-AAT GCA CTT CTG TTC CTC CAG C-3′. PCR reactions (25μL) contained 20ng of DNA, 1X GoTaq Green Master Mix (Promega), and 400nM final concentration of each primer. The PCR was performed with the following cycling parameters: 95°C for 6 min, followed by 35 cycles of 95°C for 30 sec, 55.8°C for 30 sec, and 72°C for 1 min, with a final extension at 72°C for 10 min. Ten μL of the PCR product was digested for 1 hour 37°C with 2.5 units of MseI enzyme (New England Biolabs) and run on a 1% agarose gel at 100V for 1.5 hours. Fragments were visualized under UV light with ethidium bromide stain. Fragment sizes for alternative alleles are C= 276bp and A= 135bp and 141bp.

Genotyping assays were repeated for approximately 10% of samples for all six SNPs. There were no discrepancies among genotype calls.

2.4. Dual Luciferase Assay

To determine whether allelic variation at the rs917906 affected gene expression, a dual luciferase assay was conducted by amplifying the 301bp region from two individual DNA samples (one with a C/C genotype and one with a T/T genotype for the rs917906) as described above with the exception of the final extension being extended to 30 minutes; to ensure addition of the 3′ terminal A by *Taq* polymerase for use in the T-A cloning. The resulting amplicons were cloned into the TOPO-TA vector (Invitrogen, Carlsbad, CA, USA). Inserts were excised with *XhoI* and *HindIII* enzymes to generate constructs in the forward orientation, and cloned into the pGL4.1 vector (Promega, Madison, WI, USA). The constructs were verified by sequencing.

The RN46A raphe-derived neuronal cell line was provided by Scott R. Whittemore (University of Louisville School of Medicine). Cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) 100 units/mL of penicillin, 100μg/mL of streptomycin, and 250μg/mL G418. Cell culture medium without antibodies included on the DMEM and 10% FBS. RN46A cells were cultured at 33°C in a humidified 5% CO^2 atmosphere for two days. The medium was then changed to cell culture medium without antibodies for two days. The transfection then began on the fifth day.

Two clones of each genotype (C/C and T/T) were used. RN46A cells were transfected with 1μg of the rs917906 reporter constructs plus 0.1μg of the *Renilla* luciferase pRL-SV40 control DNA using Lipofectamine 2000 and incubated for twenty minutes at room temperature. After incubation, 150μL was added to each well which contained the RN46A raphe neurons and medium. The plate was then gently mixed by rocking back and forth, then incubated at 33°C in a humidified 5% CO^2 atmosphere for 24 hours. Luciferase activity was then assayed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activity was normalized relative to the activity of the *Renilla* luciferase produced by the pRL-SV40 control vector and each construct was tested in three independent experiments.

2.5. Statistical Analyses

Multivariate general linear models were used to test associations between impulsivity subscale scores, gender and substance use problems (IBM, SPSS Statistics, 19; Chicago IL). To test associations between *NRXN3* SNPs and impulsivity and substance use problems separately for men and women we used basic allele tests in SNP and Variation Suite 7 (Golden Helix, Bozeman MT). In basic allele tests for a given phenotype, individuals are considered to have two identical phenotype scores, one for each allele. That is, each element in an individual's genotype is considered to correspond to the same phenotypic value. Basic allele tests do not enable testing of additive or dominance models. We chose to conduct basic allele tests rather than examining additive and dominance models to reduce the number of comparisons thereby preserving statistical power. A paired t-test was conducted for the dual luciferase reporter assay to determine whether rs917906 alleles differentially affected transcription.

3. Results

3.1. Descriptive Statistics

Overall descriptive statistics are shown in Table 1. Our sample of men and women did not differ significantly by age. However, men had higher mean scores on Time estimation ($p = .038$), BIS-11 Total ($p = .01$), and Motor subscale ($p = .001$), BPS Total ($p = .016$) and External Stimulation subscale ($p = .000$). The percent reporting regular use of tobacco did not differ for men and women. There was a trend for more men to be classified as having alcohol problems ($p = .092$). More men than women were classified as having drug problems ($p = .018$). Subsequent analyses of behavioral data were conducted separately for men and women in order to reduce potential confounds and to preserve our capacity to detect differences in patterns of association.

Marker statistics for the six NRXN3 SNPs tested are shown in Table 2. Genotype frequencies are shown in Table 3. All markers, but rs917906 were in Hardy-Weinberg equilibrium ($p>.05$). We genotyped rs917906 using the TaqMan assay and an RFLP method and neither method resulted in genotype calls that were in HW equilibrium. We were unable to determine the cause of this discrepancy and although we are confident with our genotype calls, we acknowledge that the lack of HW equilibrium with this marker should be

considered in interpretation. There were no linkage disquilibrium (LD) blocks in our sample or in the combined panel of Utah residents with Northern and Western European ancestry from the CEPH collection and Toscans in Italy (CEU+TSI) sample of HapMap data (see Figure 1). The pattern of LD that we observed in our sample was similar to that seen in the CEU+TSI sample.

3.2. Impulsivity and Regular Tobacco Use

For men who indicated regular tobacco use, multivariate general linear models with age as a covariate indicated significantly higher mean scores for the Attentional ($p = .005$), Nonplanning ($p = .000$) and Motor ($p = .001$) subscales of the BIS-11 and for the Perception of time ($p = .031$) subscale of the BPS (see Figure 2). For women who indicated regular tobacco use, multivariate general linear models with age as a covariate indicated significantly higher mean scores for the Attentional ($p = .01$), Nonplanning ($p = .001$) and Motor ($p = .001$) subscales of the BIS-11 and for the External stimulation ($p = .033$), Affect ($p = .004$), Perception of time ($p = .028$), and Constraint ($p = .006$) subscales of the BPS. Time estimation was not significantly associated with tobacco use in men or women.

3.3. Impulsivity and Alcohol Problems

For men who had alcohol problems (i.e., MAST > 5), multivariate general linear models with age as a covariate indicated significantly higher mean scores for the Attentional ($p = .018$), Nonplanning ($p = .000$) and Motor ($p = .000$) subscales of the BIS-11 (see Figure 3). None of the mean BPS subscale scores were different for men with and without alcohol problems. For women who had alcohol problems, multivariate general linear models with age as a covariate indicated significantly higher mean scores for the Attentional ($p = .005$), Nonplanning ($p = .011$) and Motor ($p = .000$) subscales of the BIS-11 and for the External stimulation ($p = .028$), Affect ($p = .007$), Perception of time ($p = .000$), and Constraint ($p = .035$) subscales of the BPS. Time estimation was not significantly associated with alcohol problems in men or women.

3.4. Impulsivity and Drug Problems

For men who had drug problems (i.e., DAST > 5), multivariate general linear models with age as a covariate indicated significantly higher mean scores for the Nonplanning ($p = .002$) and Motor ($p = .000$) subscales of the BIS-11 (see Figure 4). None of the mean BPS subscale scores were different for men with and without drug problems. For women who had drug problems, multivariate general linear models with age as a covariate indicated significantly higher mean scores for the Attentional ($p = .000$), Nonplanning ($p = .000$) and Motor ($p = .000$) subscales of the BIS-11 and for the Perception of time ($p = .006$), subscale of the BPS. Time estimation was not significantly associated with drug problems in men or women.

3.5. NRXN3 Association Tests

Basic allele tests identified a significant association between rs11624704 and BIS-11 Total Score ($p = .01$; see Table 4). Although this result does not survive Bonferroni correction (i.e., for each phenotype we conducted six independent association tests for each gender; $p_{crit} = .05/6 = .008$), it suggests that investigating potential associations between rs11624704 genotype and BIS-11 subscales might identify one or more subscales to be associated with the SNP. Therefore, we then conducted basic allele tests of rs11624704 on the three BIS-11 subscales in men, which indicated a significant association with Attentional impulsivity ($p = .005$; Bonferroni correction $p_{crit} = .05/3 = .017$; see Figure 5). In women, there was evidence of a weak association between Time Estimation and rs10146997 genotype ($p = .027$) that fails to survive Bonferroni correction ($p_{crit} = .05/6 = .008$).

Basic allele tests identified a significant association between rs1004212 and alcohol problems in men (p = .009; see Table 4 and Figure 4). Having a copy of the T allele of rs1004212 increased risk for having alcohol problems 2.54 times. Interestingly, in women the same allele decreased risk for having drug problems (p = .03; OR = 0.29). Although neither of these findings survived strict Bonferroni correction, they suggest that further study of this locus is needed.

3.6. Luciferase Reporter Assay

To determine whether alternative alleles at rs917906 regulate gene expression of the luciferase gene *in vitro*, a ratio of the expression of the luciferase over the expression of the control *Renilla* luciferase was calculated. Using this ratio a paired t-test was performed. No significant difference was found between the C (Mean expression= 0.0481) and T (Mean expression= 0.0480) alleles: paired t(5) = 0.027, p= 0.97. Therefore, the C and T alleles do not differentially regulate *NRXN3* transcription *in vitro*.

4. Discussion

We found suggestive evidence that *NRXN3* polymorphisms are associated with impulsivity (i.e., rs11624704 and Attentional impulsivity) and alcohol problems (rs1004212) in men. To our knowledge, this is the first report of an association between a *NRXN3* polymorphism and impulsivity. This finding may prove to be important in understanding the genetic architecture of addiction because impulsivity is an important risk factor for addictions and other behavioral disorders. Evidence is accumulating that NRXN3 may be in a pathway that influences vulnerability to addiction in general. In that sense, our findings add to the growing evidence that implicates polymorphism in *NRXN3* in risk for substance use problems and/or dependence (Lachman et al., 2007; Hishimoto et al., 2007; Beirut et al., 2007) and obesity (Heard-Costa et al., 2009). We recognize that the overlap in genetic risk for addiction to different substances and obesity is likely to be quite narrow, but it may be that this overlap includes genetic polymorphisms associated with individual differences in impulsivity. Further investigation with detailed phenotypic assessment that includes patterns of substance use, substance use disorder diagnoses, multiple measures of impulsivity and additional *NRXN3* polymorphisms should be conducted.

Novak et al. (2009) reported that in individuals with schizophrenia those with the C/C genotype of rs1004212 smoked more cigarettes per day on average than those with the C/T genotype. We did not find an association between rs1004212 genotype and regular tobacco use, which is consistent with Novak et al. (2009), who did not find an association with rs1004212 and risk for smoking. We did not assess the quantity of tobacco used for those who reported to be regular tobacco users in our study, so we cannot directly test the previously reported association. Our primary finding with substance use problems, however, was that in men, rs1004212 genotype was associated with risk for alcohol problems. We found that men carrying a T allele were at 2.54 times greater risk of having alcohol problems than C/C homozygotes. Interestingly, in women, there is a non-significant trend for the T allele to be associated with reduced risk of having a drug problem by about one third (OR = 0.39). Taken together, these findings suggest that rs1004212 should continue to be a SNP of interest in the context of addiction vulnerability.

Our finding that rs11624704 genotype was associated with a specific facet of impulsivity (BIS-11 Attentional) in men, but not in women, suggests that the influence of *NRXN3* polymorphisms may be rather specific and that the use of composite scores or other somewhat gross measures and/or statistically controlling for gender differences may fail to identify potentially interesting associations. At this time, we have no explanation for gender specific effects of *NRXN3*.

We found strong evidence that higher impulsivity is associated with regular tobacco use and problems with alcohol and drugs. Mean subscale scores from the Barratt Impulsiveness Scale (version 11) were significantly higher for both men and women for regular tobacco users, and for those with alcohol and drug problems with the only exception being Attentional impulsivity in men with drug problems. In this single case, those men with drug problems had higher mean scores than those without, but the difference did not reach significance ($p = .17$). The Barratt Impulsiveness Scale appears to assess facets of impulsivity that are relevant to addiction similarly in men and women. We observed less consistent patterns with the Boredom Proneness Scale subscales. When there were significant differences between those who used tobacco or had problems with alcohol or drugs, the substance using group had a higher mean score than the comparison group. In men, the mean BPS subscale scores were not different for those with and without substance use problems. Only the perception of time subscale differed for men grouped by regular tobacco use. The BIS-11 appears to better assess substance use relevant aspects of impulsivity. Mean scores for Time estimation were not significantly different for men or women grouped by substance use/problem. However, it should be pointed out that the direction of the effects, while not statistically different, were consistently in the expected direction (i.e., shorter time estimates for substance use/problem groups). We think that behavioral impulsivity measures show promise and we will continue to optimize their parameters (e.g., number of trials and number of seconds estimated) for use in future studies. Dick et al. (2009) present a compelling case that the construct of impulsivity may be best studied by examining its many subfacets. In the present study, we used the well-known BIS-11 subscales to examine Negative Urgency (BIS-11 Attentional), Lack of Planning (all three BIS-11 subscales) and the less well known BPS to examine Lack of Perseverance (boredom proneness). We also used the TIME paradigm to assess Judgment of Time Elapse (see Tables 1 and 2 in Dick et al., 2009 for a discussion of how these measures assess particular impulsivity subfacets). More work to characterize the pattern of association between different subfacets of impulsivity and substance use phenotypes is needed.

Men are consistently found to be more likely than women to have drinking problems (Wilsnack et al., 2000), and generally have more risk factors for alcohol use and problems than women (Nolen-Hoeksema and Hilt, 2006). There is less consistency in findings relating to differences in types of impulsivity based on gender (Reynolds et al., 2006). It does appear however, that higher levels of impulsivity in men, partially mediates their increased risk for alcohol problems (Stoltenberg et al., 2008). The present study appears to be the first report of gender differences in patterns of association with the *NRXN3* gene and behavior.

Alternative alleles at rs917906 did not differentially regulate gene expression *in vitro*. It is possible that rs917906 exerts some regulatory control over NRXN3 expression, but in our hands, the dual luciferase assay did not indicate differential expression. It may be that *in vivo* rs917906 affects gene regulation in conjunction with other SNPs (i.e., a haplotype) (Glatt et al., 2006), or that it is in linkage disequilibrium (LD) with a functional variant. Another possibility for not observing a difference in transcriptional activity in the dual luciferase assay is that because there are low levels of expression of C/EBPγ in the brain (Kuo et al., 1990) it may not be expressed in raphe neurons or if it is expressed it may be at very low levels. GABAergic Purkinje cells in the cerebellum have higher levels of C/EBPγ expression than cells in other brain areas (Kuo et al., 1990). Given that NRXN proteins have been found to be essential for normal release of neurotransmitters at GABA neurons (Missler et al., 2003) it would be interesting to better characterize the regulatory impact of rs917906 using Purkinje cells and the SHSY57 neuroblastoma cell line used by Pedrosa et al. (2010). Additional *NRXN3* regulatory region polymorphisms should also be tested for potential transcriptional regulation.

We recognize that our study has certain limitations and needs to be interpreted with some caution. More detailed assessments of the potential associations between important addiction related phenotypes (e.g., maximum number of drinks, age of onset) and genetic variation across *NRXN3* need to be conducted. In addition, other facets of impulsivity such as delayed discounting need to be examined for association with *NRXN3* polymorphisms. By current standards, our sample size is considered rather small and the p-values we report are not corrected for multiple comparisons, although we attempted to limit the number of comparisons that we made in order to conserve statistical power. In our study sample, the mean scores for the BIS-11 Total were higher than reported norms for both men and women (Patton and Barratt, 1995). We do not have a ready explanation for this observation, but note that more than a decade separates the two studies. It should be noted that our definitions of alcohol and drug problems were not meant as a diagnosis of alcohol abuse or dependence, but were used as a convenient metric of problematic substance involvement. Subsequent studies to investigate the role of *NRXN3* in substance use and problems should employ more sensitive measures.

Our findings suggest that *NRXN3* polymorphisms are modestly associated with specific types of impulsivity and substance use problems and that these patterns of association are not the same in men and women. Findings such as these, given their limitations, will be critical as we continue to improve our understanding of the pathways from genes to behavior. Impulsivity increases risk for several behavioral disorders such as addictions and therefore is an outstanding candidate trait for further study. A more complete description of the genetic architecture of impulsivity is likely to have a substantial impact on our understanding of the genetic architecture of a variety of behavioral disorders. *NRXN3* is a promising candidate gene for addiction vulnerability and merits further study.

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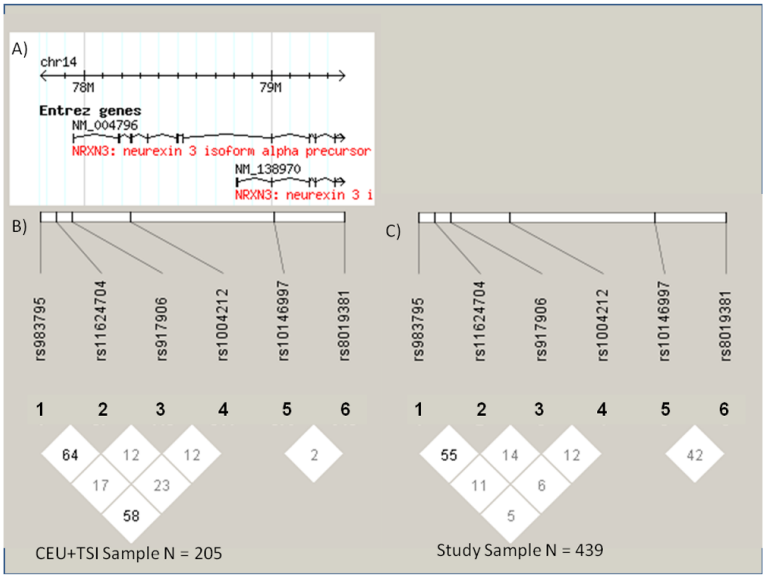

Figure 1.

Linkage disequilibrium (LD) data (D'/LOD) for the six *NRXN3* SNPs tested. A) The relative location of the two alternatively spliced forms of NRXN3. B) LD data from CEU+TSI samples from HapMap (version 3 release R2). C) LD data from this study.

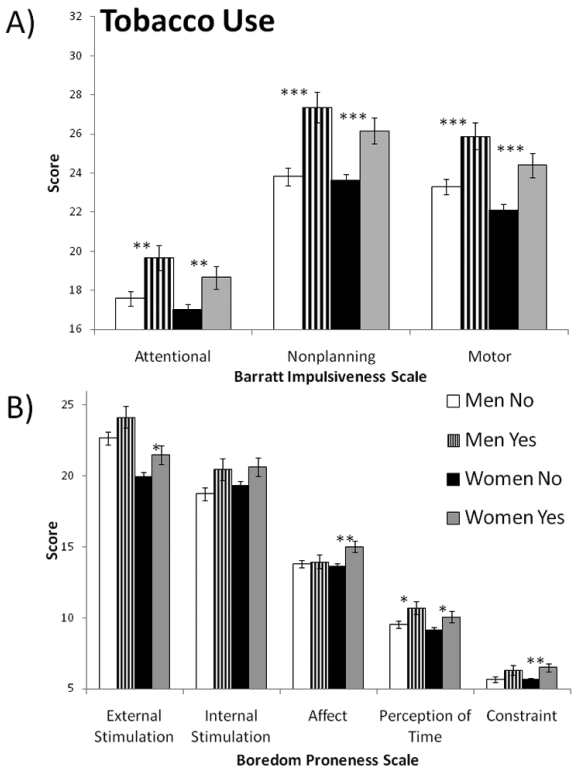

Figure 2.

Mean scores on impulsivity subscales for men and women who reported regularly using tobacco. A) Barratt Impulsiveness Scale (version 11). B) Boredom Proneness Scale. *p<.05; **p<.01; ***p<.001

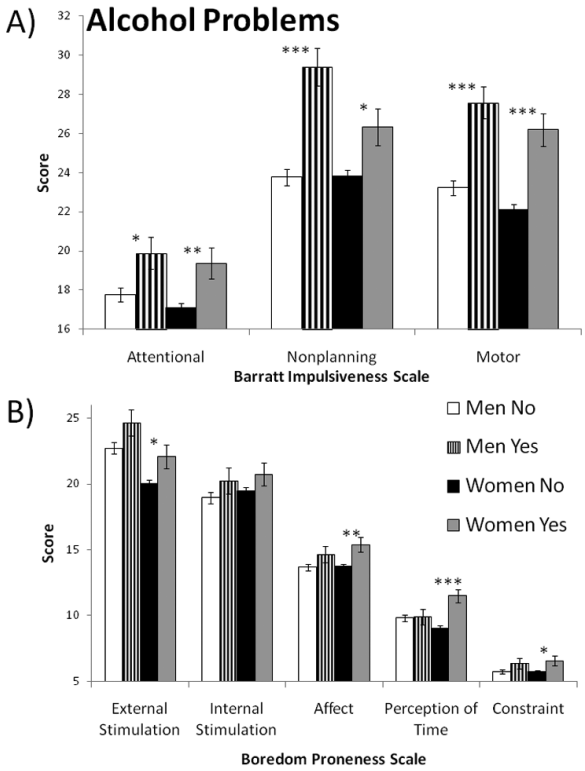

Figure 3.

Mean scores on impulsivity subscales for men and women who had alcohol problems (i.e., scoring 6 or more on the Michigan Alcoholism Screening Test). A) Barratt Impulsiveness Scale (version 11). B) Boredom Proneness Scale. *p<.05; **p<.01; ***p<.001

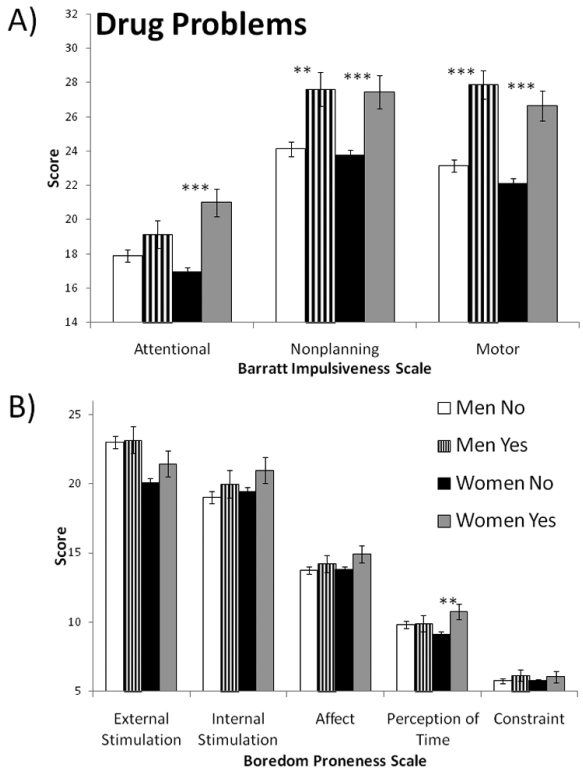

Figure 4.

Mean scores on impulsivity subscales for men and women who had drug problems (i.e., scoring 6 or more on the Drug Abuse Screening Test). A) Barratt Impulsiveness Scale (version 11). B) Boredom Proneness Scale. *p<.05; **p<.01; ***p<.001

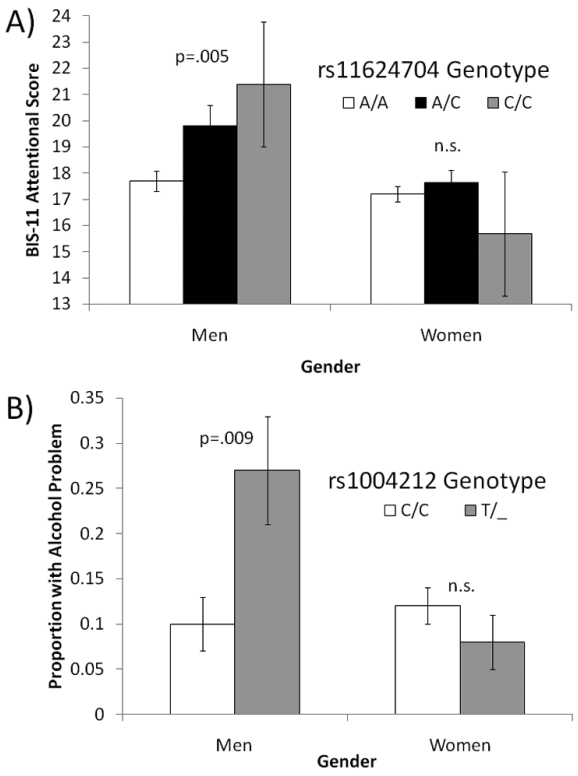

Figure 5.

Significant associations between NRXN3 SNPs and behaviors. A) Mean (±s.e.) BIS-11 Attentional score for men and women grouped by rs11624704 genotype. B) Proportion (95% CI) of individuals with alcohol problems in groups defined by rs1004212 genotype and gender. P-values shown are not corrected for multiple comparisons.

Table 1

Overall Descriptive Statistics; Mean (S. D.)

	Men (n=155)	Women (N=283)	p-value
Age	22.53 (6.28)	22.46 (6.05)	0.910
Time Estimation	63.59 (18.22)	59.79 (18.28)	**0.038**
BIS-11			
Total	66.83 (10.64)	63.96 (11.36)	**0.010**
Attentional	18.07 (4.10)	17.31 (4.14)	0.066
Nonplanning	24.75 (5.03)	24.10 (5.00)	0.197
Motor	24.01 (4.37)	22.55 (4.68)	**0.001**
BPS			
Total	74.03 (11.03)	71.13 (12.43)	**0.016**
External Stimulation	22.94 (4.82)	20.28 (4.85)	**0.000**
Internal Stimulation	19.11 (4.78)	19.56 (4.63)	0.336
Affect	13.84 (2.99)	13.93 (3.12)	0.769
Perception of time	9.83 (2.91)	9.32 (2.84)	0.072
Constraint	5.83 (2.04)	5.78 (2.08)	0.838
Tobacco Use (%)	24.5	19.4	0.207
Alcohol Problem (%)	16.1	10.6	0.092
Drug Problem (%)	16.8	9.2	**0.018**

Note: Three men did not report age and one woman did not have scores for BPS. P-values are either from independent sample t-tests or chi-square tests.

Table 2

NRXN3 Marker Statistics

SNP	Position on Chr. 14	Region	Alleles minor/major	MAF	HWE P
rs983795	77,767,309	α-promoter	A/G	0.133	0.900
rs11624704	77,855,830	α-promoter	C/A	0.134	0.459
rs917906	77,939,227	α-promoter	C/T	0.472	**0.029**
rs1004212	78,250,979	exon 5	T/C	0.172	0.106
rs10146997	79,014,915	intron	G/A	0.239	0.245
rs8019381	79,390,336	splicing site 5	T/C	0.103	0.083

Note: MAF = Minor Allele Frequency; HWE P = Hardy-Weinberg Equilibrium p-value.

Table 3

Genotype Frequencies by Gender

SNP	Genotype	Men (n = 155)	Women (n = 284)	Overall (N = 439)
rs983795	A/A	0	8	8
	A/G	37	71	108
	G/G	118	205	323
rs11624704	C/C	3	3	6
	C/A	30	75	105
	A/A	118	205	323
rs917906	C/C	29	56	85
	C/T	92	147	239
	T/T	31	79	110
rs1004212	T/T	1	7	8
	T/C	54	79	133
	C/C	98	197	295
rs10146997	G/G	9	21	30
	G/A	49	98	147
	A/A	93	162	255
rs8019381	T/T	4	4	8
	T/C	24	50	74
	C/C	127	229	356

Note: All call rates >98%.

Table 4

Basic Allele Test p-values.

	SNP	TIME	BIS-11	BPS	Regular Tobacco use	Alcohol Problem	Drug Problem
Men	rs983795	0.67	0.42	0.20	0.84	1.00	0.16
	rs11624704	0.60	**0.01**	0.43	0.23	0.63	0.48
	rs917906	0.12	0.45	0.74	0.90	0.35	0.36
	rs1004212	0.23	0.44	0.20	0.31	**0.009**	0.11
	rs10146997	0.30	0.80	0.71	1.00	1.00	0.27
	rs8019381	0.12	0.51	0.42	0.83	1.00	0.62
Women	rs983795	0.16	0.95	0.91	0.22	0.84	1.00
	rs11624704	0.75	0.85	0.07	0.17	0.33	0.40
	rs917906	0.42	0.40	0.27	0.67	0.50	1.00
	rs1004212	0.68	0.08	0.29	0.89	0.36	**0.03**
	rs10146997	**0.03**	0.75	0.75	0.27	0.12	0.87
	rs8019381	0.97	0.72	0.75	0.73	1.00	0.64

Note: Uncorrected p-values for Regular Tobacco use, Alcohol Problem, and Drug Problem are from Fisher's exact tests. Other p-values are from F-tests.

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